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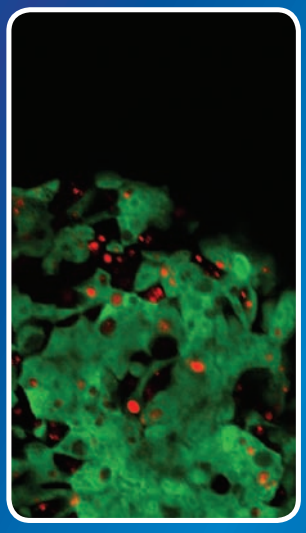
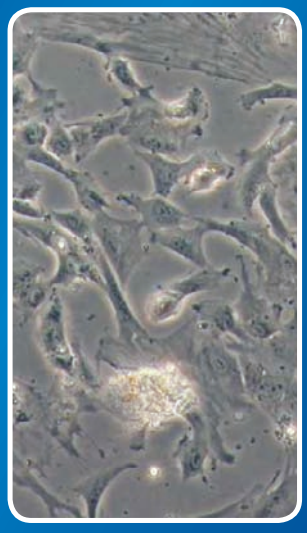
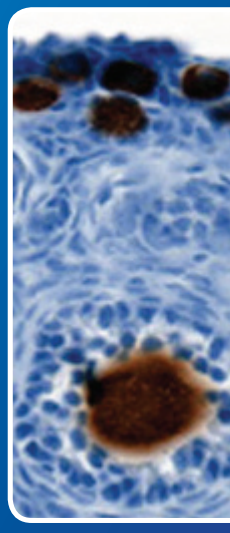
Stem Cells in Human Reproduction

Basic Science and Therapeutic Potential

Second Edition

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Stem Cells in Human Reproduction

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Preface

After the first edition published in 2007 that became a best seller, the continuous scientific developments in the field have prompted us to produce the second edition of this book. As it happens in life and science, some of the novel and promising data presented in the first edition have been confirmed, some not, and new breakthrough achievements have been accomplished.

Stem Cells in Reproductive Medicine, Basic Science and Therapeutic Potential, second edition, updates the revolutionary advances in stem cell science that may potentially impact on human reproductive medicine. From the first edition, scientists and clinicians, leaders in the field, have been invited to update their work, while new authors have also been incorporated due to the relevance of their findings.

Section I entitled the crystal ball, which introduces the clinical and the ethical views of the gamete generation from stem cells, probably one of the main key points of the stem cell field in reproductive medicine, is by two recognized opinion leaders Antonio Pellicer and Guido Pennings. Section II devoted to the female gamete updates gametogenesis by Emre Seli as a baseline to understand the differentiation of the female gamete from embryonic stem cells (ESC) from the genetic and epigenetic perspectives by the group of Orly Lacham-Chaplan and Rene Reijo Pera, respectively. The germline potential of stem cells derived from nongonadal tissues, specifically fetal porcine skin, is also presented by Julang Li. The controversial issue of the existence of germline stem cells in adult ovaries is also addressed in an exceptional chapter by Roger Gosden. Section III first describes the male gamete by Drs Garrido and Meseguer and the differentiation of this gamete from mouse ESC using two different genetic approaches by the groups of Niels Giejsen and Karim Nayernia. Herman Tournaye has produced an outstanding update of the adult stem cell population in the mouse testis. In section IV, the research on the differentiation of trophoblast from hESC has been updated by Ted Golos, and new chapters have been introduced concerning unexpected sources of pluripotent cells such as Wharton's Jelly by Ariff Bongso and amniotic fluid by the group of Antony Atala. The search for the stem cell niche in the human endometrium is presented by the groups of Caroline Gargett and Carlos Simón, and the relevance of bone marrow for stem cell production by Agustin Zapata.

The new developments in hESC research are presented in section V. The use of hESC as a model to investigate human implantation is reported by Harry Moore. The derivation of stem cell lines without causing the destruction of the human embryo is being further updated by Irina Kliminskaya, state-of-the-art cutting-edge technologies such as reprogramming is introduced by Roberto Ensenat and nuclear transfer in relation to reproductive medicine by Björn Heindryckx. Although we are still some distance away from therapeutic applications, a new service provided to IVF clinics with the creation of customized stem cells is presented by Ana Krtolica.

We hope that the readers will find the contents of *Stem Cells in Reproductive Medicine, Basic Science and Therapeutic Potential*, second edition, useful as a reference and a valuable tool for the improvement of reproductive medicine from the cell biology perspective.

Carlos Simón and Antonio Pellicer

1 Gamete Generation from Stem Cells: Will it Ever Be Applicable? A Clinical View

Antonio Pellicer, Nicolás Garrido, Erdal Budak, Santiago Domingo, A. I. Marqués-Marí, and Carlos Simón

INTRODUCTION

Stem cells (SCs) are undifferentiated cells that have the potential to self-replicate and give rise to specialized cells. SCs can be obtained not only from the embryo at cleavage or blastocyst stages [embryonic stem cells (ESCs)] but also from extraembryonic tissues such as the umbilical cord obtained at birth (1), the placenta (2), and the amniotic fluid (3). SCs can also be obtained in the adult mammals from specific niches. These somatic stem cells (SSCs) can be found in a wide range of tissues including bone marrow (BM), blood, fat, skin, and also the testis (4–6).

SCs exposed to appropriate and specific conditions differentiate into cell types of all three germ layers (endoderm, ectoderm, and mesoderm) and also into germ line cells. The latter had raised speculations that SCs may have a potential role in reproductive medicine. Thus in vitro development of germ cells to obtain mature, haploid male and female gametes having the capacity to participate in normal embryo and fetal development has been attempted for the last five years.

Infertility is a common problem in our society with a prevalence of 10% to 15% of couples in their reproductive age (7). On the basis of the 2005 National Survey on Family Growth, an American report, there was a 20% increase in American couples experiencing impaired fecundity between 1995 and 2002. Other reports have recently confirmed this tendency (8). This continuous increase is mainly due to social changes leading to women delaying childbearing to the third and fourth decades of life. As a consequence, oocyte quality is reduced (9–11), increasing the incidence of aneuploidy in human oocytes and resulting embryos, especially after age 40 (10,11). Other factors, such as a decrease in the quality of oocytes and sperm due to environmental factors, may also play an important role (12–15).

The growing demand for biological offspring among patients with impaired fertility has led them to build their hope on scientific research and obtain their own differentiated gametes. Couples seeking a child and enrolled in an assisted-reproduction technology (ART) program do not consider using donor gametes until other options have failed and after a thoughtful discussion with their doctor. Nevertheless, they face several difficult decisions, which include when to abandon treatment with their own gametes, whether to conceive with donated gametes over other options such as adoption, how to choose the donor, or whether to disclose to their children the circumstances of their conception.

In addition, the society is changing with regard to the classical concept of family. Apart from religious considerations, it is a fact that new families are being created in which two males or two females are the basis of a new family. They request their own children, and ART can only offer the use of donated gametes. However, it is obvious that the scientific developments may open new possibilities for these individuals also in our society.

The different aspects of SCs' differentiation into germ cells are covered in other chapters of this book. Although the main achievements will be reviewed from a clinical perspective, the focus will be on the needs and new hopes that this potential development will open among infertile couples, and how creation of germ line cells from SCs will impact the present practice of ART.

IN VITRO DIFFERENTIATION OF GAMETES FROM EMBRYONIC AND NONEMBRYONIC STEM CELLS

The first approach to obtain gametes from SCs was reported by Hübner et al. (16) who described oocyte-like structures from mouse ESCs. Since then, some other works have been published involving differentiation of mouse and human ESCs into germ cells and both male and female presumptive gametes. Nevertheless, the accurate functionality of these structures still needs to be demonstrated.

Germ Cells' Differentiation from Embryonic Stem Cells

Essentially, two methods have been used for germ cell differentiation from human and murine ESCs. The first method consists of spontaneous differentiation in adherent culture (16–19), whereas the second concerns the formation of three-dimensional structures known as embryoid bodies (EBs) (19–24).

Using the first method, in which factors promoting pluripotency as feeders and basic fibroblast growth factor (bFGF) or leukemia growth factor (LIF) are removed, Hübner et al (16) reported the observation of floating structures in vitro, mimicking ovarian follicles. After gonadotropin stimulation, these follicles extruded a central cell, a putative oocyte with a very fragile zona pellucida. Although the presence of the meiotic protein SCP3 indicated entry of the putative oocytes in the meiotic process, neither other meiotic proteins nor evidence of chromosomal synapsis formation was detected (17). Then, the meiotic program failed to progress correctly in vitro. Some of these structures were spontaneously activated, leading to the formation of parthenogenic embryos, which arrested and degenerated in early stages of development.

Simultaneously, other groups reported differentiation of male germ cells from mouse ESCs through formation of EBs combined with the use of knock-in cell lines with markers associated with pluripotency or germ line characteristic genes (20,21). The EBs are three-dimensional structures formed by aggregation of undifferentiated ESCs, in which not only different cell types from the three embryonic germ layers can be formed, but also cells of the germ line.

Tooyoka et al. (20) detected differentiation of germ cells from ESCs in vitro, which were separated and cultured with cells from dissociated male gonads. The resulting coaggregates were transplanted in the testes of male mice to test the developmental potential of the differentiated cells, and approximately two months thereafter, spermatozoids were detected in the seminiferous tubules of these animals. No further analysis of the functionality of these sperm was performed.

Geijsen et al. (21) used a cell line with a green fluorescent protein and employed retinoic acid (RA) to induce differentiation of ESCs. They detected expression of male germ cell–specific markers in the differentiated EBs and markers of Leydig and Sertoli cells. Although some haploid cells were found, the results suggested that meiosis was highly inefficient in the EBs' environment. Finally, the authors investigated the biological function of the EB-derived haploid cells via their capacity to fertilize oocytes by intracytoplasmic injection. About 20% of the fertilized oocytes progressed to blastocyst stage, but it was not tested if the embryos were capable of developing normally on being transferred to the uterus.

The most advanced progress in meiosis and formation of male haploid gametes was obtained following transplantation of in vitro–derived germ cells into the testis for further development into gametes (18). The authors obtained viable progeny after fertilization of normal oocytes with the putative gametes obtained after differentiation of ESCs employing RA. The cells were transplanted into testes of sterilized mice. The obtained sperm had no motility, but cells were haploid. Two hundred and ten normal oocytes were fertilized with this sperm, 65 embryos were transferred into recipient females, and 12 animals were born, although they died prematurely, presumably due to epigenetic abnormalities.

Only two studies to date have explored coculture systems to achieve oogenesis from ESCs in mice (23,24). In both, differentiated EBs were placed into biological systems. Lacham-Kaplan et al. (23) explored the effects of conditioned medium obtained from testicular cell cultures of newborn male mice on the appearance of germ cells within mouse ESC–derived

EBs. They reported that higher number of EBs produced oocyte-like cells enclosed within follicular structures when EBs were cultured in the conditioned medium, and suggested that formation of oocyte-like cells was dependent on the conditioned medium, but not on the appearance of germ cells. Similarly, Qing et al. (24) transferred EBs onto mouse ovarian granulosa cell monolayer, identifying oocyte-like cells within the EBs after 10 days of culture. Although the meiotic protein SCP3 was expressed in these cells, it was localized in the cytoplasm. In both studies, the oocyte-like cells did not contain the zona pellucida and appeared similar to gonocytes in an early developmental phase of the oogenesis process. Interestingly though, the putative oocytes obtained did not undergo spontaneous cleavage as described by Hübner et al. (16).

Attempts to derive germ cells from human ESCs resulted in similar findings as described in mice. Cells differentiated in EBs express markers for human germ cells (19,22,25), and this spontaneous differentiation seems to be line specific (19). Addition of exogenous factors to hESC cultures increases the number of germ cells, but does not necessarily induce their progress into meiosis (25).

Clark et al. (22) described expression of several germ cell markers during different stages of the germ cell development process in vitro, facilitating the characterization of the germ cells and allowing their tracing during the differentiation process.

However, among the few studies exploring the ability of hESCs differentiation into germ cells, the study reported by Chen et al. (19) was the only one to describe follicular-like structures appearing within EBs or monolayer-adherent cultures of differentiated hESCs. Disappointingly, despite the detection of GDF9 expression (post-meiotic oocyte–specific marker), the study did not explore the characteristics of cells enclosed within these follicular structures to identify if they are indeed oocytes.

Germ Cells Differentiation from Somatic Stem Cells

The potential of SSCs to differentiate into germ cells was first demonstrated by Dyce et al. (26) who obtained oocyte-like cells from fetal porcine skin. Skin SSCs in this study were isolated and cultured in follicular fluid with the addition of exogenous gonadotropins. This resulted in the formation of follicular structures containing putative oocytes. The oocyte-like cells underwent spontaneous cleavage in culture. Nevertheless, it remains unclear if the skin SSCs dedifferentiated into ES-like cells before differentiation into the germ line.

Nayernia et al. (27) showed that mouse mesenchymal stem cells (MSCs) are able to give rise to germ line SCs in vitro, but the obtained cells arrested at premeiotic stages upon transplantation into the testes of adult sterile mice.

It has also been proposed that MSCs are progenitors for oocytes in adult ovarian tissue (28,29). This revolutionary proposal has been regarded as unreliable and has sparked controversy, and several solid arguments have been raised against it (30,31). A recent work published by Liu et al. (32) showed that meiosis, neo-oogenesis, and germ SCs are unlikely to occur in normal adult human ovaries. If postnatal oogenesis is finally confirmed in mice, then this species would represent an exception to the rule. Stronger evidence is needed to confirm this new theory indicating that these SSC-derived oocytes enter meiosis or support the development of offsprings in cases of patients with allogenic BM transplant.

However, the authors of these controversial studies have come into discussion refuting the arguments against postnatal oogenesis in adult human ovary arguing, among others, that this reasoning derives from the inability of the authors to detect markers of germ cell mitosis and meiosis and that an absence of evidence does not mean an absence of the possibility (33). Curiously, a recent work published by the same group presents a mouse model in which BM transplant helps to preserve or recover ovarian function of recipient females, but all offsprings generated derived from the host germ line and not from the transplanted BM cells (34).

Recently, Drusenheimer et al. (35) have announced the differentiation of sperm from human BM–derived SCs. However, they have no method to examine the functional competence of the differentiated putative spermatocytes, the successful establishment of which will remain unexplored due to ethical constrains.

Apart from this study, it has not been clearly proven that human MSCs from the BM or any specific tissue are able to give rise to germ cells in vitro. On the contrary, Liu et al. (32) clearly demonstrated that early meiotic-specific or oogenesis-associated markers were undetectable in adult human ovaries, compared with fetal ovary and adult testis controls. These findings are further corroborated by the absence of early meiocytes and proliferating germ cells in adult human ovarian cortex, in contrast to fetal ovary controls.

Reprogramming as a New Approach to Germ Cells' Differentiation

Theoretically, newly derived gametes, genetically identical to those of the individuals whose gametes are being tried to be replaced, can be accomplished through reprogramming. The cells obtained by this technique are known as induced pluripotent stem (iPS) cells, and given that no embryos are involved in their generation, they overcome ethical, social, and legal problems and may, in future, replace ESCs for clinical therapies.

iPS cells are fibroblast cells from mouse or human tissue that are reprogrammed to the pluripotent state by introducing factors known to induce pluripotency into their genome through retroviral transfection (36–39). This new type of SCs, iPS cells, resemble ESCs in morphology and growth properties, expression of ESCs' marker genes, and teratoma formation (36–39). However, their global gene expression and DNA methylation patterns are similar but not identical to those of ESCs.

Three studies presented a second generation of iPS cells by adding a new factor (Nanog) to the cells (38–40). The selection for Nanog resulted in germ line–competent iPS cells, leading to the formation of chimeric mice. An alarmingly high proportion (20%) of chimeric mice developed tumors (40), and this result eliminated the possibility of they being currently used as prospective germ line SC progenitors. Thus, although the iPS cells have the potential to differentiate into many different cell types, including gametes, differentiation of iPS cells into germ line cells in vitro has not been described to date.

THE NEED OF FEMALE GAMETES

Oocyte donation is a very common ART procedure and different types of patients who request donated oocytes in current practice are listed in Table 1. From all ART cycles performed in 2000 in 49 countries worldwide, 32.3% of the procedures involved egg donation (41). Data published by the European Society of Human Reproduction and Embryology (ESHRE) showed that proportion of ART cycles with egg donation increased approximately by 20% from 2003 to 2004 in Europe (42,43). However, in some specialized institutions, the percentage of patients requesting such a procedure may be even much higher, as is the case at our center (Instituto Universitario IVI Valencia), where the proportion of oocyte donation cycles represents around 45% of all ART cycles (Fig. 1).

Table 1 Indications for Donated Gametes Representing Potential SCs Users

Requests for oocytes
Menopause
Aged women
Low responders
Premature ovarian failure
Spontaneous
Iatrogenic
Gay couples
Requests for sperm
Azoospermia
Obstructive
Nonobstructive
Severe oligoasthenoteratospermia
Lesbian couples

Abbreviation: SCs, stem cells.

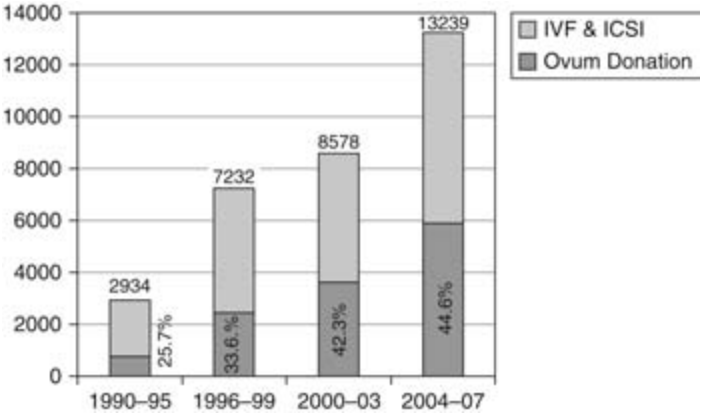

Figure 1 Relationship between ART cycles employing own and donated oocytes from 1990 to 2007 at IVI Valencia. The increase in the demand of donated oocytes has been a constant issue. *Abbreviation*: ART, assisted-reproduction technology.

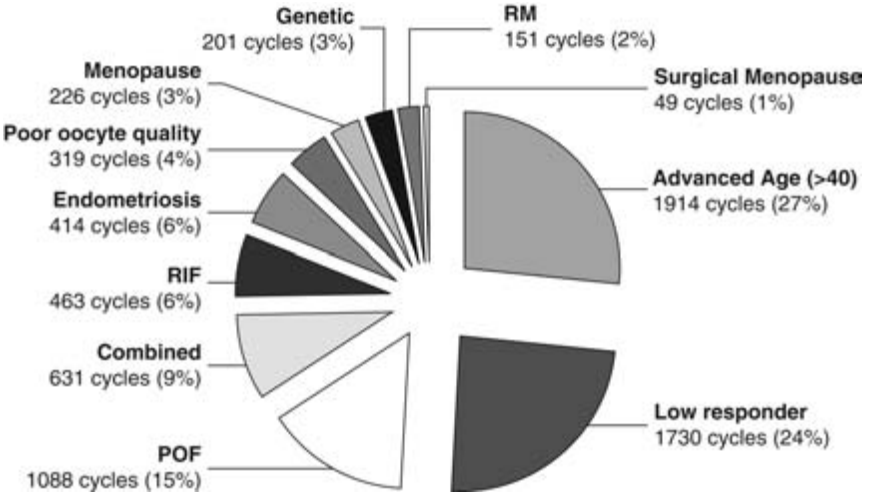

Figure 2 Indications for oocyte donation (cycles with embryo transfer = 7186). *Abbreviations*: POF, premature ovarian failure; RIF, recurrent IVF failure; RM, recurrent miscarriage. *Source*: From Ref. 47.

When the indications for oocyte donation are analyzed, it becomes apparent that there are three main indications to apply this technique: older patients, low response to ovarian stimulation, and premature ovarian failure (POF) (Fig. 2). None of these indications is expected to decrease in the near future. Conversely, there is a trend toward an increase in the request of donated oocytes because the age for childbearing has increased in our society, and age and low response are frequently, but not always, associated. Moreover, survival after cancer treatment in women in their reproductive age is increasing, and, to date, there is no established method for fertility preservation. Thus, many of the patients who will survive after cancer treatment will need donated oocytes. If new sources of gametes become available in future, all of them are certainly potential candidates.

There is an evident decline in fecundity with age, clearly observed in populations where contraception has not been employed (44). In such circumstances, fecundity decreases and infertility increases with age, suggesting that either the uterus or the ovary, or both, is responsible for this impairment of fertility with age.

When the ovary is analyzed individually, there is little doubt that the quality of the egg is affected by age. Studies performed on unfertilized human oocytes showed a significant increase in chromosome abnormalities in women aged >35 years (9). Similarly, studies employing fluorescence in situ hybridization in human preimplantation embryos have shown that aneuploidy is more frequent in women aged >40 years than in younger patients (10,11),

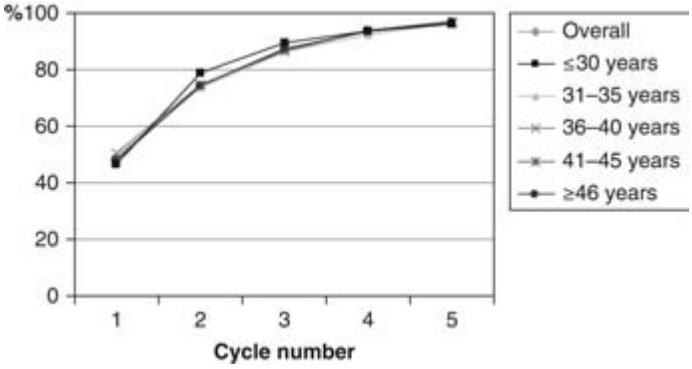

Figure 3 Cumulative pregnancy rates in relation to different age groups: similar pregnancy rate curves were observed for different age groups. *Source*: From Ref. 47.

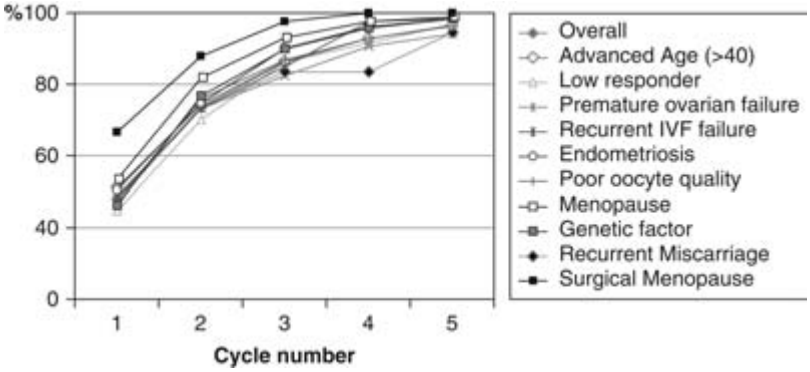

Figure 4 Cumulative pregnancy rates in relation to indication for oocyte donation. *Source*: From Ref. 47.

suggesting that the quality of the oocyte and the resulting embryo in women aged >40 years may be one of the mechanisms involved in the decline of fecundity with age.

Aging of the uterus is a more controversial subject. We have had the opportunity to analyze the highest database on oocyte donation ever published (45–47). An analysis of cumulative pregnancy rates in recent years shows that age does not seem to affect the ability of the uterus to sustain a pregnancy to term (47) (Fig. 3). However, when careful analysis of the data was performed, a small but significant decrease in implantation rates in women >45 years of age was found (46). As a principle, we do not treat women aged >50 years, although this is an issue which may raise many ethical and medical questions in future if the sources of human gametes are amplified (Fig. 4).

Women who have diminished responses to controlled ovarian hyperstimulation (COH) are usually identified as "low responders," and frequently reflect an age-related decline in reproductive performance (48), but there are other situations in which patients are within the normal age range for reproduction and prove, nevertheless, to be low responders. Some have so-called "occult ovarian failure" (49), which reflects an unexpected depletion of follicles. Others have no apparent reason for repeated low response to aggressive stimulation protocols. The etiology of low response is complex, but most of the cases suffer from seemingly depleted ovaries due to age (50).

POF is defined as the cessation of ovarian function before age 40 (51). It affects approximately 1% of the female population in the reproductive age, (52) and different etiologies have been demonstrated, although as much as 80% remain as idiopathic. The main issue involved in POF is fertility preservation because it is becoming an important topic in the management of the quality of life of prepubertal boys, girls, and young people in their reproductive age undergoing cancer treatment. The improvements in the childhood cancer treatments allow an increased number of adults who survived cancer when they were children

(53). Survival rates among young people with malignancies have reached 90% to 95% (54), but most cancer therapies produce nonreversible consequences for the reproductive system that are age and dose dependent (55).

Several strategies have been explored to overcome this unfortunate secondary effect. Preservation of oocytes or embryos is an option, but it has several drawbacks, such as the need for ovarian situation in some hormone-dependent malignancies, and the fact that only a few oocytes can be retrieved in each stimulation, which may not be sufficient to guarantee future fertility (56).

A second strategy for preserving fertility in cancer patients is cryopreservation of ovarian tissue for later auto-transplantation, which can be performed at a heterotopic or orthotopic site. To date, three pregnancies have been published in the literature, and long-term maintenance of ovarian function has not been demonstrated (57–59). Thus, the technique is considered experimental and needs further development and improvement in safety and efficiency.

There is also an important issue. Oncologists are still not familiar with these new techniques of fertility preservation. As a consequence, as much as 17% of our patients arrive to our program of fertility preservation after one of several cycles of chemotherapy, and consequently with a reduced pool of ovarian follicles (56). Therefore, the generation of own gametes from SCs is certainly an alternative for these patients.

There is also another relevant group of people who may benefit from the generation of oocytes out of SCs. As stated above, the society is changing with regard to the classical concept of family. It is a fact that new families are created in which two males are the nucleus of a newly formed family. They may request in the future, the creation of oocytes from SCs of one of the partners, whereas sperms from the other partner are used to fertilize those eggs. They will still need a surrogate to carry the pregnancy to term, but certainly they may afford their own genetically matched offspring in future if these developments reach clinical use.

An important topic, also to be discussed, is the consequences for parents, children, and parent-child relationships of nongenetic parenthood through oocyte donation. Women who finally consider oocyte donation as their method of ART, which offers the highest success rates for their particular case, face several steps in their experience such as acknowledging the desire for motherhood, accepting and coming to terms with donor oocyte as a way to achieve motherhood, navigating an intense period of decision making and living with the lasting legacy of achieving motherhood through oocyte donation (60). The results of this type of reproduction do not seem to be problematic, however, for either the parents or the children. The warmth expressed, the emotional involvement, and mother-child interaction are similar, or higher, to what is found in natural conception (61). However, it is interesting to observe that only 7% will disclose to the children the use of donated eggs, and 50% to 80% to other people, including family and friends (61). There is only some uncertainty as to how and when to disclose to their children how they were conceived. Some prefer early disclosure so that the child always knows about this issue, while others prefer to wait until family routines have been established and the child has the maturity to understand biological concepts and has developed a sense of discretion (62). Therefore, it is obvious that some concerns still exist in the use of oocyte donation as a method of reproduction, although it is a well-accepted technique.

The availability of donors and the consequences of oocyte donation for those who desire to donate oocytes also need to be addressed. Oocytes are scarce and the common picture is to find more potential recipients than donors available. Not to mention the need for fenotype matching, which is a constant demand of the recipients. As a result, waiting lists in oocyte donation programs are frequently too long. On top of this, removal of anonymity has been an adverse phenomenon to the ART method because the number of donors has decreased in the countries where this practice is done, leading to a further restriction of an already unsatisfactory service (63,64).

Safety of oocyte donation is an issue that needs to be further explored. Only psychological consequences have been studied to a certain extent, but the physical consequences of ovum donation have not yet been addressed. Caligara et al. (65) studied ovarian reserve and oocyte quality after several cycles of egg donation. They found that several cycles of ovarian stimulation do not affect number and quality of the oocytes. However, to date, nobody has addressed the impact of ovarian stimulation among other potential dangers such as infertility or cancer.

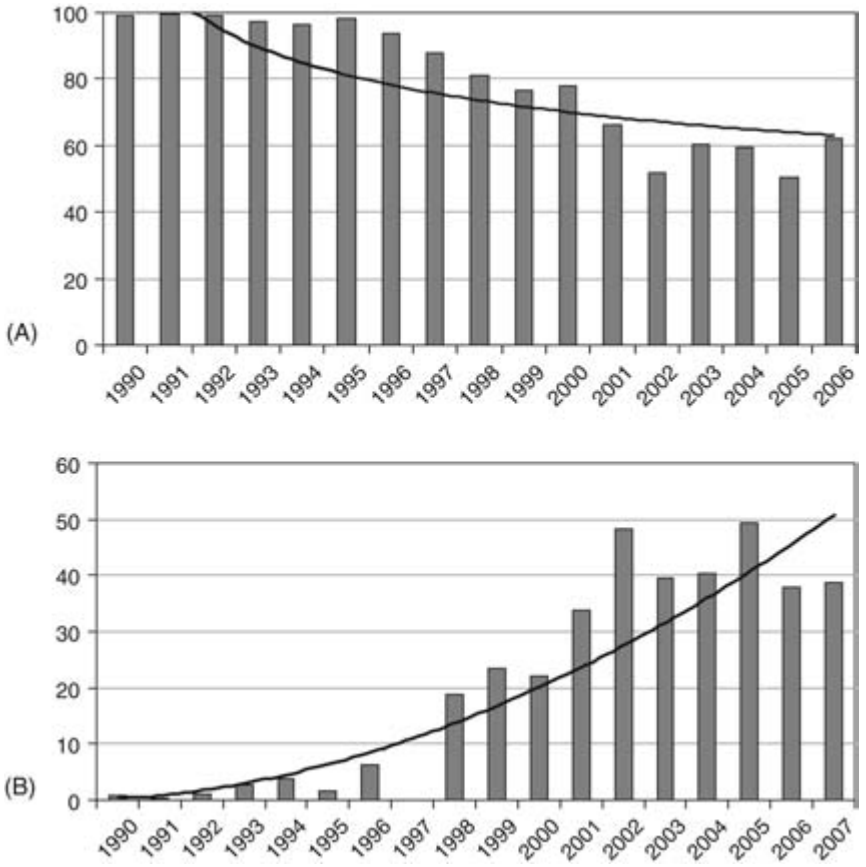

Figure 5 The evolution of the donor sperm bank in which the percentage of ART cycles employing donor sperm due to male infertility at IVI Valencia over the years has decreased due to ICSI, while the percentage of cycles performed in single women and lesbian couples has increased. *Abbreviations*: ART, assisted-reproduction technology; ICSI, intracytoplasmic sperm injection.

THE NEED OF MALE GAMETES

The growing demand of female gametes in infertility practice is not observed when male gametes are analyzed. This is mainly related to two phenomena: the different effect age has on male fertility as compared with female and to the development of intracytoplasmic sperm injection (ICSI). As a consequence, the use of donor sperm has evolved during the years, and Figure 5 explains the current situation: Donated sperm employed in couples with a severe male fertility problem represents a continuous decreasing curve, from 100% at the beginning to around 60% today. This is counterbalanced by an increase in the use of donated sperm by single women and lesbian couples, which represents as much as 40%.

Although some studies have shown a negative effect of males' age on sperm quality (66), our own clinical data confirm that paternal age has no effect on embryo quality and fertility (67). Moreover, improvements achieved in the recent years allow paternity to males, whereas this goal was unthinkable 10 years ago.

The development of ICSI by Palermo et al. (68) has been one of the breakthrough achievements in reproductive medicine in most recent years. Today the goal is to find a motile spermatozoon, and once this has been identified, these males have their parenthood options employing ICSI, either with fresh or cryopreserved samples (69).

Azoospermia is observed in approximately 1% of the general population and in 10% to 15% among the infertile male population (70,71). But azoospermia is not equivalent to the total absence of sperm production within the testes. Obstructive azoospermia (OA) is the situation

where the testes present a normal sperm production although these sperm cells are unable to reach the ejaculate, due to an obstruction in the male's genital tract, while nonobstructive azoospermia (NOA) is considered when sperm cells are produced under the threshold needed to be found within the ejaculate (72).

Accounts of the first pregnancies reported after fertilization by ICSI with testicular sperm in men with OA were published in 1993 (73,74). Testicular sperm extraction (TESE) was described for the first time in 1994 (75), initially in OA, and lately, for NOA cases (76,77).

The diagnosis of one of these two situations represents different consequences on males' chances to become fathers. In the first scenario, motile sperm can be found almost always to be employed in ART, while in the second situation, the probability of finding motile sperm will depend on several factors, but approximately in 45% to 50% of the cases, motile sperm that can be employed in assisted reproduction can be found (78).

ICSI is able to solve most of the problems related with male infertility, but still there are some inconveniences that need to be addressed, namely, the higher incidence of malformations in the newborn infants and actual success rates in severe male factor infertility.

A higher incidence of sex chromosomal aneuploidies and structural de novo chromosomal abnormalities has been found in prenatal karyotypes following ICSI compared with the general population, which could be attributed to the characteristics of the infertile men treated (79–81). Chromosome abnormalities are increasingly found in sperms of many infertile men. This makes the direct analysis of sperm aneuploidy of clinical relevance, since male infertility is now treated by ICSI, which has the implicit risk of transmitting chromosomal aberrations from paternal side. The importance of analyzing the cytogenetic constitution of ejaculated sperm is emphasized by meiotic studies, showing that 17.6% to 26.7% patients with severe oligozoospermia ($<1 \times 10^6$ sperm/mL) have synaptic chromosome anomalies restricted to the germ cell line, which are not detectable by peripheral blood karyotype (82–84). These data clearly indicate that sperm presence within the testis is not synonymous of reproductive success, especially in the cases presenting the most impaired spermatogenesis.

Those patients showing no sperm within their ejaculates are dependent on- any technique able to form sperm cells from any somatic cell containing the genetic information of the individual. Also, as stated above, the society is changing with regard to the classical concept of family and in many countries such as Spain, the marriage of two females is a reality. These couples may request their own children in future and ART can only offer the use of donated sperm, which represents 40% of the requests in our own bank (Fig. 5). However, it is obvious that the scientific developments may also open new possibilities for these individuals, and male gametes derived from SCs of one of the partners may fertilize oocytes from the other partner, allowing them to have their genetically matched offprint.

As stated in the first section of this chapter, there are two different approaches to obtain gametes from SCs. The first approach concerns reimplanting SCs within the testicular tissue. The main problem with this approach is to assume that the testicular environment will be able to maintain and differentiate these cells, when the niche has been unable to support these cell types previously because we must keep in mind that we are considering azoospermic males. Often, from the histopathological point of view, these tubules are disorganized and not structured, presumably not capable of supporting spermatogenesis.

In males, sperm cells are produced continuously during the adult life. Hence, spermatogenesis may be reestablished through progenitor germ SCs within the testes. In case of SC depletion by radiation, the damage is dose dependent, leading to transient to permanent infertility in men (85), and consequently, to the necessity of assisted fertilization treatments (86,87). The option of storing mature sperm prior to treatment is a common practice, but this possibility does not exist for prepubertal cancer patients. For these patients, transplantation of spermatogonial SCs obtained before treatment is the only possible strategy to restore fertility, although with the high risk of reseeding cancer cells back to them (88).

The second approach is to build sperm cells in vitro, with controlled media, mimicking well-functioning testes, to overcome the above-mentioned problems. This seems to be the most likely option because infertile males suffer a profound physiological disturbance of these cells, making them unable to complete the reproductive process successfully, and in vitro produced sperm cells may help to enhance fertility chances and efficiency (89). This could be even more

relevant in those males presenting with meiosis defects and severely defective sperm production, where ICSI is today the treatment of choice, but an increase in the problems exhibited by the fetuses and newborns obtained from them has been described, as mentioned previously.

The quality of parenting and psychological adjustment after donor insemination has been analyzed and compared with oocyte donation. No major differences were found, although donor insemination mothers were more likely to be emotionally over-involved with their children than egg donation mothers (90).

As in the case of egg donation, families created after sperm donation seem to be similar to families created after natural conception in terms of warmth, emotional involvement, and mother-to-child interactions. However, only 5% will disclose to the children the origin of the gametes, and 30% to 60% will never tell their relatives and friends their way of reproduction (61).

CONCLUSION

Although important advances have been achieved during the last few years in in vitro germ cells differentiation from SCs, further research is needed to obtain suitable gametes for their use in clinical purposes as reproductive medicine for infertility treatment. Some unsolved problems in gametes differentiation from SCs involve incomplete meiosis (although presence of meiotic proteins has been described), spontaneous activation of oocyte-like structures and formation of pseudoblastocysts with no further development, and lack of an appropriate imprinting status in the putative gametes obtained.

The use of iPS cells as a potential source of germ line cells in vitro offers a new alternative avoiding the social and ethical rejection, however, their ability to differentiate into putative germ cells or gametes has not been proved yet.

Even though it is expected that SCs may contribute in improving human fertility, many more progresses are required before they would be suitable and safe for use in reproductive medicine. Nevertheless, promising first steps have already been taken.

These efforts are certainly needed because the number of requests for donated oocytes is increasing. As a consequence of the delay in childbearing imposed in our society, most of the patients requesting donated oocytes are older women and low responders to gonadotropins. With regard to male infertility, age is not so critical as in women, and also the introduction of ICSI has solved many problems related to sperm. However, in severe cases there is still a need for donated sperm, and the generation of own gametes from new sources will certainly be a great solution, if proven safe and efficient.

An important group of patients may represent those suffering from malignancies in infancy or during their reproductive age. Cancer treatment may affect the gonads, and fertility preservation procedures operate successfully only in postpubertal males for whom sperms are stored in banks. However, other attempts in women and prepubertal boys are still experimental. Thus, the use of newly formed gametes may be of tremendous interest in these situations.

Moreover, society is changing with respect to the classical concept of family, and new families are being created in which two males or two females are the basis of the new families. They request their own children, and it becomes apparent that the scientific developments may open new reproductive possibilities for these couples.

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2 Gamete Generation from Stem Cells: An Ethicist's View

Heidi Mertes and Guido Pennings

INTRODUCTION

A crucial facet of human embryonic stem cell (hESC) research is to gain insight into cell differentiation and in how this differentiation can be directed to produce specific types of somatic cells. Besides differentiation into somatic cells, it became clear a few years ago that ESCs can also be coaxed into becoming both male and female germ cells in the laboratory (1–3). Not long thereafter, researchers were able to produce live offspring from sperm cells derived from mouse ESCs (4). These successes and the prospect that one day human gametes will be derived from ESCs have inspired hopes in some, fears in others. Although several technical problems will have to be solved before artificial gametes can be used in research and in the clinic, it is wise to consider prospectively the different possible applications and the ethical issues that would be involved.

POSSIBLE APPLICATIONS

Research

The most likely applications of hESC-derived gametes are in the area of research (5). While it is easy to gather sperm for research purposes, studies requiring human oocytes are hampered by a limited supply. This is due to the technical extensiveness of the procedure for oocyte retrieval and to ethical concerns about the donor's well-being. If mature oocytes could be produced using existing hESC lines, more fresh oocytes would be available whenever the researcher needs them without the need for donors to undergo the demanding procedure for oocyte retrieval. This will solve safety issues that are currently linked to oocyte donation for research purposes, most notably the possibility of donors developing ovarian hyperstimulation syndrome (6,7). One important application would probably be in hESC research itself, as stem cell–derived oocytes could be used to perform somatic cell nuclear transfer (SCNT).

"Donor Gametes" for Infertility Treatment

Not only the research setting is faced with a shortage of oocytes but also the field of assisted reproductive technology (ART). In countries where known donation is permitted, many women can rely on family members or friends to donate oocytes, but "anonymous" oocytes are scarce unless considerable amounts are offered to potential donors. Donors are reluctant to come forward for two reasons: the trying donation procedure and the idea of having genetic offspring that is unknown to them. Gametes derived from existing ESC lines could theoretically avoid the first reason. However, when existing stem cell lines or supernumerary embryos are used, there would still be a genetic link between the donor of the material and the offspring. So also with this procedure, the idea of having unknown genetic offspring might be a problem. Moreover, this procedure only makes sense when one or both of two conditions are fulfilled: (*i*) we are able to derive gametes from stem cells, but we are not capable of creating a cloned embryo; (*ii*) the infertile partner has a genetic condition, which is present in all his or her cells, and consequently, his or her DNA cannot be used. If neither of these conditions is fulfilled, it would be logical to use the infertile person's cells. Concerns for inbreeding would require that only a limited number of oocytes per stem cell line are used for infertility treatment, but this is no different from the already existing limitations for the use of donor sperm. The final possibility concerns the creation of oocytes to be used for SCNT to create customized gametes. Given the very low efficiency of SCNT, a quasi-unlimited stock of oocytes

would be handy. In that case, only the mitochondrial DNA of the donor would be present in the offspring, and this arguably does not constitute a genetic relationship. The use of nonrelated ESC-derived sperm for infertility treatment is unlikely since "natural" sperm is less scarce (although in many countries the demand remains higher than the supply) and the donation procedure is not invasive. Unless the procedure to derive gametes from stem cells becomes safe, efficient, and cheap—a combination that seems unlikely in the near future—the preferred course of action is thus likely to remain the use of natural donor sperm.

Personalized Gametes for Infertility Treatment

This possible future application is probably the least realistic at the moment; nevertheless, it is the one that has attracted most attention. People who are unable to produce gametes in a natural way might become genetic parents using ESC-derived gametes tailored to their DNA. The theory goes as follows. The nucleus of the somatic cell of the infertile person is transferred to an oocyte, which is activated to begin dividing and to form an embryo. This embryo is then used to create a stem cell line, which will match the patient genetically. If the technique is perfected, the gametes derived from this stem cell line will be indistinguishable from gametes the patient would have reproduced naturally, containing half of his or her DNA. The children resulting from these gametes will thus be genetically related to the patient to the same extent as naturally conceived children would.

ETHICAL CONCERNS

Safety

If ESC-derived gametes are ever to be used in a clinical setting, there are important safety concerns that need to be addressed. Live offspring from ESC-derived mouse sperm cells had irregular growth patterns and showed abnormalities that led to premature death (4). As a core rule of medicine is to cause no harm, it would be completely unacceptable to use stem cell–derived gametes for reproductive purposes in humans at this stage. Although profound sympathy can exist for people wanting to become parents, the right to procreate is not absolute, and the welfare of the resulting children should always remain the first concern in medically assisted reproduction. Certain steps will be necessary to ensure that the transition from research to the clinic can be made in a safe manner (8). First, further animal studies are needed, not only to assess the health of the direct offspring generated by derived gametes but also to study possible effects in later generations. However, animal models are not always transferable to humans. Even if abnormalities in animal offspring can be avoided, one should still perform preclinical research on human embryos. As the goal of this research would be to evaluate the health of embryos resulting from derived gametes, the use of spare embryos is not an option, and embryos will have to be created specifically for research purposes. This procedure is prohibited in many countries, but it is nevertheless an indispensable step to ensure safe medical applications. The next step would be clinical trials, although these should only start when animal studies and preclinical embryo research have successfully eliminated most safety concerns. Such trials would require continuous evaluation with immediate feedback to ensure fast intervention if any alarming findings present themselves. Finally, if the use of artificial gametes reaches the clinic, follow-up studies should be conducted to continually evaluate the safety of the procedure. In these follow-up studies, special attention should be given to the possibility of accumulating gene mutations (5).

While following these steps, and especially before starting clinical trials with tailor-made gametes, a thorough reflection is needed on the value of the genetic link. Most likely, gametes cultured in vitro will be less safe and much more expensive than naturally produced gametes. From which point on are the risks and costs of this technology too high compared with the use of donor gametes? In other words, when does the right and wish to have genetically related children become unacceptable? People who are unable to produce gametes naturally may regard their condition as a fundamental injustice, which should be rectified by medicine. However, their wish to have a genetically related child does not create a duty for researchers and doctors to pursue this ideal at all costs, especially not if such pursuit would endanger the welfare of the future child. Moreover, in the current stage of research, it seems very unlikely that the procedure to make tailor-made ESC lines through SCNT will become efficient any time

soon. Using oocytes derived from existing hESC lines to develop these personalized ESC lines will add an extra risk factor, and thus the technique will—at least in the initial phase—rely on a supply of donor oocytes. As these oocytes are scarce and as obtaining them requires a significant effort of the donor, it would be preferable to use the available oocytes directly, rather than to use them in an inefficient protocol with extra safety risks to produce new oocytes with a different genetic make up.

Moral Status of the Human Embryo

The moral status of the human embryo is the main point of contention in the ethical debate surrounding ESC research. Broadly speaking, three positions can be discerned. One can attribute a very high moral status to an embryo. This implies that it cannot be destroyed for research purposes, irrespective of the possible benefits that may be obtained through this research. On the other side of the spectrum, one believes that a human embryo has no moral status or a very limited one, meaning that its destruction for research is no reason for moral concern. A third intermediate position holds that a human embryo has a certain moral status but that this status is not absolute. If a research project has a reasonable chance to lead to positive outcomes, the harm that is done by the destruction of embryos can be outweighed by the benefits and can be ethically justified.

As long as artificial gametes are only used in research, no fundamentally new elements are added to this discussion. If they are used for infertility treatments, however, the ultimate purpose of the embryo destruction becomes reproduction instead of research, which further complicates the issue. Embryos are routinely sacrificed for reproductive purposes during infertility treatments since not all created embryos are replaced. The only ethically relevant difference with reproduction through ESC-derived gametes is then that embryo destruction is a mere unintended "side effect" of current ART, while it would be an essential component of the production procedure for ESC-derived gametes. People basing their moral judgment on the intention to destroy embryos (from the onset) will consider this distinction crucial, and others, basing their moral judgment on the consequences of a procedure, will consider it irrelevant. In the discarded–created discussion regarding ESC research, some representatives of the intermediate position on the moral status of the human embryo have argued that every embryo created should at least have the possibility of becoming a human being, as this situation resembles that of embryos "in nature." This criterion is fulfilled in present ART methods, but not in embryos created for ESC research or for the production of ESC-derived gametes. In conclusion, the moral status of the human embryo may be an important issue in the discussion surrounding reproduction by means of ESC-derived gametes, even for those who do not oppose current ART.

Embryonic Parents

If ESC-derived gametes are used for reproductive ends, a philosophical problem arises concerning the determination of parenthood. Is the provider of the somatic cell nucleus that is used to create the embryo from which the ESCs are taken to derive gametes, the genetic parent of the child? This person's DNA will match the resulting child's DNA to the same degree as a natural genetic parent, except for the fact that when a cultured oocyte is used, the mother's mitochondrial DNA will not correspond to the child's. The donors of the somatic cells will find their traits in the children and will pass a paternity or maternity test with flying colors. If this is the criterion for genetic parenthood, they are the genetic parents. However, this would imply that an identical twin is the genetic parent of the other twin's children, while it seems logical that one cannot be a biological parent of someone in whose creation one did not play any part. It is also unlikely that when a clone would reproduce, the "original" would be considered as the genetic parent of the child. In the case of reproduction by ESC-derived gametes, it is not as obvious as it may seem that the donor of the original genetic material is the genetic parent (9). One could argue that the gametes "belong" to the embryo of which they were derived and that the embryo comes closer to being the resulting child's genetic parent than the person who wants to have genetic children in the first place. If prospective parents feel the same way, then the use of ESC-derived gametes as a reproductive strategy misses its goal. If would-be parents make their peace with this drawback of ESC-derived gametes as a reproductive strategy, a

concern still persists about the effect on children arising from derived gametes. They might encounter psychological problems if they consider the embryo that precedes them in their family tree as their actual parent. It is debatable whether psychological problems are more likely to develop when donor gametes are used or when ESC-derived gametes are used. In the former case, knowing that one parent is not genetically related to the child, while some other person is, might drive a rift between the (social) parent and the child. In places where donor anonymity is imposed, it might lead to a frustrating search for one's genetic origin. However, when donor gametes are used, the absent genetic parent is at least a "full-grown" human being, while this is not the case for ESC-derived gametes. In the latter procedure, however, there is no missing family lineage; the child will know as much or as little as any other child about where some of his or her traits came from.

In the scenario where ESC-derived oocytes are used for reproduction by unrelated would-be parents, the resulting child might have the feeling to have come from "nowhere," as there is no person who might be regarded as the genetic mother. In many countries, there is an increasing importance given to the right to know one's genetic origin. The abandonment of donor anonymity clearly illustrates this point. One may wonder whether there would be room in this climate for ESC-derived "donor" gametes. Presuming that these gametes would be derived from a spare (or otherwise naturally fertilized) embryo, the donor would technically never have existed. Although the identities of the child's genetic grandparents could be released, the parent's identity is nonexistent and can thus neither be kept anonymous nor be released. A hypothetical situation that resembles this scenario is reproduction by means of oocytes or ovarian tissue from aborted fetuses. The use of fetal reproductive tissue for infertility treatments is shunned even by countries with liberal regulations, as the United Kingdom (10), due to concerns about safety, increased abortion rates, the psychological welfare of the resulting children and oftentimes—unfortunately—the "yuck factor" (11,12). The use of gametes derived from ESCs could meet the same degree of opposition.

Concerns for the psychological well-being of the resulting child should however not be exaggerated. Similar concerns were voiced at the advent of in vitro fertilization and conception using donor gametes, but studies have thus far not confirmed their legitimacy (13–15).

Informed Consent

The use of gametes for research purposes and infertility treatment requires consent from the person to whom the gametes belong. ESC-derived gametes can be said to "belong" to either the donor of the original body tissue (as he or she is the person who is most closely linked to the gametes) or to the five-day-old embryo that is destroyed to obtain the ESCs. In the first case, existing procedures to obtain informed consent can be applied without specific difficulties. Yet in the second case, obtaining consent is impossible. As the Human Fertilisation and Embryology Authority's (HFEA's) Ethics and Law Committee remarked: "Putting aside the genomic similarity to a living being, it must be conceded that the gametes are not the gametes of a person who, in any possible world, would be capable of giving their consent to use" (16). It would be wrong to conclude that this lack of consent presents a deadlock for the use of ESC-derived gametes. Requiring an embryo to consent to a procedure is somewhat bizarre since the duty to seek informed consent stems from the duty to respect a person's autonomy. As an embryo can hardly be called an autonomous creature, it is not clear how the doctrine of informed consent could apply to it. In other instances where medical or research procedures are performed on an embryo, it is often regarded as the "property" of one or both parents, and it is commonly accepted that the parents decide about its faith. For "in vivo" embryos in the first stages of development, the mother has the right—in most countries—to terminate a pregnancy. For "in vitro" embryos that are left over after infertility treatment, both parents need to decide together if they want to continue storage for own use, donate to other infertile couples, donate to research, or leave the embryos to perish. For the use of fetal tissue in research or therapy after an abortion, either the mother's or—what would be more justifiable both parents' consent is required, depending on a particular country's legislation (17). These precedents indicate that the genetic parents or genetic progenitors (in case of a cloned embryo) should give informed consent for the derivation of gametes from the embryo. Still, one might wonder after how many manipulations or cell divisions a certain tissue stops belonging to the

original donor—if ever. While it is defendable that an embryo belongs to its genetic progenitors, it may be more difficult to explain why the gametes that were produced from the stem cell line that was produced from the embryo that was created from one's tissue still belong to the original donor. However, respect for autonomy stipulates that people should consent to the use that is made of stem cell lines derived from their tissue. Given the possible psychological consequences of genetic parenthood, this is even more important when ESC-derived gametes are used in the clinic. It would be unacceptable if someone donating tissue or embryos for research or therapeutic purposes ended up having genetically related offspring without his or her knowledge. Having a genetic grandchild in another family may also cause a lot of distress. Explicit consent should also be obtained when ESC-derived gametes are used for research purposes. One cannot assume that people who donate an embryo to stem cell research feel comfortable about the creation of new gametes and possibly new embryos from their embryo's stem cells.

"Nonmedical" Applications

Popular articles reporting on the development of ESC-derived gametes often mention "ethical issues" that will need to be resolved before they could actually be used in the clinic, but strangely enough they do not refer to any of the issues we discussed so far. Headlines such as "The prospect of all-female conception" (18) and "Getting ready for same-sex reproduction" (19) indicate fascination with the possibility of gay or lesbian couples having children that are genetically related to both partners. Other possible applications that have received attention are the production of oocytes for women who have entered menopause, the creation of children with just one genetic parent, and the creation of genetically enhanced children. One argument that is used to denounce all of these applications is that doctors should limit themselves to curing diseases, rather than reinventing nature. Reproduction through ESC-derived gametes should then only be available for those who are faced with "authentic infertility" (20). Menopause could be described as a medical condition if it started prematurely, but this is not the case when homosexual couples cannot have children or when a person cannot impregnate himself or herself. Hence it is true that helping lesbian women to have a child together does not "cure" any disease. However, as pointed out by Smajdor, in the ART setting, healthy women are undergoing medical treatments on a daily basis because their partners are infertile, rather than being told to look for a different partner. This illustrates how difficult it is to draw a clear line between medical and nonmedical interventions. Moreover, the idea that medicine can only be used to "repair" and not to innovate implies a leap from how things normally are to how they should be. This belief that it is unethical for people to purposely deviate from the standard has no rational foundation. There is no reason to believe that nature has any moral authority, and thus we cannot conclude that medical interventions that "change the course of nature" are necessarily immoral.

However, there are some valid concerns over some of the nonmedical applications. Combining an oocyte and a sperm cell from the same person into one embryo would constitute the most extreme form of inbreeding with the related safety concerns. As doctors are required to keep the best interest of the resulting child in mind, there are good reasons not to perform this kind of social and medical experiment.

For postmenopausal women, an individual evaluation of each woman's particular situation is desirable to evaluate the risks for mother and child, a procedure that does not deviate from evaluations preceding a treatment with donor oocytes (21). Elements to be considered are health risks and whether the parents will be able to take care of the resulting child (given their age). An extra factor that will be important when ESC-derived gametes are used by menopausal women is if the age of the original somatic cell has any impact on the quality of the resulting oocyte, for example, in terms of telomere length, as the cells of cloned animals appear to be "older" than their actual age (22).

Reproduction of homosexual or lesbian couples through ESC-derived gametes requires the derivation of sperm from "female" stem cells or oocytes from "male" stem cells. While experiments in mice (23) indicate that there is a possibility to achieve the latter scenario, the former is subject to a number of technical difficulties that will be difficult to overcome.

Scientists are divided on the theoretical question whether or not it is possible to obtain viable sperm from female cells (24). Even if technical roadblocks could be overcome, safety concerns are greater in this case than when oocytes are derived from female stem cells and sperm from male stem cells. These concerns are mainly caused by fears about faulty imprinting. Besides safety issues linked to the specific technology of artificial gamete derivation, more general concerns have been voiced regarding the rearing of a child by two people of the same gender. Notably fears about the psychological welfare of the resulting children due to a lack of either a mother figure or a father figure are common and are also present in the debate about adoption by homosexual couples. Studies in this area, however, have repeatedly shown that these fears are ungrounded and that children of same-sex couples show a healthy emotional and psychological development (25).

A final possible application that raised ethical questions is the creation of genetically enhanced children. Presupposing that stem cells can be manipulated, this would facilitate germline gene modifications, which faces its own ethical problems and resulting opposition (7,26). However, genetic manipulation is in no way intrinsic to the procedure of gamete derivation from ESCs, nor is reproduction through ESC-derived gametes a necessary condition for germline modifications. It has been shown recently that the human embryo can be genetically modified directly (27). Thus, whether or not genetic enhancement of children is ethically troubling is a separate discussion that has no inherent link to the prospect of reproduction through derived gametes.

GAMETES FROM BONE MARROW STEM CELLS

Recent research by Drusenheimer et al. showed that it is possible to derive sperm cell precursors from bone marrow stem cells (28). If further progress is made in this area, efforts to produce tailor-made gametes for infertility patients will probably shift from hESCs to adult stem cells, since it would bypass the need for therapeutic cloning, which is likely to remain a very difficult procedure. If this shift does indeed take place, most of the ethical concerns voiced above will no longer apply or will be more easily overcome. This would be the case for some safety issues related to SCNT, issues surrounding the moral status of the human embryo, and the philosophical and psychological implications of ascribing parenthood to an embryo.

CONCLUSION

The derivation of gametes from stem cells has a number of possible applications in research and in the clinic. In the research setting, oocytes derived from (stem cells from) spare embryos could, for example, be used to further explore the possibilities of SCNT. The chance that ESC-derived gametes will be used in research at some point is very likely and would solve several practical and ethical problems generated by the demanding oocyte donation procedure. Ethical concerns remain regarding the moral status of the embryo and regarding informed consent. Given the current practice in other applications of assisted reproduction like embryo disposition, explicit informed consent of the donors of the original tissue (embryo or somatic cells) should be obtained. Additional ethical concerns are voiced about the use of ESC-derived gametes in the clinic. The main problem involves safety, that is, the best interest of the child. Experiments in animals have shown severe abnormalities in the offspring, which would be unacceptable in human offspring. Besides safety issues, there are ethical concerns with regard to the moral status of the human embryo, the question whether the embryo should be considered as the genetic parent of the resulting children, and if this ambiguous status poses a threat to their psychological well-being. Next, it is not clear how the basic rule that consent should be obtained from the person to whom the gametes belong should be applied. We advocate that ESC-derived gametes cannot be created—let alone used in the clinic—unless explicit consent is obtained from the genetic progenitors who donated the original tissue. Finally, reproduction through ESC-derived gametes may lead to some nonmedical applications that meet with resistance, namely, the production of oocytes for postmenopausal women who now rely on donor oocytes, the creation of children who have the same person as a mother and as a father, the creation of children with two genetic mothers or two genetic fathers, and the creation of genetically enhanced children. While many of the arguments used

to oppose these applications are weak, there are some extra safety risks involved in some of them, and caution is warranted before engaging in these experiments.

Overall, whether or not the use of stem cell–derived gametes will ever be ethically acceptable depends largely on the refinement of the production technique. Once safety concerns for the resulting offspring are solved, few major ethical obstacles remain, especially if the derivation of functional gametes from adult bone marrow stem cells can be achieved. However, as long as the welfare of future offspring cannot be guaranteed, it would be immoral to proceed with applications in the clinic.

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3 Molecular Biology of the Gamete

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INTRODUCTION

Central to biology is the accurate passage of genetic material from one generation to another. The mechanisms that control and govern our most basic yet prized function, reproduction, are tightly regulated and share common features among evolutionarily distant species. The zygote and the early embryonic cells that result from the union of gametes are totipotent as they are able to give rise to all cells in the body. Insights into the unique features of the molecular biology of both gametes and the early embryo may further our understanding of stem cell biology.

All multicellular animals whose cells become differentiated into tissues are classified into a large division of the animal kingdom, called Metazoa. Sexually reproducing metazoans, regardless of their complexity, always result from the union of two distinct gametes, an egg and a sperm. Their union forms a zygote, which will develop into a functional organism and propagate the species. Although the zygote represents an equal union of two discrete germ cell lineages containing equal amounts of genetic material, the precise cellular machinery that governs zygotic and early embryonic development is largely maternally regulated. Furthermore, control of gene expression in sperm and oocyte is significantly different from that in other, somatic, cells within an organism. It is essential to characterize and understand the mechanisms of these phases of embryonic-cellular regulation, as their implications in defining and predicting stem cell behaviors are fundamental.

OOGENESIS AND EARLY EMBRYOGENESIS

Gametes originate from primordial germ cells (PGCs), which differentiate early in embryogenesis (1). PGCs have an extragonadal origin in most metazoans and migrate to reach the somatic gonad, where they proliferate by mitosis before differentiating into gametes (1). In the female, PGCs differentiate into oocytes, which enter meiosis and arrest at prophase of the first meiotic division (Fig. 1) (2,3).

This first meiotic arrest may last as long as a few years in *Xenopus* (frog) and up to several decades in humans. During this period, large quantities of dormant mRNA are synthesized and stored in the oocyte cytoplasm (4,5). When later translated, these maternally stored mRNAs control gene expression both during meiotic reentry (6–8) and during cleavage divisions of the early embryo (9–11).

Release from the first meiotic arrest is hormonally mediated and marks the onset of a set of nuclear and cytoplasmic changes in the oocyte termed *oocyte maturation*. In *Xenopus*, meiotic reactivation can be mediated in vitro by progesterone (12,13), while in vivo, *Xenopus*, mouse, and human oocytes respond to gonadotropins (Fig. 1) (14,15). In almost all vertebrates, oocyte maturation is complete when cells arrest at metaphase of the second meiotic division and await fertilization (3). A complex network of translational activation and repression of stored maternally derived mRNAs accompanies oocyte maturation (6–8,10) when genomic transcription is downregulated.

The transcriptional silencing that begins with oocyte maturation persists during the initial mitotic divisions of the embryo. In *Xenopus*, activation of transcription in the zygote, also called *zygotic genome activation* (ZGA), occurs after 12 rapid synchronous cleavages, when the developing embryo is composed of approximately 4000 cells (16,17). In mouse and human, ZGA occurs at the two-cell and four- to eight-cell stages, respectively (18–20). Despite the earlier occurrence of ZGA, activation of maternally inherited mRNAs in mammals likely utilizes similar mechanisms to those in other vertebrates and plays a crucial role in early reproductive events (10,21).

To fully understand the unique nature of translational control of gene expression in gametes and early embryos, with its implications for stem cell biology, it is necessary to discuss

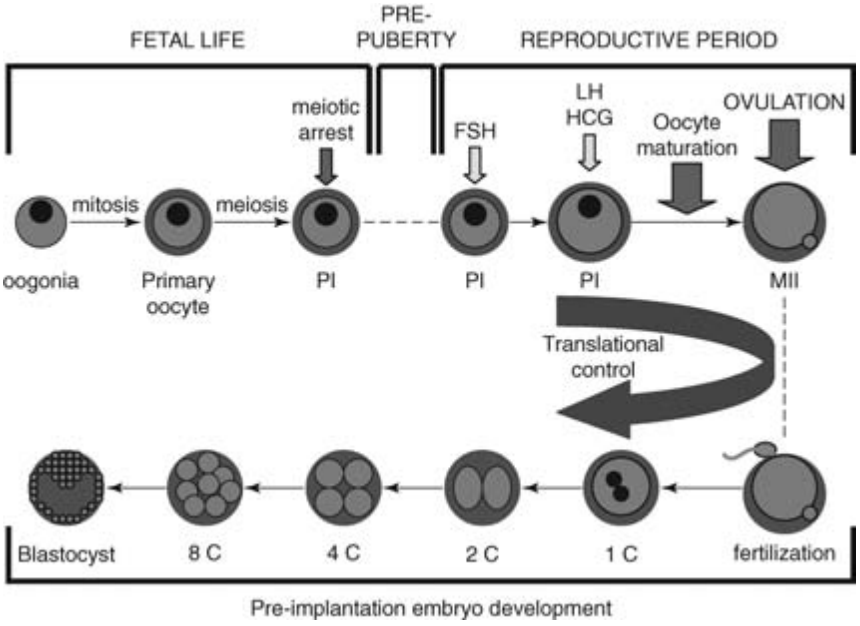

Figure 1 Regulation of gene expression during oocyte and preimplantation embryo development. PGCs proliferate by mitosis and differentiate into oocytes. Primary oocytes enter meiosis and become arrested at the prophase of the first meiotic division (PI). Release from the first meiotic arrest is hormonally mediated, and marks the onset oocyte maturation. In *Xenopus*, meiotic reactivation is mediated by progesterone, while mouse and human oocytes respond to gonadotropins. In almost all vertebrates, oocyte maturation is completed by the metaphase of the second meiotic division (MII), when oocytes become arrested for a second time and await fertilization. Oocyte maturation is associated with suppression of transcription. Thereon, until the activation of transcription in the zygote (4- to 8-cell stage in human and 2-cell stage in mouse), gene expression is regulated by activation and repression of stored maternal mRNAs. *Abbreviations*: PI, prophase I oocyte with germinal vesicle and zona pellucida; MII, metaphase II oocyte (germinal vesicle breakdown has occurred) and the first polar body; 1C, one-cell embryo; 2C, two-cell embryo; 4C, four-cell embryo; 8C, eight-cell embryo.

regulation of transcription and translation in somatic cells. By comparing gene expression in somatic cells and germ cells, we can begin to comprehend the exceptional role of embryonic translational control, the prime gatekeeper in metazoan reproductive life.

TRANSCRIPTION AND TRANSLATION IN SOMATIC CELLS

Transcription and Processing of Pre-mRNAs in Somatic Cells

From its synthesis within the nucleus through its maturation and subsequent transport into the cytoplasm, mRNA does not exist as a nucleic acid–only entity. Within somatic cells, formation and processing of pre-mRNA (Fig. 2) is restricted to the nucleus. In the nucleus, RNA polymerase II binds DNA and transcribes genes to synthesize pre-mRNAs. During transcription, when the 5′ end of the pre-mRNA becomes accessible, 7-methylguanosine (m^7Gppp) is covalently attached to the 5′ end of the pre-mRNA to form the *5′ cap* (Fig. 2). This 5′ cap protects newly synthesized pre-mRNA from enzymatic degradation (22). In addition, the cap is involved in splicing of exons, processing of the 3′ untranslated region (3′ UTR), export of mRNA into the cytoplasm, and eventually, translation of mRNA (23).

A newly capped pre-mRNA transcript continues to be transcribed until its 3′ end is cleaved at a specific sequence; subsequently, for the majority of pre-mRNAs, a series of approximately 250 adenosines [poly(A) tail] are added (Fig. 2). Cleavage site recognition within the pre-mRNA 3′ UTR and its subsequent polyadenylation are well-coordinated events. A consensus sequence, the AAUAAA hexamer, binds a multiprotein complex named cleavage

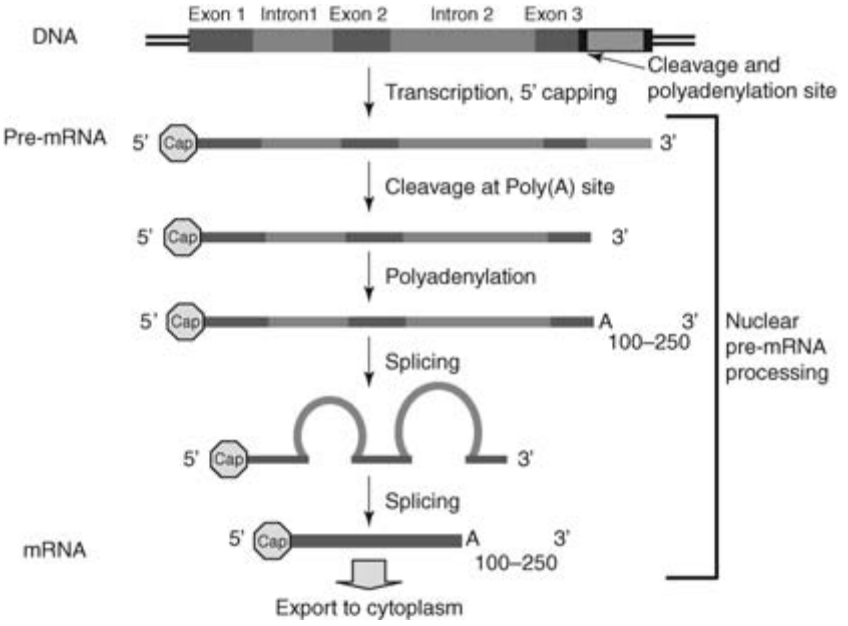

Figure 2 Pre-mRNA processing in the eukaryotes. Soon after the initiation of transcription by RNA polymerase II, the 5′ end of the nascent RNA is capped with 7-methylguanylate. Next, the pre-mRNA transcript is cleaved at the poly(A) site, and adenosine (A) residues are added. The poly(A) tail consists of approximately 250 A residues in mammals. Splicing may occur during transcription or following cleavage and polyadenylation.

and polyadenylation specificity factor (CPSF), which likely guides correct cleavage of the pre-mRNA (24,25), while the addition of the poly(A) tail is catalyzed by poly(A) polymerase (PAP).

A poly(A)-binding protein, poly(A)-binding protein nuclear 1 (PABPN1) [previously termed poly(A)-binding protein 2, PABII], rapidly associates with the poly(A) tail as the tail is synthesized. It not only provides protection from exonucleases but also enhances the polyadenylation reaction. Once the mRNA is transported into the cytoplasm, PABN1 is replaced by poly(A)-binding protein cytoplasmic 1 (PABPC1), which is significantly larger than its nuclear counterpart and protects the poly(A) tail from deadenylation. It is noteworthy that while well-conserved PABPC1 orthologues have been identified in developmentally distant species and are present in all somatic cells, they are notably absent in oocytes and early embryos.

Concomitant with these pre-mRNA processing reactions of capping, cleavage, and polyadenylation, it is necessary in the majority of metazoan pre-mRNAs to remove introns, or nonessential transcribed regions, and to splice together the remaining exons—RNA sequences that encode proteins (Fig. 2).

These complex processing steps generate a mature mRNA that encodes a functional protein. It is only after these modifications that nuclear mRNA associates with the proteins necessary for nuclear export. The 5′ cap added during the transcript's initial synthesis guides the mRNA through the nuclear pore complex in conjunction with RNA transport proteins, delivering the mRNA to the cytoplasmic translational machinery.

Translation in Somatic Cells

Translation of mRNA into the protein product is mechanistically multifaceted. The cell is prudent in its expenditure of energy, and it regulates mRNA translation to ensure that proteins are synthesized according to cellular needs (9). Translational control mechanisms impact on protein synthesis, and a familiarity with some key components is essential.

The cap structure at the 5′ end of the mRNA, involved in 3′ UTR processing, splicing, and mRNA transport into the cytoplasm, has yet another role in translation by binding the cap-binding complex (Fig. 3). This complex consists of the cap-binding protein eIF4E, the RNA

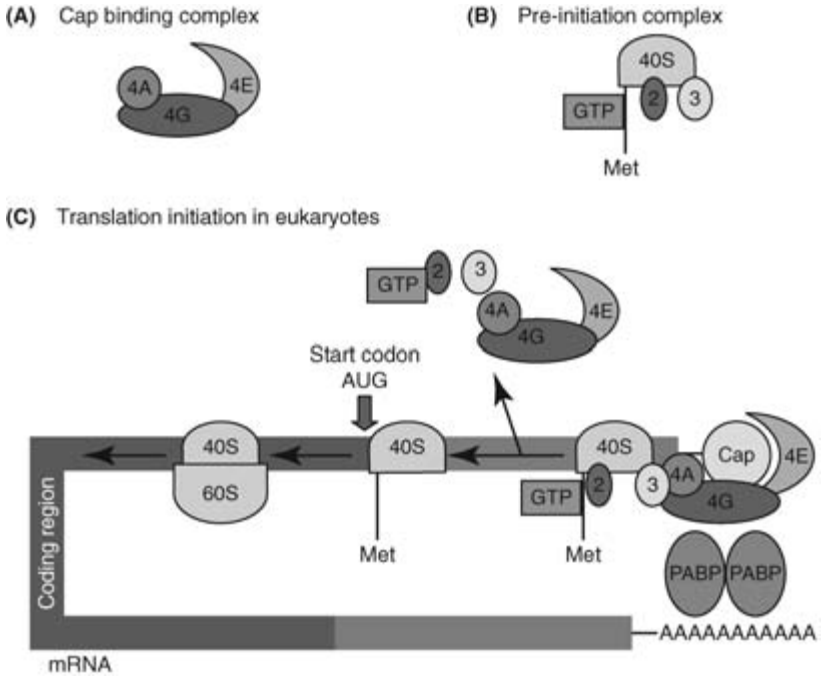

Figure 3 (**A**) Cap-binding complex consists of three eukaryotic initiation factors (eIF): the cap-binding protein eIF4E, the RNA helicase eIF4A, and the modular scaffolding protein eIF4G. (**B**) Translation preinitiation complex consists of a 40S ribosomal subunit–eIF3 complex bound by eIF1A and the ternary complex (Met-tRNA$_i$, eIF2, and GTP). (**C**) When the translation preinitiation complex becomes associated with the cap-binding complex and the mRNA, the 48S initiation complex is formed. Within the 48S initiation complex, eIF4G binds eIF4E and eIF4A, forms a bridge between the mRNA and the ribosome, and also binds the PABP to facilitate the translation of poly (A)-containing mRNAs. Once bound to the cap structure, the 40S ribosomal subunit with associated proteins scans the mRNA toward the 3′ end until it reaches the initiation codon (AUG). At this point, initiation factors become released and the 60S ribosomal subunit is recruited, initiating translation. This model is simplified for clarity and not all the initiation factors are depicted. *Abbreviations*: 4E, eIF4E; 4A, eIF4A; 4G, eIF4G; 40S, 40S ribosomal subunit; 3, eIF3; Met, Met-tRNA$_i$; 2, eIF2.

helicase eIF4A, and the scaffolding protein eIF4G (Fig. 3A). eIF4G plays a pivotal role in translation initiation by binding eIF4E and eIF4A, by forming a bridge between the mRNA and the ribosome, and by binding PABPC1 to facilitate translation of poly(A)-containing mRNAs (Fig. 3C) (26).

A translation preinitiation complex (Fig. 3B) is formed when the 43S complex that is composed of the 40S small ribosomal subunit and eIF3 complex is bound by eIF1A and the ternary complex, consisting of the transfer RNA charged with the initiator methionine (Met-tRNA$_i$), eIF2, and GTP. Cells can downregulate protein synthesis by phosphorylating eIF2 and preventing the formation of this ternary complex.

When the translation preinitiation complex becomes associated with the cap-binding complex and the mRNA, the 48S initiation complex is formed. Because mRNAs are usually produced in excess, they compete with one another for the more limited translational machinery, and so the formation of the 48S initiation complex is the rate-limiting step in translation.

Once bound to the cap structure, the 40S ribosomal subunit, with its associated proteins, scans the mRNA in the 3′ direction until it reaches the initiation codon, AUG. At this point, a subset of initiation factors is released and the 60S ribosomal subunit is recruited to form the functional 80S ribosome, thereby initiating translation (26).

Although in a strict linear sense, the poly(A) tail is the last element reached by the translational machinery, it is one of the most important factors governing the translational fate of

an mRNA. In addition to the complexes mentioned above, which bind and recruit functional ribosomes to the message, an interaction between the 5′ and 3′ ends of an mRNA plays an important role in initiating translation. In fact, the mRNA is circularized, positioning the essential components of the translational machinery [eIF4G-eIF4E at the 5′ end and poly(A)-binding proteins at the 3′ end] close to one another, which stimulates translational initiation (27).

Once translation is initiated, the polypeptide chain is elongated by bound ribosomes. This complex pauses intermittently and may offer a means for translational control at this level. Translation is completed when the message is released, after ribosomes encounter one of three stop codons (28,29). Many polypeptides undergo regulated posttranslational modifications depending on their function, localization, and other properties.

REGULATION OF GENE EXPRESSION IN THE OOCYTE

Translational Control of Gene Expression in the Oocyte by Cytoplasmic Polyadenylation

Although polyadenylation is a nuclear processing event that occurs on the majority of pre-mRNAs (30,31), cytoplasmic polyadenylation occurs during oocyte maturation and early embryo development. It plays a crucial role in translational regulation of many mRNAs.

Three key findings suggest that cytoplasmic polyadenylation regulates translation of certain mRNAs in early development. First, in sea urchin eggs, levels of poly(A)-containing mRNAs increase twofold shortly after fertilization, a time when there is no de novo transcription (32,33). Second, this poly(A) increase is a cytoplasmic event because it can occur in activated, enucleated eggs (33). Third, these polyadenylated transcripts are preferentially bound by ribosomes (32,33). Moreover, Northern analysis of specific mRNAs from the surf clam, *Spisula*, has demonstrated that polyadenylation occurs specifically on certain maternally stored mRNAs, but not on others (34,35). These observations and those in *Xenopus* (36–39) and mouse (6,10,40,41) establish a correlation between translation and polyadenylation and also demonstrate that this polyadenylation control is likely to be mRNA specific.

The regulation of maternally stored mRNA polyadenylation is also controlled temporally. In *Xenopus* where this process has been most extensively studied, it has been known for some time that certain maternally stored mRNAs are polyadenylated earlier than others (42). Recent evidence has shown that the combination of cytoplasmic polyadenylation element (CPE) and pumilio-binding element (PBE) as well as their position relative to the polyadenylation signal play a large role in regulating when mRNAs will be polyadenylated (42).

In contrast to the large number of maternally stored mRNAs that are polyadenylated during oocyte maturation and early cleavage divisions, some, such as those encoding ribosomal proteins (43,44) and actin (45,46), are deadenylated at this time. In contrast to the effect of polyadenylation, deadenylation leads to suppression of translation (47).

Regulation of Cytoplasmic Polyadenylation

Molecular mechanisms regulating cytoplasmic polyadenylation have been studied primarily in mouse (40,41) and *Xenopus* oocytes (48–51), and appear to be highly conserved between the two.

In *Xenopus* oocytes, in addition to the nuclear cleavage and polyadenylation signal AAUAAA, a second sequence in the 3′ UTR called a CPE is necessary for cytoplasmic polyadenylation (Fig. 4) (52). The CPE is located near the consensus cleavage and polyadenylation signal, usually 20 to 30 nucleotides upstream, and has the consensus sequence $UUUUUA_{1\text{-}2}U$ (52). The CPE is specific to mRNAs polyadenylated during meiotic maturation and binds CPE-binding protein (CPEB), a highly conserved RNA-binding protein with both a zinc finger and an RNA-recognition motif (RRM) (53,54). Injection into *Xenopus* oocytes either of antibody against CPEB or of excess RNA containing the CPE sequence inhibits polyadenylation and blocks progesterone-induced oocyte maturation (8).

CPEB is also present in mammalian oocytes, where its function is likely similar to that in *Xenopus* (6,55). It is therefore noteworthy that the mRNA for synaptonemal complex protein 3 (SCP3), whose absence promotes female germ cell aneuploidy and embryo death in mice (56), contains a CPE in its 3′ UTR and is probably subject to cytoplasmic polyadenylation during mammalian oogenesis and early development. Furthermore, CPEB function is critical during

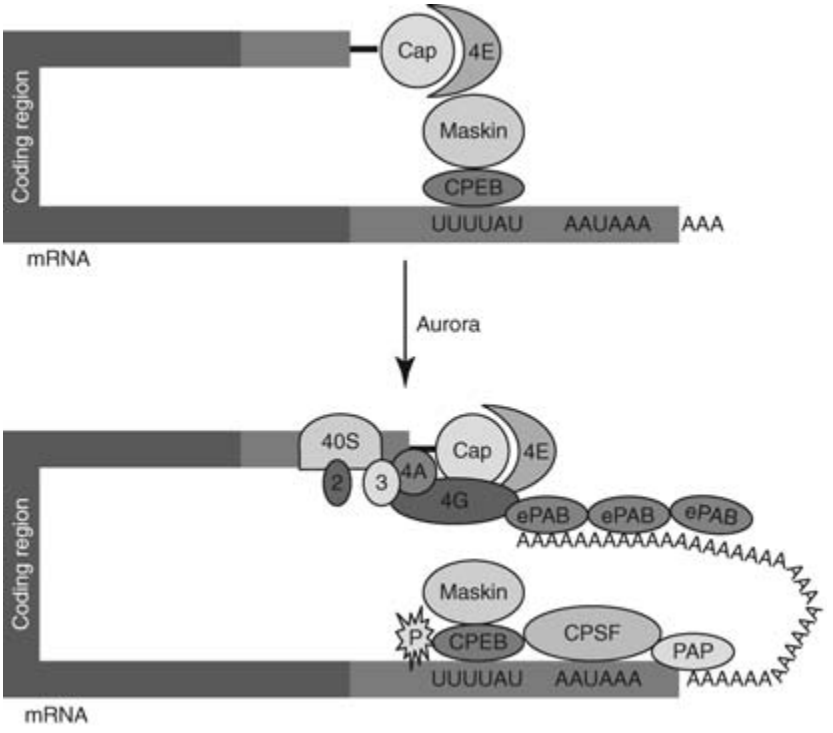

Figure 4 Model of polyadenylation-induced translation. Dormant CPE-containing mRNAs (e.g., cyclin B1) in immature *Xenopus* oocytes are bound by CPEB, which in turn is bound to maskin, which in turn is bound to eIF4E, the cap-binding factor. The binding of maskin to eIF4E precludes the binding of eIF4G to eIF4E, thus inhibiting the formation of the translation initiation complex. Following stimulation, the kinase aurora is activated and phosphorylates CPEB, an event that causes CPEB to bind and recruit CPSF into an active cytoplasmic polyadenylation complex. CPSF recruits PAP to the end of the mRNA, where it catalyses poly(A) addition. The newly elongated poly(A) tail is then bound by a PABP, which in turn associates with eIF4G. eIF4G, when associated with PABP, then displaces maskin from and binds to, eIF4E, thereby initiating translation. *Abbreviations*: CPE, cytoplasmic polyadenylation element; CPEB, CPE-binding protein; PAP, poly(A) polymerase; PABP, poly(A)-binding protein. *Source*: Adapted from Ref. 26.

oocyte development since CPEB knockdown in mouse oocytes results in both infertility and progressive oocyte loss (57).

When *Xenopus* oocytes are stimulated in vitro with progesterone, the Aurora family kinase Eg2 phosphorylates CPEB (Fig. 4) (7), helping CPEB stabilize CPSF on the AAUAAA sequence (58). However, cytoplasmic polyadenylation by a PAP GLD-2, which is recruited by CPSF in conjunction with CPEB (59,60), is insufficient to induce translation of the newly polyadenylated mRNA. This is because an inhibitory factor called maskin interacts simultaneously with both CPEB and eIF4E (61) to inhibit assembly of the eIF4G-mediated 43S translation initiation complex (Fig. 4). Displacement of maskin leads to translation initiation and requires that a PABP become associated with the newly elongated poly(A) tail (62) allowing the association of eIF4G with eIF4E (Fig. 4).

As previously described, the ubiquitous somatic cytoplasmic poly(A)-binding protein, PABPC1, which binds and stabilizes polyadenylated mRNAs in the cytoplasm, is absent in oocytes and early embryos. A cytoplasmic PABP specific to vertebrate oocytes and embryos has recently been identified in *Xenopus* (63), mouse (64), and human (65). This protein, called embryonic poly(A)-binding protein (ePAB), is present only in oocytes and early embryos prior to ZGA. ePAB binds maternally stored mRNAs and prevents their deadenylation. Moreover, it mediates the displacement of maskin and initiation of translation in the oocyte and the early embryo.

Translation Regulatory Cascades in the Oocyte

At a molecular level, meiotic reactivation depends on timely translation of specific mRNAs stored in the oocyte cytoplasm. Proteins encoded by these mRNAs include the rapid inducer of G2/M progression in oocytes/Speedy (RINGO/Spy), cyclin B1, and cyclin-dependent protein kinase 2 (Cdk2) (26,66–68).

Translation of the RINGO/Spy message is essential since the RINGO/Spy protein, a novel cell cycle regulator with unique kinase binding and activation domains, is required to activate Cdk2 (66,68–71). Cdk2 activates the protein kinase Aurora A/Eg2, which, in turn, promotes polyadenylation and subsequent translation of CPE-containing messages, including c-Mos, another serine/threonine kinase. c-Mos activates a mitogen-activated protein kinase (MAPK) cascade through a positive feedback loop, resulting in progression through oocyte maturation.

Cytoplasmic polyadenylation appears to be the predominant translational regulator of maternally stored mRNAs within the oocyte. However, to achieve timely expression of distinct genes necessary for specific steps of oocyte maturation and embryonic cleavage divisions, the presence of additional control mechanisms seems necessary.

Most recently, investigation of RINGO/Spy mRNA translational activation in *Xenopus* oocytes has revealed exciting information on additional mechanisms that may play a role in regulating translation of maternally stored mRNAs. RINGO/Spy protein is absent in the oocyte cytoplasm, while its mRNA is maintained in a dormant state. This is achieved by binding of Pumilio-2, an RNA-binding protein initially described as responsible for translational silencing (by deadenylation) in lower organisms. Pumilio-2 binds to an RNA consensus sequence called the pumilio-binding element located in the 3′ UTR of RINGO/Spy mRNA and inhibits translation. Interestingly, in addition to Pumiolio-2, the RINGO/Spy mRNA 3′ UTR is also bound by DAZL (Deleted in Azoospermia-Like protein) and ePAB. It is postulated that Pumilio-2 facilitates RINGO/Spy mRNA deadenylation and that DAZL and ePAB initially function as corepressors (72).

Hormonal stimulation (by progesterone in *Xenopus*) causes meiotic reactivation and results in dissociation of Pumilio-2 from the PBE, and potentially from the DAZL-ePAB complex. This dissociation allows the DAZL-ePAB complex to then activate RINGO/Spy mRNA translation by an as yet unknown mechanism. Once translated, RINGO/Spy leads to activation of the kinases described above. Finally CPE-dependent polyadenylation and resultant translation of c-Mos mRNA occurs and results in progression through oocyte maturation.

Recent findings pertaining to regulation of RINGO/Spy mRNA translation have two important implications. First, they suggest the presence of additional mechanisms regulating maternal mRNA translation in oocytes, other than polyadenylation. Therefore, a translational control machinery that is activated in cascades and uses specific binding sites in the 3′ UTR seems to regulate activation of maternally stored mRNAs (73). Second, the identification of Pumilio-2 and DAZL in addition to CPEB and ePAB as regulators of maternal mRNA expression suggests that an array of proteins, with homologues in evolutionarily distant animals, is involved in the regulation of maternal mRNA translation. While CPEB, ePAB, and Pumilio-2 were initially identified in model organisms, DAZL was first identified by its homology to DAZ (deleted in azoospermia), a gene on the long arm of the Y chromosome that is frequently deleted in infertile men with nonobstructive azoospermia. The widespread presence of DAZL, as well as ePAB, CPEB, and Pumilio-2 in numerous species suggests that mechanisms regulating maternal mRNA translation are evolutionarily conserved.

MOLECULAR ASPECTS OF SPERMATOGENESIS

Generation of mature male gametes (spermatozoa), or spermatogenesis, occurs in three steps. First, mitotic proliferation leads to the production of a large number of precursor cells. Then, meiotic division and recombination halve the chromosome number while generating genetic diversity. Finally, differentiation packages the chromosomes into spermatozoa for effective delivery into the oocyte. In mammals, spermatogenesis differs from oogenesis by the fact that it occurs continuously after puberty and results in the generation of millions of mature

spermatozoa daily, compared to the limited number of oocytes ovulated during a female's lifetime.

Early in the gestational life of a male embryo, PGCs are localized in the extraembryonic mesoderm. As these PGCs migrate toward the gonadal primordium, they proliferate, generating prospermatogonia that undergo cell cycle arrest at interphase. At puberty, under the influence of testosterone and supported by both Sertoli and Leydig cells, prospermatogonia enter rounds of mitosis, generating spermatogonial stem cells.

From within this reservoir of self-generating stem cells emerge, at intervals, groups of cells with a distinct morphology called *A1 spermatogonia*, which mark the beginning of spermatogenesis. Each of the A1 spermatogonia undergoes a predetermined and limited number of mitotic divisions, generating a clonal population of cells. The number of mitotic divisions that A1 spermatogonia undergo is specific for various species (6 in mouse) and will determine the total number of cells derived from that clone, although subsequent cell death may reduce this number considerably. Morphology of the cells produced at each mitotic division can be distinguished from that of parental cells, making it possible to subclassify spermatogonia. The end result of this series of mitotic divisions is a resting *primary spermatocyte*, which will in turn enter meiotic division.

During the mitotic divisions of spermatogenesis, nuclear division is successfully completed at each step, while cytoplasmic division remains incomplete. Thus, all the primary spermatocytes derived from an A1 spermatogonium are linked together by thin cytoplasmic bridges, forming a large syncytium. This syncytial organization persists throughout the further meiotic divisions, and individual cells are only released during the last stages of spermatogenesis as mature spermatozoa.

Primary spermatocytes replicate their DNA and enter prophase of meiosis I. The first meiotic division ends with separation of homologous chromosomes to opposite ends of the cell on the meiotic spindle, after which cytoplasmic division yields two *secondary spermatocytes* from each primary spermatocyte. Each secondary spermatocyte is haploid and contains a single set of chromosomes, consisting of two chromatids joined at the centromere. The second meiotic division is characterized by the separation of sister chromatids and generates two *early round spermatids* from each secondary spermatocyte. Therefore, in the case of the mouse, from a single A1 spermatogonium, a maximum of 64 primary spermatocytes and 256 early spermatids can result. However, the actual numbers are significantly less due to cell loss.

Meiosis is followed by cytoplasmic remodeling. The extensive differentiation that changes round spermatids into mature spermatozoa is called *spermiogenesis*. With the appearance of spermatozoa, the thin cytoplasmic bridges that make up the syncytium rupture and cells are released into the lumen of the seminiferous tubules (74).

It is noteworthy that the rate of progression through spermatogenesis is constant within a species. Therefore, type A1 spermatogonia within any male gonad of a given species advances through spermatogenesis at the same rate. Hormones or external agents do not seem to affect the rate of spermatogenesis, although they may influence whether or not the process occurs at all. In humans, spermatogenesis is completed in 64 days, while in rats it takes 48 days. The longest stage of meiosis in the male is pachytene of the first meiotic prophase when recombination of genetic material occurs. This is different from oogenesis, where the longest phase of meiotic division is the diplotene stage of prophase I, characterized by the first meiotic arrest in oogenesis.

Transcriptional Control in Male Germ Cells

Germ cells utilize unique mechanisms of transcription initiation including alternate transcription factors, tissue-specific promoters, and somatic gene expression silencing (75). These common characteristics are seen not only in oogenesis but also in spermatogenesis.

Transcription factors bind specific promoter regions in the DNA upstream of the protein-coding region to regulate RNA transcription. Some transcription factors are ubiquitous and regulate many genes expressed in a multitude of tissues, while others are specific for certain tissues and regulate tissue-specific gene expression. Transcription factors unique to

spermatogenic cells have been identified and include SPRM1, TAK-1, OCT-2, CREB, and CREM (76).

The best-characterized male germ cell–specific transcription factors are the cAMP-responsive element-binding protein (CREB) and the closely related cAMP-responsive element modulator (CREM), which are activated by the cyclic AMP/protein kinase A signaling pathway (77). CREM represents a regulatory element that itself is regulated during RNA processing (78). During spermiogenesis, alternatively spliced CREM mRNA encodes first a variant of the CREM protein that is a transcriptional repressor and later a variant that is a potent activator. In addition, an alternate polyadenylation site is utilized to further upregulate CREM expression (79). These data indicate regulatory control of gamete-specific gene expression at the level of nuclear splicing and polyadenylation.

There are many transcripts specific to male germ cells. Often they are the result of alternate initiation of transcription at a site different from that used in somatic cells. For example, transcription of the angiotensin-converting enzyme (ACE) gene begins within the 12th intron of the gene and produces a truncated, testis-specific protein shorter than the somatic form and with a different amino-terminal sequence. Production of this altered protein is the direct result of CREM activation (80). Examples of other proteins expressed as a result of germ cell–specific alterations to the transcriptional machinery include the proto-oncogenes c-abl and c-mos, proenkephalin, superoxide dismutase 1, and β-galactosyltransferase (81).

Translational Repression During Spermatogenesis

Translation is often controlled by repression in both meiotic and haploid spermatogenic cells (82,83). In the mouse model, both spermatocytes and spermatids express low levels of translated (ribosome-bound) mRNA and increased concentrations of untranslated mRNA-protein complexes (mRNPs) in cellular extracts (84). This is in contrast to what would be expected in an efficient, translationally active system. RNA-binding proteins that interact with the 3′ UTR of specific mRNAs seem to be important mediators of translational repression in male germ cells. One such protein is protamine 1 RNA-binding protein (PRBP), which binds a 62-nucleotide region in the 3′ UTR of protamine 1 mRNA in spermatids, where the mRNA is repressed translationally (85).

Translational repression/control occurs in a background where other translational factors are also altered. Interestingly, PABPC1 and eIF4E are expressed at higher levels in spermatogenic cells relative to somatic cells (86); however, greater concentrations of the rate-limiting proteins do not necessarily result in greater translational efficiency. This phenomenon may be due to an increased nucleotide interval between bound ribosomes in spermatogenic cells (140 nt) compared with somatic cell counterparts (100 nt) (74). In addition, the increased concentration of mRNPs may impede recruitment or may sterically hinder interactions between ribosomes and mRNAs.

Translational Activation During Spermatogenesis

Transcription terminates during spermiogenesis (87), as the remodeled condensed chromatin structure of the late-stage spermatids is incompatible with transcription. Thereafter, gene expression is regulated posttranscriptionally and is dependent on dormant paternally stored mRNAs (88) for production of functional spermatozoan proteins.

The pre-mRNAs destined to lie dormant are polyadenylated within the nucleus and exported to the cytoplasm, where they await alteration to their poly(A) tails and subsequent activation (89,90). Interestingly, unlike in the oocyte where the extension of the poly(A) tail results in translational activation, shortening of poly(A) tails in spermatids seems to trigger translation (91). Indeed, stored mRNAs in spermatids usually have poly(A) tails longer than 150 nucleotides, but these polyadenylated mRNAs are not associated with ribosomes. They appear to undergo deadenylation until approximately 30 nucleotides of poly(A) remain; this is coincident with their association with ribosomes and an increase in translation (92). However, the mechanisms regulating shortening of poly(A) tails and translational activation in male germ cells are yet to be elucidated. It is also noteworthy that translational activation of mRNAs associated with poly(A) tail truncation is exclusive to haploid cells. Dormant mRNAs

expressed in meiotic germ cells require poly(A) lengthening for activation, similar to mechanisms detailed in the oocyte.

CONCLUSIONS

Regulation of gene expression in gametes and early embryos utilizes unique posttranscriptional regulatory pathways. Identification and characterization of these pathways and associated regulatory proteins may help better understand stem cell biology. Within this context, it will be of interest to determine whether in vitro differentiation of stem cells into mature germ cells is accompanied by restoration of germ cell–specific gene expression pathways discussed above. In addition, many of the proteins identified as key regulators of translational regulation of maternally stored mRNAs in oocytes (such as DAZL and Pumilio) (93) are also expressed in stem cells (94) where their function remains to be elucidated. Further determination of the role of these proteins in gametes may provide clues to their function in stem cells.

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4 Controlled Differentiation from ES Cells to Oocyte-Like Cells

Orly Lacham-Kaplan

INTRODUCTION

Oocytes play a major role in launching the next generation either by fertilization or by nuclear transfer. Nuclear transfer is a primary requisite for embryonic stem (ES) cell production for regenerative medicine. Only a limited number of oocytes obtained from in vitro fertilization (IVF) clinics can be utilized for this purpose, hence restricting the practice of such clinical applications. In 2003, Hubner et al. were the first to report the derivation of oocytes from mouse male and female ES cells, raising anticipations of prospective unlimited source of oocyte pool. The ES cell line used in that study had a restricted germ cell expression of *Oct-4* coupled with green fluorescent protein (GFP). In that way, germ cell differentiation could be followed step by step in vitro. Cells within the ES cell cultures that were maintained in leukemia inhibitory factor (LIF)-free medium with no supporting feeder cells expressed *Oct-4* after four days of culture indicating on the appearance of germ cells. The proportion of cells expressing *Oct-4* increased with time, and by day 8 about 40% of cells were *Oct-4* positive. Large colonies with reduced *Oct-4* expression but with high *Vasa* expression appeared at about day 12 of culture. The cells within these colonies were of three types; *Oct-4*-only positive cells, *Oct-4*- and *Vasa*-positive cells, and *Vasa*-only positive cells. This suggested that the in vitro–derived germ cells were at different stages of differentiation. Culture of detached aggregates from these colonies for further two weeks resulted in the appearance of follicle-like structures from which 20% contained oocytes larger than 40 μm. The cultures were positive to estradiol and growth differentiation factor 9 (GDF-9), proposing that the follicular structures were functional and that an active interaction between the oocytes and its surrounding cells is active. By day 26, oocytes of different sizes (50–130 μm) surrounded by a fragile zona pellucida were detached from the aggregates. While the oocytes expressed the markers *Figα*, *ZP2*, and *ZP3* they did not express *ZP1*, which elucidates the fragility of the zona pellucida. In addition, oocytes with a diameter larger than 25 μm expressed the meiotic protein *SCP3*, indicative of their entry into meiosis. Larger oocytes extruded the first polar body and underwent spontaneous cleavage forming parthenogenetic embryos. Following this breakthrough study, mouse and human oocyte–like cells derived from ES cells were reported by few other research groups with varying results (1–4).

An encouraging progress in the field, however, was the production of germ cells and putative oocytes from somatic stem cells (5,6). Porcine fetal skin stem cells cultured with 5% porcine follicular fluid resulted in aggregates containing oocytes within two to three weeks of culture (5). When cultured further in medium containing gonadotropins, the oocytes expressed *GDF-9b* and *ZPA* within 5 to 10 days. These oocytes were able to undergo spontaneous embryonic cleavage cycles resulting in parthenogenetic embryos similar to that described by Hubner et al. (7) when mouse ES cells were used. Danner et al. (6) reported on the appearance of oocyte-like cells from rat pancreatic stem cells. These oocytes expressed the meiotic markers *SCP3*, *DMC1*, and *GDF-9*, but they failed to initiate embryonic cleavage cycles. The studies by Dyce et al. (5) and Danner et al. (6) suggest that future sources for the development of patient-specific oocytes need not rely on ES cell production.

While enthralling for both the scientific and nonscientific worlds, the findings provoked reservations regarding the process of gametogenesis and more specifically the process of oogenesis obtained in vitro. Criticism focused on inconsistency between studies in general and

more specifically on the short time period required for oocytes to appear in culture, but more so to mature and progress in embryonic cleavage cycles. This emphasized the fact that in vitro derivation of developmentally competent oocytes is distant.

It has been suggested that methods to produce oocytes from stem cells should utilize well-designed protocols that will establish a controlled, rather than spontaneous, oocyte growth and differentiation. These can be based on protocols developed to obtain functional oocytes from in vivo–derived primordial germ cells (PGCs) and primordial follicles. This chapter outlines these reports and their relevancy toward improving oocyte development from stem cells.

TIMED GROWTH OF OOCYTES THROUGH DEVELOPMENT

In mice, PGCs appear on embryonic day 7.25 (E7.25), and by E12.5 they reach the genital ridges where they enter the female or male germ line differentiation pathway (8). Female PGCs enter meiosis and arrest at the prophase of the first meiotic cycle. After birth, arrested oocytes grow and reach their maximum size at the germinal vesicle (GV) stage three weeks after birth. Hence, the total process of oogenesis in mice requires at least 35 days from the appearance of PGCs in order to reach the GV stage in vivo. Similarly, attempts to produce mature oocytes from PGCs and early stage follicles are based on long-term culture systems and transplantation for specified time periods (9).

Transplantation

Xenotransplantation of mouse and human ovarian tissue to congenic or immunodeficient mice has been used to obtain oocyte growth and maturation (10,11). Shen et al. (12) successfully differentiated PGCs obtained from E12.5, E13.5, and E14.5 mouse ovaries by a combination of in vivo transplantation and in vitro culture. The ovarian tissue obtained from mouse fetuses as early as E12.5 produced primary and early secondary follicles following their transplantation under the kidney capsule. These were able to reach antral follicles in vitro, containing functional oocytes. From the total number of PGCs within genital ridge grafts transplanted under the kidney capsule for 28 days, 25% developed to GV stage oocytes (12). This study indicates that successful development of oocytes from PGCs requires the support of the ovarian milieu (12), which may also be applicable for studies exploring the use of stem cells to derive oocytes.

In Vitro Follicular Cultures

Eppig and O'Brien (13) were the first to develop a step-wise system to culture mouse primordial follicles resulting in functional oocytes and offspring. In this and other studies by this group (14), primordial follicles obtained from newborn female mice were organ cultured until secondary follicles were obtained. These were isolated and individually cultured in the presence of growth factors (GF) for further development to preantral and antral follicles. GF are essential to establish oogenesis in vitro when the ovarian somatic cells are absent (15,16). Premeiotic (E11.5) PGCs can be maintained in vitro by supplementing cultures with LIF, stem cell factor (SCF), fibroblast growth factor B (b-FGF), and bone morphogenetic protein 4 (BMP-4) (16). PGCs entered meiosis when LIF was removed and other growth factors including retinoic acid (RA) were added to cultures. E13–17 mouse PGCs cultured in the presence of SCF, insulin-like growth factor 1 (IGF-1), and LIF progressed to the pachytene stage of meiosis (15). Using modified step-wise culture system to that described by O'Brien et al. (14), Shen et al. (17) successfully produced mature mouse oocytes from E16.5 fetal ovaries. This study describes that the size of oocytes obtained from antral stage in vitro–derived follicles is similar to that of the control group, which were obtained from secondary follicles cultured in vitro. Early-stage PGCs obtained from E12.5 developed to primary and secondary oocytes when cultured with the adjacent mesonephroi (18). The adjacent mesonephroi is the source of RA (19), which supports the entry of PGCs into the female germ cell path. In the study by Obata et al. (18), PGCs were cultured in Waymouth's medium supplemented with 10% FBS for 7 days. Then, the mesonephroi were removed and growth factors were added to cultures allowing the PGCs progress into oocytes within secondary follicles by 17 days. These secondary follicles were able

to develop into antral follicles for another week. Oocytes obtained from these follicles underwent maturation and embryo development.

The effects of cocultures or the addition of conditioned medium prepared from gonadal cell cultures and granulosa cells on oocyte differentiation from ES cells was examined by Lacham-Kaplan et al. (1) and Qing et al. (3). In the study by Lacham-Kaplan et al. (1), embryoid bodies (EBs) that were grown in conditioned medium collected from testicular cell cultures of newborn males for over two weeks transformed into ovarian-like structures containing putative oocytes (1). It is likely that factors within the medium secreted by the testicular cells allowed this differentiation (20–22). Attempts to coculture mouse ES cell–derived EBs with granulosa cells resulted in the formation of early-stage oocytes (3). Differentiation of oocytes from ES cells cocultured with granulosa cells and conditioned medium prevents oocytes exceeding 35 μm in size and spontaneous embryo division cycles. This may suggest that there is more control over the oocyte growth phase by slowing spontaneous events of meiotic progress.

Slowing down the growth phase and, hence, the length of in vitro culture time may consequently have a negative effect on oocyte quality. Obata et al. (23) found that oocytes within primordial follicles cultured in vitro double in their size by 10 days (about 52 μm) and 70 μm after 21 days in culture. About 20% of these oocytes undergo the first meiotic division but they fail to fertilize. Oocytes obtained from secondary follicles from 10-day-old females were of similar size to those obtained from primordial follicles that were cultured for 10 days, which also reached the size of 70 μm within 10 days of culture. About 35% of these oocytes underwent the meiotic division and after IVF 40% fertilized with restricted preimplantation embryo development. When GV stage nuclei from oocytes developed in vitro were transferred to in vivo–produced GV oocytes obtained from Graafian follicles, the maturation of the reconstituted oocytes was 80% to 95% and blastocyst formation was over 90%, regardless of the length of culture (23). More so, live pups in rates of 30% were born from reconstituted embryos. These findings indicate that cytoplasmic maturity is more likely to be influenced by lengthy in vitro culture periods. Thus, the combination of in vivo/in vitro systems as used by Shen et al. (17) may be more suitable for derivation of functional oocytes from stem cells.

Evidently with the culture systems used in published report, ES cell–derived oocytes do not progress through meiosis in a normal way, and more recent studies showed that their chromatin fails to generate normal axial core structures and the chromosomes are not synapsed (24). ES cell cultures producing oocytes secrete estradiol, due to the activation of *Figα* (1,2,7). Yet, this microenvironment is not optimal in supporting the oocytes to complete normal meiosis. This may be due to suboptimal communication between the oocyte and the surrounding cells via gap junctions, which is mediated by Connexin 37 and c-Kit (25,26).

GAP JUNCTIONS AND OOCYTE GROWTH AND DEVELOPMENT

In vivo, oogenesis is closely regulated by paracrine and endocrine factors and cell-cell interaction. This interaction is initiated once PGCs enter the genital ridges during their homing period and is continued until fertilization. PGCs and the somatic cells proliferate rapidly at the genital ridges. Each PGC, termed gonocyte, become surrounded by a single layer of somatic cells. With the onset of primordial follicles, pre-granulosa cells change in morphology and become cuboidal and proliferate while the oocyte grows in size (27). For successful development of oocytes, the presence of gap junctions is required for exchange of metabolites between the viable oocyte and the granulosa cells. Gap junctions are intercellular membrane channels composed of membrane proteins known as connexins. Two of these proteins, connexion 43 (Cx-43) and connexion 37 (Cx-37), are the most important during folliculogenesis. The *Cx43* gene is known to be expressed in oocytes obtained from E13.5 ovaries (28). Disruption of the gap junctions between the oocyte and the surrounding granulosa cells at preantral follicles results in spontaneous GV breakdown and escape of the oocytes from cell cycle control (29) partially because it affects adjustments of paracrine factors such as Kit and Kit ligand (KitL) that are associated with oocyte growth. This reaction of oocytes to disruption of gap junctions may explain the spontaneous maturation of stem cell–derived oocytes and their uncontrolled embryonic cycles.

Paracrine Factors of Folliculogenesis

In vivo, folliculogenesis is regulated by endocrine and intraovarian factors coordinating oocyte growth and proliferation of the somatic cells. These factors that are produced locally within the follicle are important for the ongoing and vital interaction between the oocyte and the soma, which is facilitated through gap junctions (30). Key role for this function is held by KitL/SCF and the receptor tyrosine kinase Kit, both identified in oocytes and granulosa cells (31–33). The genes for these two proteins are already expressed in mouse oocytes at E16.5 (34). In fetal ovaries, Kit-KitL interaction encompasses an antiapoptotic effect on PGCs, oogonia and oocytes, which in postnatal ovaries is associated with follicular growth (35,36). The action of KitL is altered by gap junctions that control the optimal exposure of the oocyte at different growth stages to the KitL (37–41).

Other key players in oocyte growth are GDF-9 and BMP-15, which are important for granulosa cell proliferation and differentiation (42–44). GDF-9 and BMP-15 are secreted by the oocyte at all stages of follicular growth. The two factors have significant interaction with KitL, and the three are regulated by FSH (41). FSH also regulates the contact between the granulosa cells and the oocytes by affecting the numbers of the granulosa cell extensions at different stages of folliculogenesis, and in turn the establishment of gap junctions (45). Interestingly, receptors for GDF-9 and BMP-15 have not been identified in mouse oocytes yet and it is possible that their action is through the surrounding granulosa cells. Mutant mice lacking Cd43, GDF-9, or KitL present folliculogenesis disorders resulting from the inability to coordinate the factors' activity as happens within wild-type ovaries (39). The regulatory loop is also disrupted in the double mutant mice $Gdf9^{+/-}Bmp15^{-/-}$ resulting in impaired cumulus expansion and expression of the gene *Has2* (46).

Recently, Dole et al. (47) reported that glial cell line–derived neurotrophic factor (GDNF) plays a role in the regulation and development of primordial follicles in rat ovaries. Ovaries from four-day-old females grown in organ cultures in the presence of GDNF increased the number of developing follicles in a similar way to KitL. GDNF found to be predominantly produced in oocytes; however, it also appears in follicular granulosa cells.

It is clear that the oocyte through the secretion of paracrine factors plays a role in its own fate. The well-organized structures of oocyte-soma complexes within the ovary allow the oocyte via gap junctions to control the differentiation of the surrounding cells, which in turn supports its well-timed growth and differentiation phases (48). This is important for later events where the oocyte is required to support the development of the embryo independently. A way to allow these structures to keep intact in vitro is growing follicles in three-dimensional culture systems.

Three-Dimensional Culture Systems

The importance of three-dimensional structures to support ovarian tissue development to allow normal oogenesis and folliculogenesis has been recognized and may be a key instrument in in vitro production of oocytes. A variety of three-dimensional culture systems including membrane inserts and alginate hydrogel matrix as a scaffold for follicle growth have been used (49–51). Using alginate hydrogel matrix in a specific concentration (0.25–0.5%) resulted in meiotically competent oocytes enclosed in 100 to 130 μm secondary preantral follicles, which were able to participate in the development of live offspring (51). The three-dimensional system allows physical contact between the granulosa cells and the oocyte, reinforcing the importance of normal structure of the developing follicle (38,39).

In the majority of studies on stem cell differentiation to germ cells, progenitor cells are obtained from EBs. However, the three-dimensional structure is lost following germ cell isolation. For oocytes production keeping the complete EB structures until oocytes are formed may be beneficial (1,52). So far there are no histological evidences that gap junctions between the putative oocytes and the surrounding cells exist. Even though GDF-9 and estradiol are secreted by the oocytes and the surrounding cells, respectively, it is not known how optimal the interaction between the cells is (7). Mixing in vitro produced oocytes with in vivo sourced ovarian cells at the early stages of the in vitro oogenesis may be required to obtain their optimal functionality. Primordial follicles isolated from fetal or neonate mice can be

reconstituted in-vitro and progress in folliculogenesis, suggesting that gap junctions can be reestablished (28,53). It has been also shown that addition of recombinant GDF-9 to Cx-43-deficient granulosa cells results in restoration of their proliferation (54). The close interaction of the oocyte with the soma allows its morphological and genetic changes during growth and differentiation. These also entail epigenetic modification.

CONTROLLING EPIGENETIC MODIFICATIONS DURING OOCYTE GROWTH AND DIFFERENTIATION

In mice, epigenetic modifications begin before the germ cells enter the genital ridges (55). The maternal and paternal alleles are marked during gametogenesis by differential DNA methylation patterns. Hence, epigenetic may be the most difficult to control when growing oocytes from stem cells. However, proper epigenetic profile may be indirectly obtained in oocytes obtained from stem cells via optimization of culture conditions that will enhance timed oocyte growth and proper oocyte-soma interaction.

While going through the growth phase in vivo, oocytes express several specific genes, but as it approaches the GV stage, transcription seizes until fertilization takes place (56). This is accompanied by changes in the oocyte chromatin structure. At early stages, growing oocytes have a noncondense chromatin scattered all over the nucleus (57). As the oocyte grows the chromatin starts condensing and is concentrated around the nucleolus. The change in the chromatin configuration plays essential role in the alteration of gene expression during oocyte growth and is involved with genome remodeling and epigenetic modifications (58). Epigenetic modifications are different in male and female germ cells as indicated by different expression time and activity of the DNA cytosine-5 methyltransferases, DNMT1, 2, and 3a,b,L (59,60). These enzymes are more active in oocytes after birth. De novo and imprinted methylation is initiated at postnatal oocyte stages resulting in global transcriptional repression (60–63), allowing the appropriate chromatin configuration changes to take place (64). Successful imprinting patterns are also associated with the oocyte size (65,66). Chromatin configuration changes in mice oocytes are linked to the follicular growth and metabolic changes in the oocyte and the surrounding cells (64). Links between meiotic and epigenetic abnormalities are well documented in mouse models (67). Oocytes obtained in vitro following organ culture of E16.5 ovaries have an abnormal methylation pattern of the imprinted gene *Igf2r* that may account for the reduced preimplantation embryo development following fertilization (17). A protein that links chromatin configuration and epigenetics has been identified by De La Fuente et al. (64). Lymphoid-specific helicase (Lsh), which is part of the SNF2-helicase family of chromatin remodeling proteins, is required for meiotic chromosome synapsis in female germ cells. Deletions in the *Lsh* gene result in mortality shortly after birth (68,69). Oocytes carrying deletions in the gene show incomplete synapsis although *SYCP3* and *SYCP1* gene expression is similar in wild-type and $Lsh^{-/-}$ oocytes (64). Thus, *SCP* gene expression and protein detection are not indicative of normal meiosis. This is also the case in ES cell–derived oocytes where *SCP3* is expressed but oocytes have chaotic chromosomal synapses (24).

SUMMARY

Reports on the derivation of oocytes from mouse male and female embryonic stem (ES) cells raised anticipations of prospective unlimited source of oocyte pool. However, the accumulative findings provoked reservations regarding the process of in vitro oogenesis. The findings on oocyte derivation from stem cells were criticized for inconsistency related to the time of oogenesis in the in vitro cultures, and overlooking the interaction between the putative oocytes and the surrounding cells. The active granulosa cell-oocyte interaction via gap junctions and the association with paracrine factors may be lacking during oogenesis in vitro, resulting in dysfunctional oocytes with inability to undergo normal timed meiosis. There are proven published protocols to derive functional oocytes from early stage primordial germ cells (PGCs) and primordial follicles. In these protocols, PGCs and primordial follicles are exposed to a variety of growth factors, are cocultured with ovarian cells or transplanted into females for a specific length of time. Utilizing these published protocols when attempting to obtain oocytes from stem cells may improve the oocytes' capacity to participate in normal embryonic development.

CONCLUSION

The above details explore the fact that development of oocytes from stem cells has not yet optimized. However, established protocols are proven to support the growth and differentiation of functional oocytes from germ cells as early as PGCs. The most promising outcomes in establishing developmentally competent oocytes, however, were obtained when PGCs and primordial oocytes were transplanted into females for a specific period of time. Future studies may explore these possibilities.

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5 Germ Cell–Specific Methylation Pattern: Erasure and Reestablishment

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INTRODUCTION

Both genetic and epigenetic information are passed on from one generation to the next, and it is the germ cells, the precursors to eggs and sperm, that provide this link between generations. While genetic information is transmitted in the form of DNA, epigenetic information is saved in the form of DNA modifications that do not alter the DNA sequence. To date, three main types of epigenetic mechanisms are known to exist, including (*i*) DNA methylation, (*ii*) RNA-mediated silencing, and (*iii*) histone modifications. However, DNA methylation is the predominant mechanism studied to date. Two waves of DNA methylation erasure and subsequent de novo DNA methylation occur during the life of an individual. The first wave of methylation erasure takes place during early embryo development, and methylation is subsequently reestablished in a wave of de novo methylation at the time of implantation (1). The second wave of DNA methylation erasure solely affects primordial germ cells (PGCs) and prevents epimutations from being passed on to the next generation.

This chapter focuses on the mechanisms and enzymes that are involved in the process of de novo DNA methylation and DNA methylation maintenance. The association of impaired gametogenesis with imprinting defects as well as the association of imprinting disorders with assisted reproductive technology (ART) will be outlined to stress the importance of the correct establishment of methylation patterns. In addition, this chapter will review the diverse methods for the reprogramming of somatic cells and the importance of the epigenetic reprogramming of the donor nucleus.

MAMMALIAN GERM CELL FORMATION

Germ cell formation in mammalian species is initiated by cell-cell induction (2). Human germ cell specification takes place after the implantation of the embryo and has, therefore, not been studied in depth. However, what is known about germ cell allocation and development in mammals has been extrapolated from model organisms, predominantly the mouse. In the mouse embryo, germ cells can first be identified at 7.2 days post coitum (dpc) as an extraembryonic cell cluster that is localized at the base of the allantois. The secretion of bone morphogenetic proteins (BMPs) 4 and 7 from the extraembryonic ectoderm has been shown to induce germ cell specification in the proximal epiblast. Therefore, it is the position of the respective cells that determines their fate as germ cells rather than their origin. These so-called PGCs subsequently migrate through the hindgut to the dorsal body wall and finally into the genital ridges where they become nonmigratory gonocytes (2). Colonization of the genital ridges by human PGCs takes place at approximately 4.5 weeks of gestation (3).

After migration, the gonocytes in the female enter meiosis and arrest, thereby becoming primary oocytes and follicles. The gonocytes in the male, on the other hand, arrest in the G0/G1 stage of mitosis as prospermatogonia and maintain their proliferative potential. These prospermatogonia migrate to the basement membrane of the seminiferous tubules after birth and differentiate into spermatogonial stem cells. The spermatogonial stem cells form the basis for spermatogenesis throughout male adult life (4).

EPIGENETIC MECHANISMS: DNA METHYLATION

Of the different epigenetic mechanisms, DNA methylation is the most widely studied and best-characterized epigenetic modulator. The methylation of DNA takes place at the 5-position

of cytosines within CpG dinucleotides and plays a role in gene inactivation and chromatin remodeling (5). While CpG dinucleotides are underrepresented throughout the mammalian genome, they are common in distinct genomic regions termed "CpG islands." The majority of the approximately 45,000 CpG islands are located close to or within promoter regions or the first exon of genes. These CpG islands are mostly unmethylated in normal cells with the exception of CpG islands on the inactivated X chromosome in females (6). It has been shown that genes containing methylcytosines in their promoter region are transcribed at a lower level compared with that in unmethylated regions. DNA methylation inhibits the binding of transcription factors and is involved in recruiting methyl-CpG-binding proteins, thereby preventing the transcription of the respective genes (7).

Characteristics and Function of DNA Methyltransferases

The methylation of DNA is mediated by a family of DNA methyltransferases (DNMTs), which catalyze the transfer of the methyl group to the 5-position of cytosine residues within CpG dinucleotides. DNMTs are classified according to the similarity of their catalytic domain and play a role in the establishment and/or maintenance of DNA methylation patterns. DNMT1 was the first DNA methyltransferase to be described and was shown to be essential for normal embryonic development, imprinting, and X-inactivation (8–10). Since DNMT1 has a preference for hemimethylated DNA, can be detected at replication foci, and interacts with proliferating cell nuclear antigen (PCNA), the DNA polymerase cofactor, it was suggested that DNMT1 mainly functions as a maintenance methyltransferase (11,12). As such, it copies and maintains the DNA methylation patterns established in both DNA strands early after embryo implantation (13). Data showing that the homozygous deletion of *Dnmt1* leads to an approximately 70% reduction in 5-methylcytosine content support this hypothesis (9). Interestingly, *Dnmt1* also encodes for an oocyte-specific isoform termed "Dnmt1o," which was initially thought to be involved in the establishment of DNA methylation patterns in oocytes (14). However, studies with *Dnmt1o* null mice demonstrated that Dnmt1o is not required for acquisition of oocyte DNA methylation and that other Dnmt family members may play a role (15).

The observation that embryonic stem cells (ESCs) carrying a *Dnmt1* null allele preserve the ability to acquire new or de novo DNA methylation patterns led to the identification of the DNMT3 subfamily. Unlike Dnmt1, Dnmt3a and Dnmt3b show an equal affinity for hemi- as well as for un-methylated DNA and are thought to be essential for the wave of de novo methylation, which takes place after embryo implantation (16,17). While *Dnmt3b* constitutive Ig-null mice are not viable, *Dnmt3a* constitutive Ig-null mice are runted and die approximately four weeks after birth (9,17). In addition, *Dnmt3a* and *Dnmt3b* double homozygous null mice lack somites and do not undergo embryonic turning, suggesting that their growth and development is arrested shortly after gastrulation. Moreover, ESCs from *Dnmt3a* and *Dnmt3b* double mutant mice completely lack de novo methylation activity, supporting their role as de novo methyltransferases (17). The targeted disruption of *Dnmt3a* and *Dnmt3b* in germ cells by conditional knockout technology demonstrated, however, that only Dnmt3a is required for embryo development, spermatogenesis, and the methylation of both paternally and maternally imprinted loci. Conditional *Dnmt3b* germ line null animals, on the other hand, demonstrate no detectable phenotype. In support of this, Dnmt3a was shown to increase prenatally in the male germ line of the mouse when new methylation patterns were established, whereas Dnmt3b expression was not detected in mouse male germ cells until shortly after birth, suggesting that Dnmt3b may be important for methylation maintenance (18).

Although similar in sequence to Dnmt3a and Dnmt3b, Dnmt3L, another member of the Dnmt3 subfamily, lacks the catalytic domain that is critical for methyltransferase activity. Nevertheless, it has been shown that Dnmt3L functions as a regulatory factor in germ cells where it plays an important role in the establishment of maternal DNA methylation imprints in oogenesis, for instance (19,20). Transcription of the *Dnmt3L* gene can be initiated from one of three different promoters allowing for the regulation of methylation events. While a transcript encoding the full-length protein is produced in prospermatogonia, three short transcripts without open reading frames prevail in late pachytene spermatocytes. Finally, a third promoter, located upstream of those used in male germ cells, is used in growing oocytes,

resulting in the translation of a full-length Dnmt3L. The different promoter regions therefore facilitate the production of full-length Dnmt3L protein exclusively at times of de novo methylation (21).

Interestingly, the phenotype of mice with a deletion in the *Dnmt3L* gene is highly reminiscent of those with *Dnmt3a* germ line mutations, suggesting an interaction between Dnmt3a and Dnmt3L (19,20,22). Indeed, a recent study has identified a complex formed by Dnmt3a and Dnmt3L, in which the central Dnmt3a dimer binds two Dnmt3L monomers. This discovery led to the suggestion that the dimerization of two Dnmt3a proteins might facilitate the methylation of two CpG sites separated by one helical DNA turn (8–10 bp) at the same time. Earlier observations showing that Dnmt3a methylates DNA in a non-processive way can be explained by the large interface between the Dnmt3a complex and DNA (23). This pattern of DNA methylation was suggestive of DNA-sequence specificity, and a subsequent study revealed a remarkable overrepresentation of CG dinucleotides with an eight-base-pair periodicity throughout the genome, providing an optimal substrate for a Dnmt3 dimer (24). Aiming to identify additional mechanisms of genome recognition, it was shown that the interaction between the methylated core histone H3 lysine 4 with the PHD-like domain of Dnmt3L inhibits the interaction of Dnmt3L with DNA. These results demonstrate that the modification of histones is able to control the DNA methylation machinery (25).

DNA Methylation Erasure and De Novo Methylation

Two waves of DNA methylation erasure and subsequent de novo DNA methylation occur during the life of an individual. At the morula stage of early embryogenesis, methylation is almost completely erased, allowing for the reactivation of genes that had been silenced during gametogenesis. Methylation is subsequently reestablished in a wave of de novo methylation, which takes place at the time of implantation (1). While these changes in methylation affect the entire genome, CpG islands appear to be protected by *cis*-acting sequences (26). Following this wave of de novo methylation, the newly established methylation pattern is maintained during subsequent DNA replication. The second wave of DNA methylation erasure solely affects PGCs and prevents epimutations from being passed on to the next generation. At later stages of human development, sex-specific methylation patterns are reestablished during male and female gametogenesis, albeit at different times. Human germ cell specification takes place after the implantation of the embryo and has not therefore been studied in depth, unlike mouse germ cell specification and development. Mouse germ cells can first be identified at 7.2 dpc as an extraembryonic cell cluster that is localized at the base of the allantois. These so-called PGCs are characterized by a somatic-like genomic methylation pattern. They subsequently migrate through the hindgut to the dorsal body wall and finally at 10.5 to 11.5 dpc, arrive at the genital ridges, where the vicinity of the genital ridges appears to induce global loss of DNA methylation. Consequently, the PGCs that colonize the gonads at 12.5 to 13.5 dpc are globally hypomethylated (27). Female germ cells subsequently enter meiosis, which is arrested at birth. De novo methylation in oocytes only occurs later during oocyte maturation, just before ovulation (28). PGCs in males, on the other hand, proliferate until 14.5 dpc, when they enter mitotic G0/G1 arrest. These so-called prospermatogonia undergo de novo methylation from 15.5 dpc until birth when they become spermatogonial stem cells (29).

Genomic Imprinting

Besides being associated with the epigenetic silencing of genes, DNA methylation has been shown to be important for repressing repetitive genomic regions, such as parasitic transposable elements (TEs) and clustered repeats (primarily pericentric satellite DNA) as well as regulating the expression of imprinted loci. Genomic imprinting is the variation in the expression of a subset of genes according to the maternal or paternal origin, which necessitates the presence of both parental genomes for normal development in mammals (30). Imprinted genes are characterized by the presence of differentially methylated regions (DMRs), which contain imprinting control regions (ICRs). While the methylation status of DMRs can change during the course of development, differential methylation of ICRs is maintained following its establishment in germ cells. Imprinted genes mentioned in this chapter are listed in Table 1. The allele-specific expression of imprinted genes is regulated with the help of proteins such as

Table 1

Imprinted genes	Maternally imprinted	Paternally imprinted
ATP10C		X
CDKN1C		X
Gtl2/Meg3		X
H19		X
GTL2		X
Igf2r		X
KCNQ1		X
TSSC5		X
UBE3A		X
Dlk1	X	
IGF2	X	
KCNQ1OTI/KvDMR1/LIT1	X	
MEST/PEG1	X	
PEG3	X	
Peg10	X	
PLAGL1/ZAC	X	
Rasgrf1	X	
SNRPN	X	
SNURF	X	

The X indicates whether the corresponding gene is maternally or paternally imprinted.

CCCTC-binding factor (*CTCF*). The *CTCF* gene encodes an 11-zinc-finger protein that has been shown to solely bind to the ICRs of the unmethylated parental allele where it controls the binding of enhancer elements and prevents DNA methylation of the corresponding region (31).

Establishment of Methylation Patterns in Imprinted Genes

Although the majority of DMRs are methylated on the maternal allele and unmethylated on the paternal allele, paternally imprinted genes have been described as well (32,33). Kerjean et al. investigated the establishment of methylation patterns, focusing on the paternally imprinted gene *H19* and the maternally imprinted *MEST/PEG1* genes during male germ cell differentiation. The findings of the study show that the DMRs of both genes are completely unmethylated in fetal spermatogonia. However, the paternal methylation pattern of the *H19* DMR is partially established in human adult spermatogonia and de novo methylation is completed before meiosis. This process of de novo methylation might therefore be associated with the transition from proliferation to differentiation of spermatogonial stem cells. As expected, the maternally imprinted *MEST/PEG1* DMR remains unmethylated throughout male germ cell differentiation (30). A more recent study has analyzed the methylation status of the DMRs of the paternally imprinted genes *H19*, *Gtl2*, and the maternally imprinted genes *Dlk1* and *Rasgrf1* at different time points during mouse embryo development. The results demonstrated that the methylation of imprinted DMRs occurs gradually after E14.5 and is finally completed in prospermatogonia on postnatal day 0 (29). These studies suggest slight differences concerning the time of methylation establishment during mouse and human germ cell maturation.

Methylation imprints of *H19, Rasgrf1*, and *Gtl2* in the male germ line are partially established during embryonic germ cell development and are completely established in postnatal pachytene spermatocytes (34–36). In the female germ line, however, *Igf2r*, *Snrpn*, *Peg1*, and *Peg3* methylation imprints are not established at the same time but rather in a gene-specific manner (37). Female germ cells undergo meiosis during fetal development and subsequently arrest in the diplotene stage. In the adult, meiosis is then resumed and is followed by oocyte growth and development. During this hormonally regulated maturation process, changes in gene expression take place, leading to changes in Dnmt3 protein levels, which function in the establishment of germ line imprints (17,38). Methylation analysis of the DMRs of *Snrpn*, *Igf2r*, *Mest*, and *Peg3* showed that maternal imprints are established during the nongrowing phase of oocyte maturation and are completely established at the metaphase II stage. While the methylation of imprinted genes takes place during the replication process in

spermatogonia and is only completely established during meiotic prophase, the methylation process in the female is initiated and completed in nondividing oocytes (39). There are indications that hormonal changes during oocyte maturation influence the correct methylation of imprinted genes. It could be shown that oocytes retrieved after superovulation show an increased *H19* methylation and a loss of *PEG1* methylation. Since experiments using the mouse model lead to similar results, these methylation defects can be ascribed to the superstimulation (40).

Association of Impaired Spermatogenesis with Genetic Imprinting Defects

Recent studies indicate that individuals who have been conceived in vitro have an increased likelihood of a loss in methylation patterns and that assisted reproductive technologies (ARTs) may interfere with the correct methylation of key ICRs (41). It has been suggested that the use of testicular spermatozoa and the lack of natural selection during the intracytoplasmic sperm injection (ICSI) procedure might be responsible for methylation defects in the resulting embryo. However, investigation of the methylation status of the paternally imprinted *H19* gene in patients with impaired spermatogenesis revealed that even early germ cells such as spermatogonia and spermatocytes have the same methylation pattern as ejaculated spermatozoa from normozoospermic men (42,43).

In contrast, other studies investigating the methylation status of multiple maternally and paternally imprinted genes found a correlation between poor sperm quality and aberrant DNA methylation levels. Houshdaran et al. analyzed ejaculated spermatozoa from 69 patients seeking infertility treatment for parameters such as sperm concentration, total motile sperm count, and morphology as well as for methylation patterns in repetitive elements, promoter CpG islands, and DMRs of imprinted genes. The results demonstrated elevated methylation levels in samples with poor sperm quality, particularly of the *NTF3*, *MT1A*, *PAX8*, and *PLAGL1* genes (43). Yet, a third study investigated the DMR methylation status of the paternally imprinted genes *H19*, *GTL2* and the maternally imprinted genes *PEG1*, *LIT1*, *ZAC*, *PEG3*, and *SNRPN* in ejaculated sperm of 97 men undergoing infertility treatment. In 14% of the samples, abnormal methylation was found at the *H19* and the *GTL2* locus, whereas 21% showed defects in the maternally imprinted genes. Most methylation errors were detected in the DMR of the maternally imprinted *PEG1* gene. Overall, a higher incidence of methylation defects was present in men with moderate-to-severe oligospermia compared with patients with normal spermatogenesis. These observed methylation defects were constricted to the DMRs of imprinted genes since the global methylation appeared to be normal in the individuals who were tested. Hence, there appears to be an association between abnormal spermatogenesis and an increased methylation of maternal imprints or a disruption in the erasure of methylation imprints in PGCs during fetal development. Consequently, some cases of male factor infertility might have fetal origin (43). Interestingly, it has been demonstrated that superovulation in women is also associated with demethylation of the *PEG1* DMR in superovulated oocytes, suggesting that the DMR of the *PEG1* gene is particularly prone to methylation errors (44).

Association Between Imprinting Disorders and Assisted Reproductive Technology

Since its introduction in 1978, in vitro fertilization (IVF) technology has become a successful treatment for both female and male factor infertility. In 1992, ICSI was introduced as an additional ART, whereby a spermatozoon is directly injected into the oocyte, thus allowing the treatment of couples with low sperm counts. Animal studies as well as follow-up studies on children conceived with the help of ART indicate that ART procedures might lead to an increased risk of imprinting disorders. One factor responsible for this association could be the manipulation of gametes during germ cell maturation, a time that is essential for de novo methylation. ART might also interfere with the maintenance of methylation imprints in preimplantation embryos. Furthermore, it is unknown whether other ART procedures such as ovarian stimulation, IVF, ICSI, and early or late embryo transfers may increase the risk of methylation defects.

Recent findings suggest that there is a higher incidence of the genetic imprinting disorders, Angelman syndrome (AS) and Beckwith-Wiedemann syndrome (BWS), in children conceived in vitro. In the case of BWS, certain studies indicate a 4.2- to 9-fold higher risk in children conceived via ART (45,46). Children with BWS have a predisposition to embryonal tumors, especially Wilm's tumor, and may be affected by fetal and postnatal overgrowth,

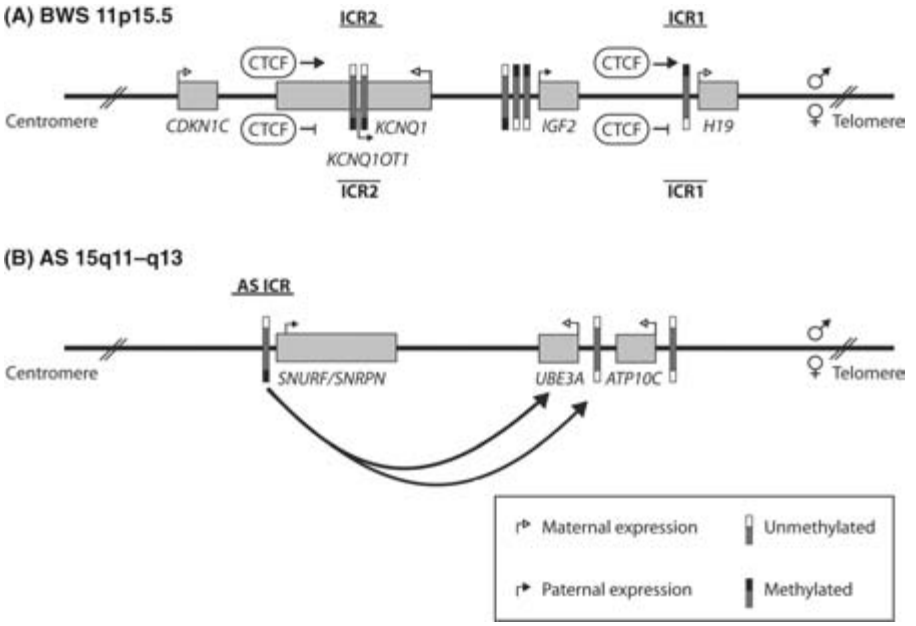

Figure 1 Organization of the genomic regions associated with BWS and AS. (**A**) BWS is associated with methylation defects in chromosome region 11p15.5. This region contains two imprinting domains. ICR1 controls the expression of the maternally imprinted gene *IGF2* and the paternally imprinted gene *H19*. ICR2 controls the expression of the paternally expressed *KCNQ10TI*, the maternally expressed *KCNQ1*, as well as other maternally expressed genes such as *CDKN1C*. Methylation defects in the ICR2 have been associated with BWS. *Abbreviations*: BWS, Beckwith-Wiedemann syndrome; ICR1, imprinting control region 1; ICR2, imprinting control region 2. (**B**) Methylation defects in the genomic region 15q11–q13 have been associated with AS. This region contains the *SNURF/SNRPN* gene as well as the maternally expressed *UBE3A* and *ATP10C* genes. The *SNURF/SNRPN* transcript has been shown to inhibit the expression of UBE3A and ATP10 on the paternal allele as indicated by the black arrows. Loss of the *UBE3A* expression has been associated with AS. *Abbreviation*: AS, Angelman syndrome. *Source*: Modified from Ref. 13.

neonatal hypoglycemia, anterior abdominal wall defects, and renal abnormalities in addition to numerous other characteristics.

Both genetic and epigenetic changes can be the underlying causes of BWS, however, in the majority of cases, BWS is associated with methylation defects in chromosome 11p15.5 (Fig. 1A). This region contains two imprinted domains, the first of which contains the maternally imprinted gene *IGF2* (insulin-like growth factor 2), the paternally imprinted *H19*, as well as the ICR1, which controls the expression of both genes. The second domain contains the paternally expressed *KCNQ1OT1* (*KCNQ1*-overlapping transcript 1), the maternally expressed *KCNQ1* (potassium voltage-gated channel, KQT-like subfamily, member 1), and other maternally expressed genes such as *CDKN1C*. Expression of these genes is controlled by the ICR2. Under normal conditions, the paternal allele of ICR1 and the maternal allele of ICR2 are methylated. Recent data suggest that methylation defects in ICR2 are associated with BWS, while defects in the methylation of ICR1 may be associated with tumorigenesis (47). Geuns et al. demonstrated that the maternally imprinted *KvDMR1* gene located within the ICR2 showed a loss of methylation in patients with BWS who had been conceived using ART (48). These results suggest that the ART procedure either influences the maternal imprint establishment during gametogenesis or the imprint maintenance during early embryo development (49,50).

While the majority of AS cases are associated with a deletion in chromosome 15q11–q13, there are instances of methylation imprint defects in this chromosomal region in AS-diagnosed children (Fig. 1B). AS affects approximately 1 in 15,000 children in the normal population and is characterized by severe mental retardation, delayed motor development, poor balance, and the absence of speech. One study demonstrated that the loss in the expression of the maternally expressed gene *UBE3A* may be associated with AS. *UBE3A* encodes for an

E3 ubiquitin ligase and is solely imprinted in the brain, which may explain the symptoms of AS (51). Analogous to BWS, an association between AS and ART has also been suggested (52,53). Cox et al. demonstrated an abnormal methylation pattern at the *SNRPN* DMR, with either a complete or partial loss of normal maternal allele *SNRPN* methylation (52). The suggestion that ICSI might be a factor in these cases is consistent with the observation that the *SNRPN* methylation imprint is established at fertilization (54). Thus, ART procedures might interfere with the correct methylation pattern of key imprinting control centers and might increase the likelihood of developing genomic imprinting disorders such as AS and BWS.

EPIGENETIC MECHANISMS: HISTONE MODIFICATIONS

A second type of epigenetic mechanism that is important for gene regulation is histone modifications. Histones are the main protein components of chromatin and comprise two classes: core histones and linker histones. The core histones, together with approximately 146 base pairs of tightly wrapped DNA, form repeating units called "nucleosomes." Linker histones connect nucleosomes together and have been implicated in the formation of higher orders of gene repression and chromatin structure. Chromatin organization is further influenced by mechanisms such as ATP-dependent chromatin remodeling and the exchange of histones and histone variants (55).

Core histones are unique in that they have a greater propensity to undergo posttranslational modifications, including methylation, acetylation, phosphorylation, ubiquitination, sumoylation, citrullination, and ADP-ribosylation, which alter their interaction with DNA and nuclear proteins. While the methylation of DNA involves the addition of methyl groups to cytosine bases in genomic DNA, histone methylation is associated with the addition of methyl groups to specific lysine (K) residues. Methylation of certain lysine residues on histones H3 and H4, for instance, leads to a rather compact DNA-protein structure, with the methylation of H3-K9 being a characteristic of heterochromatin. Methylated lysine 9 also serves as a docking site for the heterochromatin protein 1 (HP1), which has the ability to attract methyl transferases (56). This mechanism has been suggested to ensure the histone-modification pattern during DNA replication as well as during the associated assembly of nucleosomes. In contrast, the acetylation of lysine residues on histones H3 and H4 leads to a more open DNA-protein structure.

Chromatin Remodeling During Spermatogenesis

The process of male germ cell maturation from spermatogonial stem cells to mature sperm is called "spermatogenesis" and takes place in the seminiferous tubules of the testis. Spermatogonial stem cells provide the basis of spermatogenesis, since they are characterized by their ability to self-renew and to give rise to differentiating daughter spermatogonia (57). The differentiating daughter spermatogonia undergo meiosis to become primary spermatocytes. Finally, the secondary spermatocytes produce haploid spermatids that differentiate into mature sperm. Once the second meiotic division is completed, complex morphological and biochemical changes take place, resulting in cytoplasm elimination and nuclear reshaping. This process of nuclear reshaping involves the replacement of somatic histones with DNA transition proteins, and subsequently with protamines (58). Following histone removal, the transition proteins, including transition proteins 1 and 2, account for approximately 90% of all chromatin proteins (59). This replacement process seems to be partially regulated by dephosphorylation of the transition proteins, thereby allowing for DNA binding and chromatin condensation (60). However, the replacement of transition proteins with protamines appears to depend on the phosphorylation of transition protein 2, which is associated with a rather open DNA-chromatin structure (60). Protamines, which are comparably small proteins, are characterized by a high arginine content, which facilitates an increased flexibility in the formation of hydrogen bonds, with the DNA backbone allowing for higher chromatin condensation. Protamines 1 and 2 have been described in both mice and humans, demonstrating their equal importance for male fertility. With regard to the transition proteins, phosphorylation of protamines appears to also allow for the incorporation of protamines into DNA (61). The latter replacement leads to a high degree of DNA compaction followed by cytoplasmic ejection as well as acrosome and flagellar formation. During this process of

spermatogenesis, approximately 85% of the nucleosomes of elongating spermatids are first replaced by transition proteins and finally by protamines. These changes in chromatin structure facilitate the dense packaging of the DNA necessary for its transportation to the oocyte (62). In mature human sperm, the following histones have been described: H2A, H2AX, H2AZ, H2B, H3.1, H3.3, CenH3, and H4 (63). Also, a sequence-specific chromatin conformation seems to be conserved between individuals, indicating its importance for the three-dimensional organization of the sperm nucleus and a function in the transcriptional activation of paternal genes in early embryogenesis (64).

Following gamete fusion, intense remodeling of the sperm chromatin takes place. The protamines and potentially the nucleosomes are removed from the DNA and are replaced with maternally derived histone H3.3 to H4 dimers. While histone variants H3.1 and H3.2 are part of the nucleosomes that are assembled during DNA replication, H3.3 is part of nucleosomes that are assembled during times with no DNA replication. Approximately eight hours after insemination of the human zygote, at the beginning of zygotic S-phase, the deposition of maternal H3.1 and H3.2 begins. Consequently, all H3.1 and H3.2 that can be detected in paternal chromatin before the onset of zygotic S-phase must be of paternal origin. Recent studies have shown that nucleosomes containing H3.3 are less stable and are associated with actively transcribed genes, whereas nucleosomes containing H3.1 and H3.2 are associated with silent regions (65–67).

Histone Modifications During Female Gametogenesis

In contrast to mature male germ cells that shed most of their cytoplasm, human oocytes have an enriched cytoplasm. The first division of the primary oocyte leads to the formation of two unequal daughter cells, with the secondary oocyte containing most of the cytoplasm, while the polar body possesses very little cytoplasm. This enriched cytoplasm of the primary oocyte is maintained during the subsequent process of meiosis II.

Changes in chromatin structure appear to influence gene expression during the process of oocyte growth. While acetylation of most lysine residues in H3 and H4 as well as the methylation of H3K4 result in increased transcription, methylation of H3K9 leads to lower transcription levels (68). These findings were contradicted by the observation that even though all of these histone modifications occur during oocyte growth, transcriptional activity is decreased. The acetylation of histones and the methylation of H3K4 might therefore lead to changes in chromatin structure that do not lead to increased transcription but might be essential for achieving meiotic and developmental competence. It was also shown that increased di- and tri-methylation of H3K9 during oocyte growth might play a role in the suppression of gene expression in euchromatin as well as in the formation of heterochromatin, resulting in silencing of the genome. This silencing process might be essential for the repression of transposons during oocyte growth (69).

While phosphorylation is an important regulating mechanism during spermatogenesis, acetylation appears to be the predominant variant of histone modification in oogenesis. Maturing oocytes are characterized by a global decrease in acetylation, presumably leading to reprogramming via the information erasure on active genes. The chromatin composition does not change as dramatically as during the process of male germ cell development. However, as the oocyte develops, the somatic histone H1 is replaced with the maternal variant H1Foo (H1 histone family, oocyte-specific; formerly named "H1oo"), which is heavily polyadenylated and is characterized by a high lysine content. H1Foo might play a role in the process of chromatin condensation and may thereby lead to transcriptional repression. This assumption is supported by data showing that chromatin in mouse oocytes has a high H1Foo content during the period with low transcription levels, whereas H1Foo is removed as soon as the zygotic gene activation takes place (70).

EPIGENETIC MECHANISMS: RNA-MEDIATED SILENCING

The observation that Dnmt3a-Dnmt3L complexes methylate DNA in a non-processive manner suggests that DNA methylation is sequence specific. This specificity might be mediated through small RNAs that are able to direct DNA methylation to target loci. In the germ line,

this class of small RNAs is called "Piwi-interacting RNAs" (piRNAs) as it is Piwi proteins that bind the piRNAs. piRNAs have been identified in *Drosophila*, *Caenorhabditis elegans*, zebra fish, and mammals and differ from microRNAs (miRNAs) and small interfering RNAs (siRNAs) in several ways. Therefore, piRNAs represent a third distinct group of small RNAs (71).

Recent studies suggest that piRNAs are involved in DNA methylation–dependent repression of retrotranspons in fetal male germ cells undergoing de novo methylation. In particular, it was shown that piRNAs isolated from male germ cells during the pachytene stage of meiosis at approximately 14 days postpartum (dpp) consisted of various sequences that were transposable element (TE)-poor and mapped to unobtrusive genomic sequences (72,73). However, piRNAs isolated before approximately 10 dpp were highly rich in TEs, suggesting that the piRNA system provides a germ line defense against TE activity. On the basis of these findings and additional studies, a model concerning the function of piRNAs has been suggested in which Piwi-piRNA complexes have the ability to detect the synthesis of TE transcripts during spermatogenesis. Subsequent to this recognition, Piwi-piRNA complexes are thought to interact with histone-modifying proteins, resulting in the binding of Dnmt3A-Dnmt3L complexes, de novo methylation, and TE silencing (74–76).

Maintenance of Pluripotency and Cell Lineage Determination in Human ESCs

The derivation of hESCs from the inner cell mass (ICM) of blastocysts cultured in vitro was first described by Thomson et al. (77). The resulting pluripotent hESCs are characterized by their ability to proliferate indefinitely and by their potential to differentiate into derivatives of all three embryonic germ layers in vitro. Other characteristics of hESCs include a high nuclear-to-cytoplasmic ratio, elevated telomerase activity, and the ability to form teratomas following the injection into severe combined immunodeficient (SCID) mice (77).

As previously mentioned, hESCs are characterized by their ability to self-renew and differentiate into derivatives of all three embryonic germ layers, both in vitro and in vivo. Recent studies suggest that epigenetic mechanisms may play a role in the regulation of self-renewal and differentiation (78). Bivalent histone modification has been suggested as a mechanism to hold both developmental and lineage-control genes in a transcriptional-permissive status, thereby maintaining ESCs in a pluripotent state. This bivalent status is achieved through repressive H3-K27 methylation and the simultaneous activation of H3-K4 methylation of the transcriptional start site of certain lineage-control genes. Upon differentiation of ESCs to a specific lineage, the repressive histone modifications are removed from corresponding lineage-control genes while the activating modifications remain, resulting in the transcription of essential genes. Only a limited number of lineage-control genes have been shown to be regulated through bivalent histone modifications. It is possible, however, that the majority of lineage-control genes use the same regulating mechanisms, but technical limitations do not allow for their detection (79).

Despite numerous advances in the developmental biology field, the question as to how cell fate is decided within the cells of the ICM at the blastocyst stage remains to be answered. A pulsing model suggests dynamic and asynchronous modifications of chromatin proteins as an explanation for cell fate decisions in ESCs (48). On the basis of this model, pluripotent cells of the blastocyst would be able to respond differently to environmental factors depending on their current histone modification status at a particular developmental or lineage-control gene. The dynamic and asynchronous histone modifications suggested by the pulsing model might therefore be of importance for not only maintaining pluripotency but also for initial cell fate decisions during early embryo development (48). The pulsing model offers interesting ideas on how self-renewal and differentiation might be regulated in pluripotent ESCs. However, further studies are necessary to analyze the mechanisms involved.

Regulation of OCT4 Expression

OCT4/POU5F1 (POU class 5 homeobox 1) is a member of the POU transcription factor family, members of which are characterized by the presence of a POU domain. OCT4 functions as an activator or a repressor of its target genes and has been shown to regulate ESC pluripotency and differentiation (80–82). During early mouse and human embryo development, OCT4

expression can be detected in all blastomeres. At the blastocyst stage, however, OCT4 is highly expressed in the ICM and only at low levels in the TE and primitive endoderm (83,84). Following implantation, Oct4 is inactivated and maintained in this state via DNA methylation of the promoter region (85). As cell differentiation proceeds, Oct4 expression becomes restricted to the developing germ cells (81,86). However, it has been shown that demethylation of the regulatory region and transcriptional activation of the *Oct4* gene occur upon dedifferentiation of somatic cells (87). Thus, key regulators of pluripotency such as *Oct4* need to be reactivated in order to reprogram cells of somatic tissues back to a pluripotent state.

Reprogramming of Somatic Cells to a Pluripotent State

Somatic cells can be reprogrammed to a pluripotent state via somatic cell nuclear transfer (SCNT) in oocytes, fusion with ESCs or, in the case of male germ cells, via culture conditions alone. In addition, factor-based reprogramming has been successfully used to generate induced pluripotent stem (iPS) cells with ESC characteristics from mouse as well as from human fibroblast cells (88–90). The latter two mechanisms of reprogramming allow the generation of patient-specific pluripotent cell lines without the ethical issues associated with SCNT and ESC research.

SOMATIC CELL NUCLEAR TRANSFER

SCNT has been applied in recent years to reprogram somatic cells to a pluripotent state. While SCNT has been successfully used in mammals, human reproductive cloning has not been achieved thus far. One explanation for this might be the incomplete epigenetic reprogramming of the donor nucleus following injection into the enucleated oocyte. Somatic cells have specific epigenetic modifications, which are maintained during cell divisions. Germ cells and the early embryo, on the other hand, undergo genome-wide epigenetic reprogramming, a process essential for the gain of developmental potential (91). Following normal fertilization, reprogramming of DNA methylation and histone modifications occurs. This process, however, appears to be impaired during SCNT since a change in DNA methyltransferase expression, abnormal methylation patterns of imprinted genes, and an incomplete demethylation of the *Oct4* promoter have been demonstrated in SCNT-derived embryos (92,93). Interestingly, a study comparing epigenetic reprogramming of global DNA methylation and histone H3-K9 acetylation in SCNT-cloned and IVF rhesus monkey embryos demonstrated that SCNT embryos are not completely reprogrammed. In particular, SCNT embryos displayed higher DNA methylation levels at the two-cell stage as well as at the eight-cell stage. Moreover, at the eight-cell stage, H3-K9 acetylation levels were decreased in SCNT embryos compared with IVF embryos. This increased level of DNA methylation in SCNT-cloned embryos was also detected at the blastocyst stage, suggesting that reprogramming is not completed in SCNT embryos (94). Since global epigenetic reprogramming is essential for normal development and successful cloning, these results might explain the lack of or the tremendously low efficiency of blastocyst formation achieved after SCNT in humans and nonhuman primates, respectively.

ESC-like Properties of Male Germ Cells

Recent studies have demonstrated that spermatogonial stem cells isolated from adult mouse testes can be propagated in vitro and acquire embryonic stem cell (ESC) properties under certain culture conditions. The pluripotency of these cells was determined by their ability to spontaneously differentiate into derivatives of the three primary germ layers in vitro and form teratomas after injection into SCID/beige mice. In addition, their developmental potential was investigated by injecting the spermatogonial stem cells into 3.5-day-old blastocysts, which were subsequently transferred into the uterus of pseudopregnant mice and resulted in the birth of several chimeric animals (95). Imprinting pattern analysis of the reprogrammed ESC-like cells was performed by investigating the methylation status of the *Oct4* promoter and the DMRs of the paternally imprinted genes, *H19*, *Meg3*, *IG*, and *Igf2r* and of the maternally imprinted genes, *Rasgrf1* and *Peg10*. The ESC-like cells were hypomethylated at the DMR of the *Oct4* promoter, demonstrating their undifferentiated state. Moreover, the paternally methylated DMRs showed diverse levels of methylation, whereas the maternally imprinted

DMRs were predominantly unmethylated. In contrast, while ESCs displayed higher levels of methylation in both maternally and paternally imprinted DMRs, male germ stem (GS) cells were characterized by an androgenetic imprinting pattern, with methylation of the paternally imprinted DMRs and demethylation of the maternally imprinted DMRs. Therefore, pluripotency appears to be a shared characteristic of hESCs, the epiblast, PGCs, and, under certain conditions, of spermatogonial stem cells (77,96).

Generation of Induced Pluripotent Stem Cells

Recently, factor-based reprogramming has been successfully used to generate induced iPSCs with ESC characteristics from both human and mouse somatic cells (88–90). Initially, a selection of genes known to function in the establishment or maintenance of pluripotency, including *OCT4*, *SOX2*, *KLF4*, *MYC* and *OCT4*, *SOX2*, *NANOG*, and *LIN28*, respectively, were cloned into lentiviral or retroviral vectors (88–90). Transgene-expressing lentivirus/retroviruses were then used to transduce human somatic cells, resulting in the formation of ESC-like colonies 12 to 25 days later. These so-called iPSCs are characterized by their ESC-like morphology and the expression of the pluripotency markers, TRA-1-60, TRA-1-81, SSEA3, SSEA4, OCT4, NANOG, and alkaline phosphatase. In addition, iPSCs show high telomerase activity and have the potential to give rise to derivatives of the three primary germ layers as demonstrated by embroid body and teratoma formation. As demonstrated by SCNT experiments, cell differentiation depends on epigenetic rather than genetic changes and can, therefore, be reversed. This process of reprogramming involves the promoter demethylation of pluripotency genes such as *OCT4*. Thus, the methylation pattern of the *OCT4* promoter was investigated in iPSCs and found to be demethylated, which is analogous to hESCs. Patient-specific iPSCs are valuable as they facilitate the investigation of disease mechanisms and the screening of drugs, while avoiding the use of human embryos. However, before iPSCs can be used for clinical applications, reprogramming mechanisms using nonintegrating virus, thereby avoiding harmful genetic modifications, need to be established.

SUMMARY AND CONCLUSIONS

Epigenetic modifications of the genome are essential for the regulation of diverse cellular processes, including germ cell formation. In this chapter, the mechanisms and enzymes involved in de novo DNA methylation and DNA methylation maintenance, RNA-mediated silencing, and histone modifications were reviewed. The importance of the correct methylation pattern for normal cellular function was demonstrated by the association of aberrant DNA methylation with human diseases. In addition, the successful reprogramming of somatic cells to a pluripotent state was shown to depend on epigenetic reprogramming. Future studies will lead to a more detailed understanding of epigenetic gene regulation, thereby providing information about the etiology of diseases associated with methylation defects, as well as about the necessary epigenetic changes required for somatic cell reprogramming.

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6 Germ Line Stem Cells and Adult Ovarian Function

Roger Gosden, Evelyn Telfer, and Malcolm Faddy

THE ZUCKERMAN THEORY

One of the defining moments in the history of reproductive biology was in 1950 at the Laurentian Hormone Conference held in Franconia, New Hampshire. Probably the audience, and possibly even the keynote speaker, did not realize that a foundation stone was being laid, which would profoundly affect the course of scientific endeavor. Solly Zuckerman, a British primatologist from the University of Birmingham (Fig. 1A), presented a paper with a deceptively simple title, "The number of oocytes in the mature ovary," which appeared in print the following year (1). He argued, contrary to the popular view of his time, that mammalian oocytes are only formed before birth and cannot be replenished afterward, a theory that has held sway ever since and has only recently received its first serious challenge. His theory posits that the early fixing of the oocyte population sets an upper limit on the potential number of ovulations and the length of the reproductive life span and hence has become central to our understanding of the biology of menopause. Moreover, the longevity of oocytes in the late reproductive years provides a plausible explanation for the dramatic effects of ageing on oocyte quality, at least in human ovaries, namely, accumulated effects of reactive oxygen species and other harmful factors. Thus, the Zuckerman theory has had tremendous explanatory power for ovarian physiology as well as being a major influence in human and animal medicine.

Zuckerman was not however the originator of this theory, though he did provide a more robust basis than his predecessors. A German anatomist-embryologist, Waldeyer (Fig. 1B), is usually credited with being the first to propose that oocytes are only created before birth, and then from the ovarian surface epithelium that was called (we now believe erroneously) the "germinal epithelium" (2). As the author of the neuron theory and other major scientific contributions, Waldeyer's views on oogenesis were authoritative, though some of his contemporaries held that oocytes were renewable, like spermatozoa in testes, and this alternative theory gained popularity after the 19th century. Primitive germ cells had already been described migrating along the embryonic gut to the gonadal anlagen, an interpretation turning out to be substantially correct (3) and consistent with an early onset of oogenesis. But there were doubts whether this first wave of germ cells persisted after birth, and Brambell (4) suggested another population partly or wholly replaced it for adult fertility. Such confusion is understandable in light of the enormous wastage of follicles occurring by atresia during the postnatal period.

Allen (5), attempting to link ovarian anatomy with its physiology, showed cyclical follicle growth and degeneration occurred in tandem with the mouse estrous cycle using the then new method of monitoring by vaginal lavage. Of the four stages in the cycle, he noted most mitotic activity occurred around the time of estrus (when estrogen levels are high) and "young ova" appeared a few days later at diestrus under the surface epithelium. Chromosomal synapsis during zygotene-pachytene of meiosis is a highly conspicuous and germ cell–specific transition that lasts several days in fetal mice (6) (Fig. 2), so it is surprising that its absence after birth did not upset Allen's belief in formation of oocytes in adult ovaries. A speculative explanation was offered a few years later by Evans and Swezy (7) who suggested that meiosis might be modified in adult ovaries. They thought the entire life span of oocytes from their emergence to ovulation or demise in atretic follicles occurred during the span of a single four- to six-day estrous cycle, whereas we now believe a primordial follicle takes about three weeks to grow to the Graafian stage (8). Gregory Pincus, who later pioneered contraceptive steroids, summed up the general consensus in 1936 as follows: "all our recent knowledge . . . militates against the assumption of a single large initial store of ova gradually being exhausted throughout sexual maturity" (9).

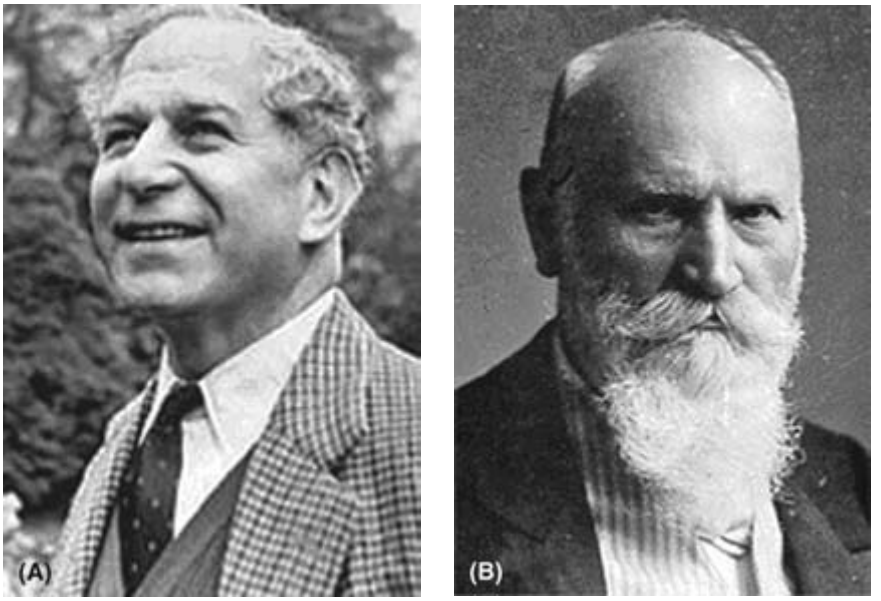

Figure 1 (**A**) Lord Solly Zuckerman (1904–1993), (**B**) Heinrich Wilhelm Gottfried von Waldeyer-Hartz (1836–1921).

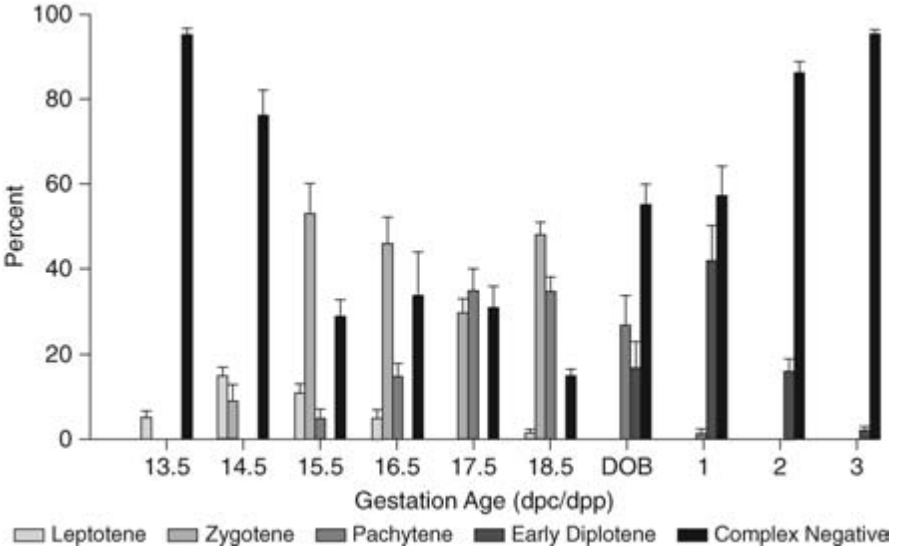

Figure 2 In mouse ovaries germ cells enter meiosis fairly synchronously between days 13 to 16 of pregnancy, reaching diplotene by birth (day 20) and undergoing no further nuclear maturation unless they grow to full size in a follicle (6).

Zuckerman was therefore bucking the view of many of his senior peers when he claimed that Waldeyer was correct after all. He succeeded by marshalling arguments from different experimental quarters and systematically analyzing the older papers that had often not taken into account the pool of primordial follicles (10–12). Zuckerman's conclusion was quickly adopted and developed by many researchers (13–15) who showed by morphology and DNA labeling that oogenesis was confined to fetal ages in mice. Other lines of evidence also supported oogenesis limited to fetal life including dynamic studies of follicle growth based on mathematical models (16,17). The idea of a fixed population became firmly entrenched in dogma until it received its first serious challenge in 2004.

The decline in follicle numbers found by Zuckerman and his colleagues was continuous and approximately exponential with respect to age. Some of the older studies were misleading because they omitted the tiny primordial follicles representing 90% of the total, and this oversight led the authors to conclude that new oocytes were being formed because the numbers of larger growing follicles varied during estrous or menstrual cycles. Besides, none of the early studies provided reliable estimates of follicle degeneration, which was a precarious basis on which some claimed that high atresia rates supported a mechanism of follicle replacement (18). In truth, Zuckerman could not rule out this possibility, though his data were entirely consistent with continuous loss of oocytes from a fixed stock, like an hourglass. His findings that the oocyte population is both age dependent and cycle independent have been repeatedly confirmed in a wide range of species, including human (19,20), monkey (21), and mouse (22). Zuckerman argued that if oocytes were rapidly turning over and were replaced from stem cells at the ovarian surface, the absence of an epithelium in ovarian grafts or its destruction should precipitate sterility. However, after bathing in corrosive chemicals (23,24) or X-irradiation (25), the number of oocytes remained unchanged in accordance with his theory. More subtle experiments using doses of radiation high enough to damage germ cells but too low to affect somatic cells have been shown to lead to lifelong infertility (26,27). Such experiments seemed to rule out a role of the surface epithelium for oogenesis.

It was already well known that when one ovary is surgically removed, its remaining partner undergoes compensatory hypertrophy because the declining output of ovarian hormones triggers an increase in pituitary gonadotrophin secretion. Zuckerman (1) pointed out that there is no corresponding rise in oocyte numbers after hemi-ovariectomy, the greater mass being entirely accounted for by multiplication of somatic cells (28). The older literature harbors a number of reports of ovarian tissue regeneration after bilateral ovariectomy, sometimes called "ovarian remnant syndrome," but there is little doubt that the results were due to conservative surgery in which the hilus was ligated. Ovarian regeneration from incomplete ovariectomy is a well-known problem in chickens, and in mammals too a more radical operation is often needed to guarantee a profound and permanent hypoestrogenism, e.g., by also removing the tubes.

Zuckerman realized that if early meiotic stages existed in adult ovaries, these would be key indicators of neo-oogenesis, but they had never been observed and he conceded only one exception to the rule, and that a qualified one. Oogonial stem cells are present and can undergo replication in mature prosimians (lemurs and bush babies), but they never differentiated further (29). Summing up he said, "the evidence, both histological and experimental, implies that the view that oogenesis continues throughout reproductive life is very insecurely based" (1). During the discussion after his paper, the audience, which included Gregory Pincus, did not demur from this conclusion, which quickly became absorbed into the corpus of ovarian biology.

GERM LINE STEM CELLS IN ADULT OVARIES—BLIND ALLEY OR NEW PARADIGM?

The scientific community therefore received a jolt when a group led by Jonathan Tilly at Harvard University recently claimed in two high impact journals that oocytes are continually being renewed throughout adult life (30,31). This news claiming a scientific revolution quickly attracted attention from the media, and *The New York Times* for one proclaimed: "Study of Mice Reproduction Discovers Egg Regeneration—Data Challenges a Central Idea of Biology." In fact, the Bukovsky group in Tennessee had already made an earlier challenge to the conventional doctrine, but based on a different mechanism, and it had received little attention at the time (32,33). After 2004, a struggle began between two sides of the debate (34–42) and only four years later did it begin to settle.

Commentators wondered if the conventional doctrine about oogenesis was wrong, and why so many distinguished biologists over several decades had been misguided. According to Peter Medawar, "What shows a theory to be inadequate or mistaken is not, as a rule, the discovery of a mistake in the information that led us to propound it; more often it is the contradictory evidence of a new observation which we were led to make *because* we held that theory" (43). He adhered to Karl Popper's principle of empirical falsification as opposed to the classical inductive philosophy of science, which would interpret scientific error as a

misapprehension of the facts. The controversy in ovarian biology is an interesting case study in itself and one we believe is consistent with both philosophies: the challenge to convention arose from a conflicting observation but at the same time interpretations of some of the new data may have been mistaken.

NUMBERS OF OOCYTES AND FOLLICLES

In an interview, the leader of the Harvard group reported his doubts about the conventional theory of oocyte formation, which began to surface when his laboratory was counting follicles in C57BL/6 mouse ovaries. They found stocks of 2500 to 5000 healthy follicles in immature ovaries from different strains, much the same as other groups had found, but they noted the numbers of atretic follicles rose dramatically to 1200 per ovary and remained high for several months after puberty (30). Since atretic follicles are cleared within a few days (44), they concluded that the high rate of wastage was being compensated by new follicles from germ line stem cells (GSCs). This suggestion harked back to literature of more than half a century ago, but their data were much more solid. Besides, they recognized the implication for ovarian ageing and menopause, which, if new oocytes were forming, would be explained by progressive exhaustion of stem cell activity rather than by wastage of a limited follicle reserve.

Byskov et al. (38) have pointed out the problems of numerically comparing mean follicle counts at a given age and actual turnover, partly because atresia can only be defined subjectively and the Harvard group had found higher rates of atresia than most other studies. Nevertheless, based on differences in counts between 16 and 40 days after birth, the follicle depletion rate of another mouse strain (17), and a rather circular argument (30), they estimated that 77 new follicles were formed every day in young mice (although this estimate is rather imprecise with a large standard error of 30). This deduction seemed to draw support from data in their study and an independent report showing an apparent stabilization or even slight increase in follicle number at certain ages.

However, the actual data for both total and primordial follicles in C57BL/6 mice in Figure 1A of Johnson et al. (30) are entirely consistent with a *decline* in mean follicle numbers with increasing age postpartum. A statistical test for goodness of fit of a linear regression model does not indicate any significant lack of fit, the p-value being almost 0.50 for total follicle numbers (Fig. 3A). Thus, there are no statistical grounds for doubting that mean follicle numbers decline with increasing age, which we estimate from their data at 48 ± 18 follicles per day. There was an observed mean increase of about 1000 follicles between days 4 and 8, but the standard error of this estimate is rather large (920), so this change is not significant ($p > 0.10$). Similar inferences can be drawn for other apparent "increases" in mean numbers of total or primordial follicle with increasing age in the same data, including the observed 20% rise for a different strain (AKR/J) between days 4 and 42 in which the estimated mean increase in toto was about 500 with a standard error of 580 ($p = 0.20$). In other words, there was no statistical basis, at least from these data, for inferring anything other than a decrease in mean follicle numbers associated with increasing age, in agreement with numerous classical studies. Bristol-Gould et al. (45) reached a similar conclusion from their own data and from testing a mathematical model in which recruitment of new follicles failed to adequately describe their data.

In another study of the same strain of mice, Kerr et al. (46) reported that the decline with age in mean follicle numbers was interrupted, leading them to argue that their data "provide qualified support for an as yet unknown mechanism for follicle renewal." But in fact these data are quite uninformative about the relationship between number and age and provide no confirmation of a hypothetical lack of change in mean numbers with respect to age. Linear regression analysis of the data showed total follicle numbers declined at an estimated mean rate of 2.5 per day over 7 to 100 days (Fig. 3B), but the standard error of this estimate was 3.8, so the data are consistent with all three possible hypotheses, namely, declining or constant or increasing mean numbers! Furthermore, the low replication levels of 3 to 5 used in the studies by Johnson et al. (30) and Kerr et al. (46) led to these studies having very low statistical power because of the very high variation in age-specific follicle numbers; something of the order of 10 times as many replicates would be needed at each age to reach more informative inferences or even to discriminate between a simple linear and the more realistic exponential decline.

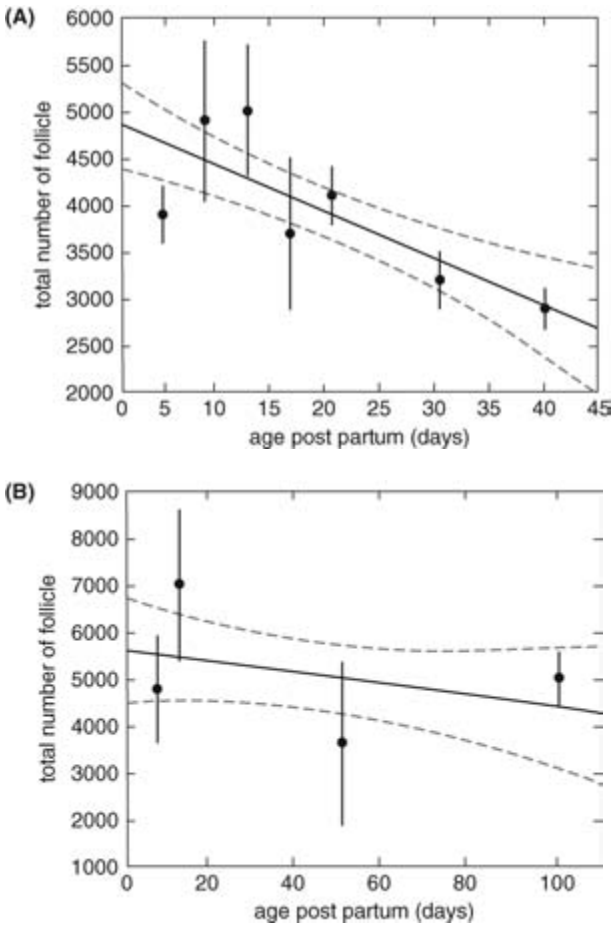

Figure 3 Mean total numbers of follicles (●) ± SE (vertical bars) in mouse ovaries at different ages with linear regression (——) ± SE (- - -) fitted to data from (**A**) Johnson et al. (30) and (**B**) Kerr et al. (46).

While the absence of evidence of new follicle production is not logically the same as evidence of absence of new follicle production, we have noted above that the inverse relationship between follicle numbers and age has been remarkably consistent across many studies *as if* no new follicles were being produced, and just as Zuckerman pointed out from the limited sets of data then available. However, the debate about oocyte renewal cannot be settled purely from counts of healthy and atretic follicles, and its chief virtue has been to stimulate more critical discussion and efforts to increase the wealth and quality of experimental data.

POTENTIAL SOURCE(S) OF POSTNATAL GERM LINE STEM CELLS

Johnson et al. (30) described cells embedded in the surface epithelium of postnatal mouse ovaries that appeared as candidate GSCs based on techniques that were not available in Zuckerman's day. Occasional spheroidal cells among the majority of cuboidal ones expressed *Mvh*, and some of these had incorporated the thymidine analogue, *BrdU*, which implied they were mitotically active oogonia (GSCs). This gene is specific to germ cells and strongly expressed at all stages of oocyte development in the adult mouse ovary (Fig. 4). Furthermore, several genes known to have a role in early meiosis (*Dmc1*, *Spo11*, and *Scp3*) were also expressed in the same ovaries, and levels of mRNA for the synaptonemal protein *Scp3* were remarkably high (a quarter of those in adult testes). Collectively, these findings indicated that all the early stages of oogenesis found in fetal ovaries were being recapitulated after birth and, moreover, Johnson and colleagues estimated that there were about 63 of these candidate GSCs corresponding roughly to their estimate of daily production of 77 new follicles.

Germ cells have been reported in the surface epithelium earlier, though a different interpretation was given. They are rarely found after puberty in mice and have disappeared by

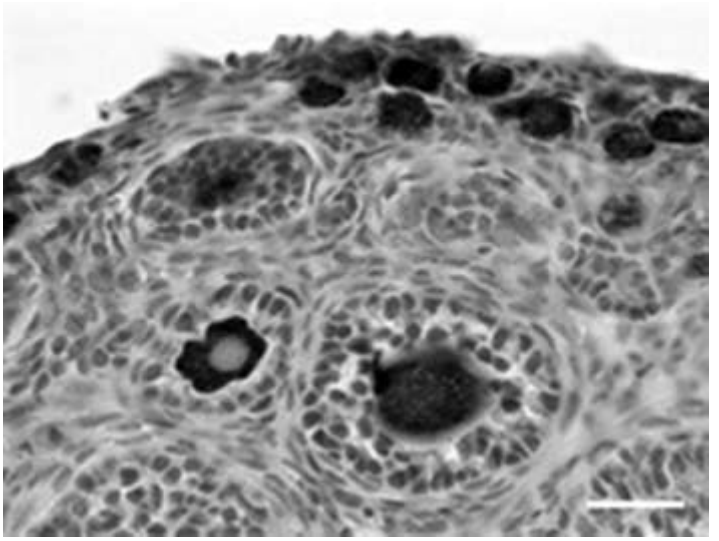

Figure 4 Immature mouse ovary showing expression of the germ line marker, Mvh, in oocytes of primordial follicles under the ovarian surface as well as growing follicles in the medulla. *Source*: Courtesy of Dr Katia Manova (bar = 50 μm).

birth in humans. Ultrastructural studies suggested that they are either premeiotic or meiotic germ cells crossing the surface epithelium and perishing in the peritoneum after leaving the ovary (47). Apparently for this reason Johnson et al. (31) withdrew a hypothetical role for ovarian germ cells in favor of a novel reservoir of GSCs in adult bone marrow, which continuously repopulates the ovary (31). This explanation neatly accounted for the absence of early meiotic stages and also for the remarkably rapid replacement of follicles they had observed after treating mice with an ovotoxic drug.

When Johnson et al. used the anticancer drug doxorubicin to destroy follicles, there was such a prompt rebound in numbers that they reported "adult mouse ovaries can produce hundreds of new oocytes within 24 hours." In two months, the population was restored to that of vehicle-injected controls. This very surprising result is at odds with many old studies showing that chemical- or radiation-induced sterilization is irreversible in female mice (48,49) as well as contrary to much clinical oncology experience that high-dose chemotherapy or total body irradiation causes permanent sterility (50). Even accepting the possibility of regeneration, this was still an astonishing rate of recovery because generation of small follicles from GSCs normally takes more than a week in fetal ovaries, but if germ cells started development elsewhere before migrating to the ovary, this objection is vitiated.

In searching for extrinsic sites of germ cell production, the investigators were guided by the knowledge that hematopoietic and germ line stem cells share a common environment at early stages of development after they emerge in the proximal epiblast (51). Several experimental strategies were designed to test the hypothesis that adult GSCs originate in bone marrow and are then carried in the bloodstream to the ovaries. In support of their hypothesis, *Oct4*, *Mvh*, *Dazl*, *Stella*, *Fragilis*, and *Nobox* were expressed in bone marrow, though it is open to debate whether these genes should all be considered strictly as germ cell markers rather than stem cell markers. And as Jaenisch and Young (52) point out, expression of molecular markers of pluripotency or differentiation is not a stringent test for functionality as it can be caused by cellular stress. Meiosis and, above all, generation of live offspring after fertilization are far higher in the hierarchy of criteria for germ cell identification, but neither has yet been confirmed from a bone marrow precursor. Nevertheless, the molecular evidence was echoing results of a number of concurrent studies showing oocyte-like cells being generated in vitro from embryo stem cells (53) and even from unexpected sources like fetal skin (54). Moreover, there was provocative evidence of variation in *Mvh* expression in bone marrow and peripheral blood during the mouse estrous cycle, which peak at met-estrus, suggesting that a hormonal feedback loop exists between the ovary and bone marrow (31). But

since the majority of cells with a molecular germ cell signature were negative for *Sca-1*, germ cells were evidently not being derived from hematopoietic stem cells.

Transplantation provided a more rigorous experimental test of a bone marrow site for oogenesis. After semi-sterilizing mice using busulphan and cyclophosphamide, bone marrow transplantation (BMT) restored follicle numbers though not to pretreatment levels (31). Marrow from male donors was predictably ineffective. Even more compelling evidence was obtained by injecting nucleated peripheral blood cells from donors expressing green fluorescent protein (GFP) from an *Oct4* promoter. The recipient mice were congenitally sterile as a result of the *Atm* mutation but, following for several months after BMT, the ovaries contained growing follicles enclosing GFP-positive oocytes according to immunohistochemistry (31). Unfortunately, the abundance of these oocytes was not reported, nor in another striking experiment that generated chimeric follicles (30). In the latter, ovarian tissue was grafted from wild-type mice into the ovarian capsules of recipient animals, ubiquitously expressing GFP transgene from an actin promoter. A few weeks later, GFP-positive oocytes of the host were found inside a GFP-negative envelope of granulosa cells of the donor, which was attributed to ongoing oogenesis.

At first sight, the experiments reported by Johnson et al. (30,31) provide seemingly watertight evidence for the formation of new follicles, but most of them are open to alternative interpretations, including the apparently robust interpretation of chimeric follicles. The mouse ovary is a plastic organ that can be disaggregated into single follicles or even into cell suspension and afterward recombined to reform functional follicles (55). Thus, there is a possibility that damaged primordial follicles at the graft interface had intermingled to create follicles with combined genotype. Such a chance event is likely to be much rarer than the hypothetical production of new follicles from stem cells, so it is regrettable that the incidence of chimeric follicles was not reported.

The published debate has focused on formation of new oocytes but to survive and function they need to interact with granulosa cells, a fact so obvious that the debate has been almost completely overlooked. Interestingly, the chimeric follicles described by Johnson et al. imply that although oocytes are derived from external precursor cells the granulosa cells originate in the ovary (30). During normal development, granulosa cells are thought to derive from stem cell–like progenitors in the rete system of the fetal ovary (56). We are unaware of studies demonstrating that these somatic cell progenitors persist after birth or that mature granulosa or luteal cells can dedifferentiate to recreate them, though ovarian regeneration depends on a source of new granulosa cells. This knowledge gap can be addressed by experimentally testing whether GSCs from fetal ovaries can complete full development to oocytes after introducing them into adult ovaries, using genetic markers to trace the lineage of granulosa cells in any newly formed follicles. If these follicles were absent and the transferred germ cells died, there would be more doubt about ovarian regeneration, but if the experiment succeeded this test could serve as quality control of the efficiency of regeneration.

Perhaps the single most significant gap is the lack of direct evidence of fertile oocytes from donor somatic cells. According to this most stringent test, green fluorescent oocytes should be ovulated from wild-type recipients after BMT or peripheral blood transfer from transgenic donors, or wild-type oocytes from the reciprocal experiment. An independent group tested this prediction using both BMT and parabiosis between immunologically compatible strains of wild-type and GFP-expressing mice (57). A common circulation is formed in two to three days between pairs of parabiotic mice, and blood chimerism quickly approaches 50:50 with cross-engraftment of bone marrow. A conditioning protocol of busulphan and cyclophosphamide/X-irradiation was used to deplete the ovary and create "niches" for new oocytes to form, just as Johnson et al. (31) had done. Eggan et al. (57) superovulated animals either two weeks or two months after BMT or parabiosis, but the genotype of oocytes remained true to the animal of origin, i.e., wild-type mice generated only non-GFP oocytes and GFP-mice produced only GFP oocytes. This study provided some of the clearest evidence that circulating cells cannot generate ovulatory oocytes in adult animals.

In a new study, the Tilly group revised their hypothesis based on similar findings. After BMT to semi-sterilized animals, Lee et al. (58) confirmed oocytes of donor origin in small follicles of recipient ovaries, though the proportion was low (1–2%). These structures were clearly different to the donor-derived cells expressing CD45 found in recipient ovaries by

Eggan et al. (57) who presumed that the cells were committed leucocytes. The fertility of the recipients given busulphan and cyclophosphamide was impaired, but BMT significantly boosted their fertility, especially when given soon after treatment with the anticancer drugs (58). The mechanism of this improvement is obscure, assuming it was not a nonspecific effect on general health, but the authors favored the explanation that the profertility effects of BMT were mediated by the new follicles, which, although incompetent to ovulate, helped residual oocytes to realize their potential. In a recent study, wild-type ovaries were transplanted under the kidney capsule or into the evacuated ovarian capsule of a strain of mice expressing GFP under a histone promoter. Begum et al. (59) examined over 800 follicles in these grafts up to eight weeks, and mostly at the primordial stage, but did not find a single case of an oocyte exhibiting green fluorescence, which would be expected if new oocytes had formed.

The balance of evidence in our opinion rests solidly in favor of Zuckerman's theory, since the alternative hypothesis depends on claims advanced by mainly one group in recent times, and independent studies have failed to confirm them.

AFTERTHOUGHTS

Before ending, it is helpful to consider whether any other studies not designed to test oocyte formation in adult ovaries can enlighten the debate.

The mechanisms that regulate the utilization of primordial follicles from a "resting" pool are largely unknown, and recent studies using knockout mice have shed light on the regulatory mechanisms as well as providing evidence against the regeneration theory. One such study was based on *FoxO3*, a member of the evolutionarily conserved forkhead transcription factor family of genes involved in longevity, metabolism, and reproduction. When this gene was deleted in mice, the animals were born with normal ovaries and remained healthy but their ovaries ran out of oocytes by 18 weeks (60,61). A similar phenotype has been described for *Pten* deletion (62). Evidently, the phosphoinositol-3-kinase pathway is required to maintain inhibitory tone on the resting pool of primordial follicles because its disruption had triggered all the small oocytes to grow prematurely, leading to their early death. Unless it is posited that GSCs are unaffected in fetal ovaries but not in adults, the permanent sterility of these model animals provides strong evidence of the lack of ovarian regeneration in adults.

In another study, Haas et al. (63) showed that the C57BL/6 ovary is highly sensitive to the reproductive toxicant 4-vinylcyclohexene diepoxide, follicles being completely destroyed six weeks posttreatment. This damage too became permanent and the lack of follicle renewal was unlikely to be due to destruction of any GSCs because spermatogonial stem cells in testes survived even larger doses of the same compound (64).

In addition to these genetic and toxicological studies, Hirshfield (65) provided unintentional corroboration of the conventional theory while measuring follicle growth in rats. She slowly infused tritiated thymidine from osmotic minipumps into adult females for up to seven days using autoradiography to detect labeled granulosa and stroma cell nuclei, but never found any labeled oocyte nuclei (AN Hirshfield, personal communication). This observation was consistent with older studies showing the final S-phase in mouse oogenesis occurs during the third week of gestation (13).

The case against ovarian regeneration is largely based on negative findings (necessarily so) that are more open to criticism and alternative interpretations than positive results. Yet the volume and quality of the studies are now so overwhelming and consistent that no reasonable doubt remains in our minds that the lifetime supply of oocytes is formed before birth. It is human nature of course for investigators who have committed time and resources to a scientific venture to be tempted to resist challenges that could undermine a fundamental premise, and this is true for ourselves as biologists and statisticians for whom Zuckerman's theory has been a foundation. Yet it is also true that one of the greatest joys of the scientific enterprise is to witness, and preferably participate in, discoveries that open fresh opportunities for research, especially where there are promising applications in human and animal health. The concept of ovarian renewal is enthralling as it suggests new strategies for combating ovarian ageing, reversing menopause, and even generating oocytes for research. The likely reality though is an inexorable decline in the follicle reserve, which presents a continuing challenge to find ways of maximizing the value of a limited supply of oocytes and identifying the most competent gametes for assisted reproductive technologies.

Up to this point, the arguments have been based mainly on studies in rodents. Extrapolation between species can be risky but in the closing section we find evidence from humans and primate models leading to the same conclusions.

GERM LINE STEM CELLS AND ADULT HUMAN OVARIAN FUNCTION

The experimental limitations on research in humans have left more room for speculation about oogenesis than in animals, although the embryology and physiology are broadly similar even if the time courses are distinctive. In humans, large numbers of oocytes in meiotic prophase have appeared by the end of the first trimester of pregnancy, and at seven months mitotic germ cells and most prediplotene oocytes have disappeared with the remaining germ cells incorporating into follicles, some of which start to grow (66,67). The total number of germ cells peaks at five months but afterward degeneration dominates over replacement and the number of healthy cells declines from nearly seven million to about one million at birth, and the losses continue afterward until the last follicles disappear soon after the menopause (19). While a rare stem cell might be overlooked in an adult human ovary, it is unlikely that large pachytene oocytes have gone unnoticed by the countless pathologists and biologists who have studied ovarian histology over the decades.

Molecular evidence too is consistent with a lack of oogenesis. Liu et al. (68) studying premenopausal ovaries aged 28 to 53 years could not detect mRNAs for several genes associated with germ cells/stem cells/early meiosis or proliferating germ cells, whereas all of these were present as expected in fetal ovaries. Some studies have however struck a discordant note based on immunohistochemistry. Stem cell markers were recently reported by Virant-Klun et al. (69) in the ovarian surface epithelium, including *OCT4*, *NANOG*, *c-KIT*, and the germ cell–specific marker, *VASA* (an orthologue of *Mvh* in mice), the results being more remarkable from postmenopausal ovaries. Scrapings of the ovarian surface produced cells after extended culture that grew to 95 μm in diameter, reinforcing their suspicion that ovarian epithelial cells had a "primordial germ cell ancestry."

These findings extended an earlier, substantial study by Bukovsky et al. (32) who also found large "oocyte-like" cells after culture bearing a superficial morphological resemblance to healthy human oocytes but expressing zona pellucida proteins normally associated with growing oocytes, as well as a gene involved in meiotic recombination *SCP3* (70). The latter gene may not however be strictly germ cell specific (68) and was absent in the other study (69). But based on immunohistochemistry evidence of cytokeratins and immune regulators, they proposed a mesenchymal origin for new germ cells *and* granulosa cells in the surface epithelium providing oocytes for the reproductive years up to the last decade before the menopause. According to this hypothesis, residual oocytes subsequently accumulate damage, rendering them less fertile and subject to aneuploidy (71). While this hypothesis satisfies the need to explain the origin of both cell types, it rests precariously on limited data of a descriptive character.

It is likely that resistance toward Zuckerman's theory for humans will persist longer than for animals because there are fewer experimental tools available. There is also more room for controversy because the standards of evidence are lower. For example, it has been argued that occasional reversals of sterility in patients after chemotherapy may be due to formation of new oocytes owing to a weak compensatory response of the kind described by Lee et al. (58) in mice. As yet only one informative clinical case has been reported and that after an allogeneic bone marrow transplant in a patient who delivered a baby genetically related to her birth mother rather than the bone marrow donor (72). The simplest explanation is often best (Occam's razor), and is in this case the survival of a few follicles, although the probability of this in a Fanconi anemia patient after cytotoxic drugs and radiation is admittedly low even in a young patient.

PROSPECTS FOR GENERATING OOCYTES DE NOVO

Adherence to Zuckerman's theory does not rule out the possibility of rare pluripotent stem cells in ovaries, although they had never been confirmed in an adult tissue (52). Pluripotent cells are more likely to be realized under culture conditions than in vivo where we might presume that evolutionary selection has suppressed such a biologically hazardous cell type

that could cause cancer. Even so, generating competent human oocytes from such cells will not be easy since they have a long and complicated life history, and the opportunities for generating culture artifacts is great. Germ cells are generalists able to generate totipotent gametes and yet, at the same time, are highly specialized for meiosis and fertilization, characteristics that require repression of somatic differentiation, cellular interactions in a unique environment, and specific networks of RNA regulation (73). More rapid progress is being made to produce male cells from stem cells (74), and a normal haploid nucleus can then be injected into a fertile oocyte to create a zygote. A fertile oocyte, on the other hand, requires competent cytoplasm and membranes, a nucleus at the correct stage of meiosis with appropriate epigenetic modifications and, obviously, the highest standards of safety if it is ever to be used in fertility treatment. But although there are poor prospects of creating fertile oocytes from stem cells in the near future, there appears to be no biological reason for discounting the possibility or halting the quest and a very recent paper upholds this hope (75).

SUMMARY

A consensus emerged over 50 years ago that the total population of oocytes in mammalian ovaries is fixed before or around the time of birth. No further increase in number can occur because of the disappearance of germ line stem cells. Atresia and ovulation progressively deplete the stocks of follicles until the organs become sterile, marking the onset of the menopause in primates. This theory was challenged recently by claims that follicles in adult mouse and human ovaries are continually being renewed, either by "reseeding" from germ cell precursors in the bone marrow or in the ovaries. These claims attracted widespread attention because of the profound implications for ovarian biology and reproductive medicine. But in a wide-ranging review of evidence from experimental biology and genetics, we have found no basis for overturning the conventional theory, and only negative results from independent studies designed to verify whether germ line stem cells generate oocytes de novo in adult ovaries or contribute to fertility. The lack of a physiological mechanism for replacing lost oocytes poses an interesting evolutionary conundrum, but we cannot rule out the existence of pluripotent cells in adult ovaries, which, under certain conditions, might be induced to generate new germ cells.

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7 Somatic Stem Cells Derived from Non-Gonadal Tissues: Their Germ Line Potential

Paul Dyce, Katja Linher, and Julang Li

SOMATIC STEM CELLS AND THEIR POTENCY

Although the potential to utilize the pluripotent nature of embryonic stem cells (ESCs) is attractive in terms of treating diabetes, muscular dystrophy, heart disease, and various neurological disorders, as well as regenerating bone marrow (BM), an alternate source of equally plastic stem cells from adult tissues could circumvent the ethical issues associated with their application to regenerative medicine. It is, therefore, encouraging that certain somatic stem cells isolated from adult, fetal, and placental tissues have been shown to possess a far greater differentiation potential than was previously thought possible. Indeed, exciting recent evidence suggests that a rare population of highly plastic cells derived from somatic tissues are able to differentiate into cell types other than their tissue of origin (1–4) and, more importantly, into cells of all three germ layers (5–8). Somatic stem cells comprise a low percentage of the total cell population within a given environmental niche. Until recently, they were thought to play a role only as progenitors within their tissue of origin, regenerating a very specific pool of functional cells required to maintain homeostasis, and replacing cells that are damaged due to disease/injury or lost during aging. As an example, a single stem cell of the hematopoietic system may differentiate into all mature blood components (9). In addition to BM, other tissue-specific, lineage-committed somatic stem cells have been isolated from the central nervous system, endometrium, intestine, kidney, liver, lung, muscle, pancreas, retina, skin, and thyroid (10–23), and it is possible that multipotent cells will be identified in niches throughout the adult organism.

It is unexpected but of considerable interest that Oct4, which encodes an early embryonic transcription factor that is silenced as cells differentiate into the various germ layers during development (24), has been shown to be expressed in certain rare populations of somatic stem cells (25). The expression of Oct4 provides a common link between these distinct cells and ESCs, cells of the epiblast, and germ cells. To date, a small number of Oct4+ somatic cells have been identified in the BM, bronchial epithelium, cord blood, endometrium, pancreas, and skin [reviewed in (25)]. Unlike other lineage-restricted somatic stem cells characterized to date, these cells are thought to be pluripotent, contributing to the formation of meso-, endo-, and ectoderm, including BM, pancreas, skin, and germ cell–like cells. It has been suggested that the existence of these rare, highly plastic cells may arise from remnants of ESCs, the function of which has been preserved into adulthood (25). One proposed hypothesis is that these "special" somatic stem cells may, upon organ damage, be mobilized from their specific niches and trafficked in the peripheral blood to the site of injury, thereby playing a role in tissue repair (26). This model of mobilization/trafficking could also provide a means by which cancer stem cells contribute to various metastases. Conversely, it is possible that certain tissue-specific stem cells are able to differentiate into lineages not normally present in their native niche in response to changes in certain environmental conditions (27).

While the discovery of the rare Oct4+ subsets of cells within somatic stem cell populations is exciting in terms of advancing regenerative medicine, further studies are required to substantiate their pluripotency. This can only be achieved conclusively if a single such cell could not only regenerate itself, contributing to other functional cells occupying its niche as well as cells found in an unrelated tissue, but also repopulate these different tissues in vivo. Of interest, multi- and pluripotent somatic stem cells have been purified following in vitro culture from BM, muscle, skin, and umbilical cord blood (4–8,19,28–30). In addition, a homogenous population of rare very small embryonic-like (VSEL) cells has been isolated directly from murine BM without prior culture (4–8,31). While these cells have been shown to

contribute to more than one embryonic germ layer following in vitro culture under the proper conditions, it remains to be determined whether their plastic nature is observed in vivo, or whether it is a culture-induced phenomenon resulting from reprogramming in response to the in vitro environment. This section of the current review summarizes the findings that rare populations of highly plastic somatic stem cells share many of the characteristics of ESCs.

Bone Marrow–Derived Pluripotent Cells

Multipotent adult progenitor cells (MAPCs) are fibroblast-like Oct4+ stem cells that have been isolated following adherent culture of adult BM, muscle, or brain cells (5). Human, mouse, and rat MAPCs have been induced to differentiate into various mesenchymal cell types (5), as well as endothelial-like cells (32), hepatocytes (33), and neurons (34). MAPCs expressing Oct4 and two additional pluripotent markers, SSEA1 and Nanog, have also been isolated from adult porcine BM, differentiating into adipocytes, chondrocytes, endothelium, hepatocyte-like cells, neuron-like cells, osteoblasts, and smooth muscle cells (35). Importantly, the plastic nature of MAPCs was substantiated in vivo, as it was shown that a single β-galactosidase-marked cell injected into a murine blastocyst was able to give rise to cells of all three germ layers in the resulting chimeric offspring (5,36). Furthermore, in studies involving the engraftment of MAPCs into irradiated mice, these cells contributed to the gut, hematopoietic system, liver epithelium, and lung, albeit at low levels. Cells that were subsequently collected from the BM were able to differentiate into multiple cell lineages in a secondary recipient, providing evidence of their pluripotent nature in vivo. A recent comparative transcriptome analysis of mouse and rat BM–derived MAPCs, ESCs, and a lineage-restricted subset of mesenchymal stem cells (MSCs) revealed that MAPCs are a unique group of pluripotent Oct4-expressing cells, and that the substantial differences identified between MAPCs and MSCs are not due to variations in culture conditions. While MAPCs do not express Sox2 or Nanog, two markers typically associated with an ESC phenotype, these cells do express Klf4 and Myc-n, which together with Oct4, Sox2, and, indirectly, Nanog are required for cultured fibroblasts to acquire ESC characteristics (37). However, this particular study did not clarify whether MAPCs are part of the native in vivo BM cell population, or whether they arise in response to in vitro culturing (37).

Another set of putative pluripotent stem cells isolated from human BM are the marrow-isolated adult multilineage inducible cells (MIAMIs). MIAMIs were identified following modifications of the culture conditions originally reported for MAPCs (5), including a higher fetal bovine serum (FBS) concentration in the media, low oxygen tension, and growth on fibronectin (7). In contrast to MAPCs, MIAMIs express Rex1, a zinc finger protein thought to be a hallmark of pluripotent stem cells (38). It is possible that the subtle changes in marker expression between the two cell populations are due to variations in the respective methods used to isolate and subsequently expand them in vitro. MIAMIs are able to differentiate into endo-, ecto-, and mesodermal cell lineages, although their plasticity has not been evaluated in vivo. Human BM–derived multipotent stem cells (hBMSCs) comprise another population that appears to undergo unlimited self-renewal, differentiates into cells of all three germ layers in vivo, and may be able to regenerate cardiomyocytes, smooth muscle, and endothelium after myocardial infarction (8).

Interestingly, a very rare population of SSEA1+ cells that also express Oct4 and Nanog, as well as several markers thought to be associated specifically with the germ line (Mvh, Stella, Fragilis, and Nobox) have been sorted directly from adult-derived murine BM without prior in vitro culture (31,39). As already mentioned, these VSELs (31) are highly plastic, demonstrating the ability to differentiate into several mesenchymal cell types as well as cells of all three germ layers following in vitro culture. In addition, a subpopulation derived from a single progenitor was able to differentiate into diverse mesenchymal cell types in vivo (39). VSELs form spheres similar to embryoid bodies after coculture on a murine myoblast feeder layer. Such spheres have also been formed from fetal liver-, spleen-, and thymus-derived VSELs, although it should be noted that they could not be generated from mice older than two years (31). It is possible that, since VSELs closely interact with BM-derived fibroblasts (26), the identification of the various putative pluripotent BM-derived stem cell populations discussed above may in fact be due to the tight adherence of VSELs to other cells during isolation. In other words, VSELs may be

infiltrating other heterogeneous cell populations previously thought to be endowed with ESC characteristics and therefore be the actual cells conferring the observed high levels of plasticity. This hypothesis has not been verified, and it has been argued that MAPCs, which resemble primitive endoderm, are a unique pluripotent cell type, as their phenotype is only maintained under specific in vitro culture conditions (37). While it is nevertheless possible that MAPCs could be induced to acquire pluripotency through reprogramming, not all mesenchymal stem cells cultured under the same conditions give rise to Oct4+ cells (37). This suggests that only a rare percentage of all cells from BM either are already pluripotent (VSELs) or have the intrinsic ability to be successfully reprogrammed (MAPCs).

It is known that BM cells are mobilized into the fetal circulation during the onset of labor, and human cord blood is an established source of cells that is able to contribute to the formation of diverse tissues [reviewed in Ref. (25)]. The umbilical cord matrix from pig and human cord blood has been shown to harbor Oct4, Nanog, and SSEA3/4+ stem cells (6,40). Human cord blood–derived CD45-negative cells, termed unrestricted somatic stem cells (USSCs) (6), have been differentiated in vitro into adipocytes, chondroblasts, osteoblasts, as well as hematopoietic and neural cells, and have also been shown to contribute to populations of parenchymal hepatic cells and cardiomyocytes when transplanted into preimmune fetal sheep (6). Interestingly, a rare population of cells similar to murine BM–derived VSELs has been purified from human cord blood, although it remains to be shown whether these cells are pluripotent and how to define their relationship with the existing cord blood stem cells (41).

Putative Pluripotent Cells from Other Somatic Tissues

A growing number of studies have reported the isolation of Oct4+ stem cells from tissues other than BM, including the pulmonary epithelium of the murine neonatal lung (42), rat pancreas (43), human amniotic fluid (44), human endometrium (45), the human hair follicle (46,47), and the skin of porcine fetuses (19). Recently, Oct4+ cells were shown to be present at the bronchoalveolar junction of the neonatal murine lung in vivo. Following in vitro culture under serum-free conditions, these cells grew in colonies and expressed, in addition to Oct4, other stem cell markers such as SSEA1, stem cell antigen 1 (Sca1), and Clara cell secretion protein, but not c-kit, CD34, or p63 (42). This particular expression profile suggests that these cells are most likely a subpopulation of Clara cells, which were previously identified as putative lung stem cells residing in the bronchiole in lung injury models (48). When Oct4+ Clara-like cells were removed from the mesenchymal stroma surrounding in vitro–produced colonies, they terminally differentiated into alveolar type 1 and 2 pneumocyte-like cells. Their identification has provided an exciting link between cells observed in vitro and the existence of an actual Oct4+ lung stem cell population in vivo. Various other cell types that may have the potential to repopulate the injured lung include the secretory cells of the trachea, type 2 pneumocytes of the alveolus, and mucus gland duct cells of the bronchus and trachea (48).

Oct4+ cells exhibiting a high potential for self-renewal and multilineage differentiation have also been isolated and characterized from adult rat pancreas (43). Purified cells from either single colonies or from single-cell clones expressed several other pluripotency markers including SSEA1 and nestin. These cells were able to give rise to cells of all three germ layers as well as putative germ cells in vitro, and also formed teratoma-like structures that contained cells from various lineages. Furthermore, exposing the pancreas-derived stem cells to specific factors resulted in the generation of defined cell types. For example, alpha smooth muscle actin expression was upregulated in cells cultured in the presence of retinoic acid.

Human and rodent Oct4+ and SSEA1/4+ amniotic fluid–derived stem cells have been maintained in an undifferentiated state in vitro for more than 250 population doublings while also retaining their telomere length and a normal karyotype (44). Clonal human lines were induced to differentiate into cell types representing all three embryonic germ layers, including fat, bone, muscle, endothelial tissue, neurons, and liver. Examples of differentiated cell types generated from human amniotic fluid cells that exhibit physiologically relevant functions include neutrotransmitter-secreting neurons and hepatocytes that produce urea.

Recently, it was shown that a rare population of Oct4+ cells reside in the endometrial stroma of 44% of women (45), supporting the long-held notion that somatic stem cells are present within the human endometrium. During a woman's reproductive years, endometrial

tissues undergo monthly cycles of shedding, regeneration, and differentiation. While there are currently no appropriate phenotypic stem cell markers, functional studies have provided evidence that putative human epithelial and stromal stem cells of endometrial origin exist. These rare cells have been identified using clonogenic analyses (49,50), and a subpopulation of stromal cells isolated based on coexpression of CD146 and the PDGF-β receptor was shown to differentiate into multiple lineages, including bone, cartilage, fat, and muscle. The finding that transplanted human endometrial cells contribute to the generation of endometrium in recipient animals also suggests that some of these cells may indeed correspond to somatic stem cells [reviewed in Ref. (21)].

Due to the high turnover of mammalian dermis, hair follicles are a niche somatic stem cells. Recently, using in vitro conditions typically applied to culture human ESCs (46), it was shown that a distinct Oct4+ population of adult stem cells resides in the human hair follicle that expresses several neuronal stem cell markers and Nanog. These very small cells undergo self-renewal, proliferate as spheres, and are able to differentiate into multiple lineages (adipocytes, endothelial cells, muscle cells, neurons, and osteoblasts) as assessed by analyzing the expression of appropriate lineage-specific markers. In addition to these studies, our work has shown that cells (a subpopulation of which are Oct4+) isolated from the skin of porcine fetuses also possess multilineage potential, as clonal populations expanded from individual cells were able to form neuron-, astrocyte-, and adipocyte-like cells upon induced differentiation (30). Furthermore, a subpopulation of these cells differentiated into oocyte-like cells (OLCs) (19). It will be of interest to determine whether the Oct4+ cells identified by Yu et al. (46) and those isolated using our model are in fact one and the same population of putative pluripotent somatic stem cells.

CURRENT FINDINGS REGARDING THE GERM CELL POTENTIAL OF SOMATIC STEM CELLS

In mammals, it is evident that cells inherit the intrinsic machinery that determines germ cell status, and that extracellular factors and/or cell-to-cell interactions induce the formation of germ cell precursors (51–54). It has been demonstrated that the differentiation of grafted embryonic cells depends not on their origin but rather on their placement. For example, β-galactosidase-labeled distal epiblast cells, which normally give rise to neuroectoderm, are able to contribute to the primordial germ cell (PGC) population when placed in the proximal epiblast (51).

We have previously reported on the isolation of stem cells from fetal porcine skin. These stem cells proliferated as nonadherent spheres and were able to differentiate into neurons, astrocytes, and adipocytes in vitro (30). Recently, we reported that these fetal-derived cells possess the intrinsic machinery to support induced differentiation into OLCs (19). Previous studies have shown that mouse ESCs form cells with germ cell characteristics spontaneously when cultured in ESC medium in the absence of LIF (52,55). To determine whether our porcine fetal skin–derived stem cells possessed such potential, we used a similar base medium, which was supplemented with follicular fluid collected from ovarian follicles. We reasoned that key autocrine/paracrine factors required for germ cell development may be present in follicular fluid. Prior to differentiation, the suspended stem cell aggregates were completely dissociated into single cells and plated on poly D-lysine/laminin-coated culture dishes. The cells initially attached and proliferated to form a confluent monolayer of cells (Fig. 1A). During differentiation, half the medium was changed every four to five days. At approximately day 10 to 25, denser colony-like areas began to form in the cultures (Fig. 1B, C). These areas continued to develop and by approximately day 15 to 30, some "shiny," round cells appeared within the colonies. These cells gradually detached, forming aggregates composed of several cells. At this point, no large cells were detectable within these aggregates. Upon further culture, the aggregates disappeared, possibly reattaching to the monolayer of cells that remained adherent during this process. At approximately day 35 to 40 of differentiation, some aggregates again detached from the surface, and at this time a larger cell (~45–60 μm) was visible in the center of some of the aggregates, resembling the morphology of a cumulus-oocyte complex (Fig. 1D, E). Upon further culture, a small subset of the large cells grew significantly larger, reaching approximately 80 to 100 μm and morphologically resembling an oocyte with a

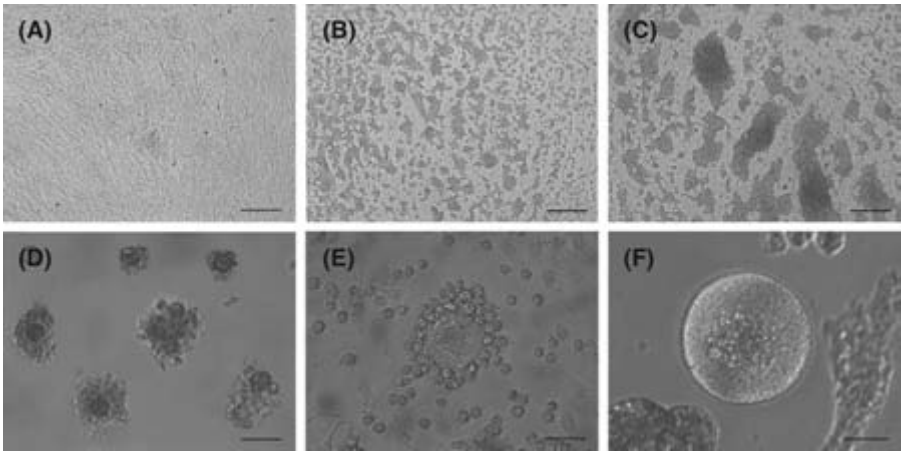

Figure 1 Morphological characteristics of skin-derived porcine fetal stem cells during induced germ cell differentiation. (**A**) Phase contrast micrograph depicting a monolayer of cells following eight days of differentiation. (**B**) At approximately 20 days of differentiation shiny cells begin to aggregate on the culture surface. (**C**) These shiny cells continue to aggregate and form colonies in the differentiation culture around day 25 to 30. (**D**, **E**) Aggregates released from the culture surface with a centrally located large cell resembling an oocyte-cumulus complex. (**F**) A phase contrast micrograph depicting an oocyte-like cell produced in vitro from porcine fetal stem cells. Size bars: **A**–**C** = 200 μm, **D** = 100 μm, **E** = 50 μm, **F** = 40 μm.

zona pellucida–like membrane (Fig. 1F). At advanced stages of culture, some cells spontaneously appeared with the distinct morphology of a blastocyst. While this was a rare event, we nevertheless were able to demonstrate that the expression of oocyte markers such as ZPA, ZPC, SCP3, Vasa, and GDF9b were turned off in the blastocyst-like cells, while Oct4 expression was maintained, suggesting that the expression of a set of germ cell markers was reprogrammed.

We have also compared the relative levels of mRNA present in the OLCs with those of natural porcine oocytes isolated from small follicles (<3 mm in diameter). Interestingly, the expression of some oocyte markers, such as Oct4 and Scp3, were elevated in the OLCs [3.87 ± 1.53 (fold ± SEM) and 21.72 ± 8.52, respectively] compared to natural oocytes. Some markers such as Vasa and GDF9b were expressed at a similar level in the OLCs when compared to natural oocytes (0.85 ± 0.15 and 1.02 ± 0.63, respectively). Other markers (cMos, ZPA, and ZPC) were expressed at significantly lower levels (0.33 ± 0.23, 0.001 ± 0.0003, and 0.2 ± 0.04, respectively). The low levels of ZPA and ZPC may partially account for the fragility of OLCs, which were easily broken and unable to withstand interaction with sperm during attempts at in vitro fertilization (IVF). The extreme fragility of the OLCs appeared to be linked with their overall size, as those over 100 μm broke very easily when experimentally manipulated. Interestingly, the expression of ZPB, another member of the zona pellucida family, was not detectable in our OLCs. The variability in the expression of key oocyte markers observed between OLCs and natural oocytes suggests that these large cells are by no means "perfect" oocytes. The true test will be to demonstrate the function of the OLCs by assessing their ability to support early embryo development following fertilization. Currently, both the overall rarity of OLCs generated in vitro and their inherent fragility have made it challenging to generate embryos using standard oocyte activation protocols.

Together with the oocyte, other major components of an ovarian follicle are granulosa and theca cells, which are characterized by their concerted production of estrogen. In natural follicles, androgen precursors produced by theca cells are aromatized into estrogen in the granulosa cells [reviewed in (56)]. In addition, granulosa cells also produce progesterone. We collected aliquots of spent medium from day 0, 50, and 55 (10 and 15 days, respectively, after cell aggregates were transferred to and cultured in oocyte growth medium) of differentiation to determine the presence of estradiol and progesterone by ELISA. While both of the steroid hormones were absent in the oocyte growth medium at day 0 (control), estradiol was detected at

a concentration of 108 ± 19 pg/mL and 153 ± 70 pg/mL, and progesterone at 882 ± 186 pg/mL and 1156 ± 309 pg/mL at day 50 and 55, respectively. Furthermore, mRNAs for $p450_{arom}$, $p450_{c17}$, and StAR, which are key enzymes involved in estrogen production, were found to be present at day 40 of induced differentiation. These data suggest that the granulosa- and theca-like cells differentiated along with the OLCs in our culture are indeed functional.

In addition to our work investigating the germ cell potential of fetal porcine skin–derived stem cells, several other groups have also demonstrated that somatic stem cells possess the potential to differentiate into germ cells (57,58). Danner et al. have shown that pancreatic stem cells may be induced to form OLCs in vitro (58). Similar to the stem cells isolated from fetal pig skin (30), pancreatic stem cells initially express Oct4 (43) and differentiate into cells morphologically resembling oocytes that express appropriate markers including Scp3, DMC1, Vasa, GDF9, and Oct4. Furthermore, Scp3 and DMC1, which are associated with the onset of meiosis, were confirmed to be present in the OLCs at the protein level (58). Another study has reported on the isolation of a population of stem cells from adult goat ear skin that express Oct4 and display the ability to form OLCs in vitro (57). The main index for assessing germ cell formation in this study was based on morphology, although germ cell marker expression data was not provided to fully demonstrate that these in vitro–produced OLCs express genes in a manner consistent with natural oocytes.

Another source of cells that possess germ cell potential from non-ovarian tissue is BM. It has been shown that a subpopulation of BM cells express germ line markers such as Vasa, DazL, Stella, and Fragilis (59,60). Following BM transplantation (BMT) into chemotherapy-sterilized recipients, as well as in ataxia telangiectasia–mutated gene-deficient mice that are incapable of producing germ cells, oocyte production was restored. In addition, transplantation studies using peripheral blood from transgenic female mice expressing the green fluorescent protein (GFP) driven by the Oct4 promoter and its germ cell–specific enhancer revealed that GFP-positive OLCs formed in chemotherapy-treated recipients. This suggests that the BM may release germ cell progenitors into the peripheral circulation, which travel to the ovaries, contributing to oocyte production (60). Using a preclinical mouse model, the same group performed BMT on high-dose chemotherapy-treated mice to study whether BM cells could rescue female reproductive function. BM from transgenic germ line–specific GFP-expressing mice was transplanted into chemotherapy-treated wild-type recipients. It was found that BMT enhanced long-term fertility, and cell-tracking studies showed that donor-derived GFP-positive oocytes were generated in the ovaries of recipients (59).

Similarly, Oct4+ stem cells isolated from BM have been shown to differentiate into male germ cells in vitro (61). To facilitate the analysis of male germ cell formation, a transgenic mouse line expressing GFP under the control of the Stra8 promoter, which is known to be activated specifically in male germ line stem cells, was utilized (62). Following 21 days of retinoic acid treatment, a population of Stra8-GFP cells was isolated and tested for male germ cell marker expression, revealing that positive cells coexpressed Oct4, Vasa, and Dazl as well as spermatogonial stem cell markers including Ingb1, Inga6, c-kit, Piwil2, and HSP-90a (61). Of note, the expression of Scp3, Pgk2, or acrosin, which are markers for late-stage sperm development and meiosis, were not detectable, suggesting that the cells were arresting at premeiotic stages in vitro. When the cells were transplanted into germ cell–depleted mice, the same premeiotic arrest was observed. It is therefore likely that additional factors are required for BM-derived germ cells to further differentiate to a stage at which meiosis is appropriately initiated and more mature male gametes are formed. Similar findings were reported in an in vivo study in which stem cells isolated from the BM of GFP-expressing transgenic mice were transplanted into the seminiferous tubules and interstitial space of recipient testes (63). One group of recipient mice used for these experiments was treated with busulfan to ablate the resident germ cell population and the other represented a c-kit receptor mutant transgenic line that is characterized by the complete absence of endogenous germ cells within the gonads. The recipient testes were analyzed several weeks following transplantation. It was found that a population of GFP-positive donor cells resembling Sertoli cells and coexpressing the follicle-stimulating hormone (FSH) receptor survived in the testes. In addition, some GFP-positive cells presented a phenotype similar to spermatogonia or spermatocytes and coexpressed Vasa. Interestingly, no GFP-positive spermatids were observed, further confirming the existence of a

premeiotic block likely resulting from in vitro differentiation. Therefore, it appears that cells residing in the BM have the potential to differentiate into somatic and germ line cells when placed into the correct environment but are unable to complete meiosis to form functional gametes. It was speculated that inappropriate genetic imprinting or stage incompatibility with the endogenous Sertoli cells may have contributed to the arrest of donor-derived germ cells at the spermatocyte stage (63).

POSSIBLE MECHANISMS REGULATING THE IN VITRO FORMATION OF GERM CELL–LIKE CELLS FROM SOMATIC STEM CELLS

Why are cells derived from somatic tissues able to differentiate into OLCs in vitro? In mammals, the germ lineage is believed to begin in the epiblast. PGCs are present in the posterior wall of the yolk sac and migrate to the gonads (51). Several hypotheses have recently emerged to explain the highly plastic nature of somatic stem cells, particularly in the context of their ability to differentiate into OLCs in culture. One possibility is that some of the PGCs are allocated to differentiating niches other than the gonads early during embryonic development and thereby end up residing in somatic tissues, where they retain germ cell potential. However, this is inconsistent with the fact that the estimated number of PGCs in mice ranges from approximately 100 to 5000 during migration (64), while approximately 400 to 1000 spheres, each composed of 100 to 400 cells, can be isolated from one square centimeter of skin (30). A less likely possibility is that a small population of PGCs "goes astray" during migration to the genital ridges and proliferates in the tissue that these "lost" PGCs eventually populate. Should this be the case, a logical question that arises is why the microenvironment within somatic tissues would sustain the survival and proliferation of PGCs when there are already an excess number of germ cells residing in the ovary? It is well known that the vast majority of oocytes within the mammalian ovary do not give rise to a dominant follicle from which an oocyte is ovulated. Only a small percentage of resident oocytes therefore play a role in transmitting genetic information to subsequent generations, and it does not make physiological sense for PGCs to proliferate in the skin. It is likely that these cells may never form germ cells in their native skin niche either due to negative regulation or due to the lack of appropriate extracellular signals that are simply absent from the microenvironment. Thus, it may be the process of in vitro culturing that "unlocks" their germ cell potential. In addition, it is worth noting that in our laboratory, although all skin sphere cells are exposed to the same differentiation medium, only a small population are able to develop into OLCs (on average, 6 to 70 large cells are typically extruded from aggregates generated from a culture originally started by plating 50,000 skin stem cells). Therefore, it appears that only a small fraction of skin stem cells possess the intrinsic machinery required to give rise to germ cells.

A recent report proposed that during embryonic development, the epiblast acts as the source of all somatic stem cells following gastrulation. The epiblast, derived from the inner cell mass of the blastocyst, contains pluripotent epiblast stem cells (EPSCs) that also retain germ line potential (65). It was suggested that some EPSCs may be deposited in peripheral tissues during gastrulation and that a very small population of these cells retain their pluripotent nature throughout development (25,65). Thus, the migration of PGCs to various niches mentioned above may link with this hypothesis, as PGCs and EPSCs are thought to be closely related pluripotent cell types. The possibility that a subpopulation of skin-derived cells remains uncommitted and is able to differentiate into multiple cell types upon induction is also in line with this hypothesis. While skin cells are of ectodermal origin, we and others have shown that sphere cells isolated from skin are able to not only differentiate into cells of the ectoderm (neurons, astrocytes) but also of the mesoderm (adipocytes) (4,19,30). Moreover, the expression of the neuronal stem cell marker nestin has been detected in the dermis of mice (4), suggesting the in vivo presence of uncommitted cells in skin. Although morphologically similar to each other, the skin sphere cells isolated in our laboratory are by no means homogeneous. When skin spheres were dissociated into single cells and plated under limiting dilution, on average 56% of single cells divided into two cells, and 11% and 5% of the individual cells proliferated into as many as 10 and 20 cells, respectively during the 24-hour culture period, suggesting that they possessed different proliferation potentials (unpublished data). Immunocytochemistry revealed that only a very small population of the

skin sphere cells expressed Oct4. It will be of interest to determine whether these Oct4+ cells also express other pluripotent markers. Further experiments using genetically labeled Oct4-expressing cells would help to address the question of whether the Oct4+ skin sphere cells are indeed able to undergo germ line specification. It is possible that cells similar to the VSELs described by Ratajczak et al. (25) are present in fetal skin, which, upon being placed into an appropriate environment, may first give rise to PGC-like cells and subsequently differentiate into OLCs. A logical question is, are some of these skin cells with germ cell potential a physiologically relevant source of oocytes? Skin cells are less mobile than cells from other tissues such as BM, are closer to the external environment, and are therefore more prone to genetic mutations. If putative pluripotent cells are indeed present in somatic tissues, skin cells may be the least likely source from which cells are recruited to contribute to the germ line in vivo.

Recently, mouse fibroblasts were reprogrammed into a state resembling pluripotent ESCs using retroviral-mediated transduction of four transcription factors, Oct4, Sox2, c-myc, and Klf4, followed by selection for activation of the Oct4 target gene Fbx15 (although Fbx15-induced pluripotent stem cells seem to represent an incomplete state of reprogramming) (66). When activation of the endogenous Oct4 or Nanog genes was used as a selection criterion to assess pluripotency, the resulting induced pluripotent stem (iPS) cells, Oct4-iPS or Nanog-iPS, were fully reprogrammed into pluripotent ESC-like cells that were able to form viable chimaeras and contribute to the germ line (67–69). It has been suggested that during the acquisition of pluripotency, Oct4 may be the most upstream gene to initiate reprogramming. In our case, it is possible that the rare Oct4+ skin–derived stem cells are maintained in a suppressive microenvironment in vivo, and that this block serves as a self-protection mechanism to prevent uncontrolled growth or inappropriate differentiation. When they are released from the inhibitory niche and are cultured in vitro, a subset of these cells may then be reprogrammed into progenitor cells that are able to differentiate further into OLCs.

The function of our in vitro–generated porcine OLCs remains to be demonstrated. As mentioned, the fact that estradiol was detected in the spent culture medium, levels of which increased in response to FSH stimulation, suggests that functional granulosa and theca-like cells were generated in parallel with OLCs through the process of induced differentiation. It is particularly intriguing to observe the spontaneous formation of structures that strongly resemble cumulus-oocyte complexes in culture. Is the presence of a germ cell a prerequisite for the generation of these somatic support cells (granulosa and theca cells), which are essential for the assembly of a functional ovarian follicle in vivo? What triggers the formation of these distinct follicle-like structures? Are the actions of chemokines and their receptors involved in directing the assembly in vitro? If yes, what are the specific factors/receptors and their associated signaling networks that may be involved in this process? Do induced germ cells secrete factors that directly influence the surrounding cells to differentiate into granulosa and theca cells? Future studies utilizing genetic labeling with markers specific to oocytes, granulosa cells, and theca cells will hopefully facilitate the monitoring of these processes in vitro, potentially helping to answer the array of developmental questions on how oogenesis and follicle formation are orchestrated within the in vitro environment.

CHALLENGES, LIMITATIONS, AND FUTURE POSSIBILITIES

In the context of regenerative medicine, consideration of the therapeutic potential of pluripotent cells has emerged largely due to two developments, the derivation of ESCs from human blastocysts (70) and the cloning of mammals from somatic cell nuclei (71). While the use of ESCs remains controversial, the growing number of studies reporting on the existence of ESC-like somatic stem cells is lending support to their use as a potential alternate source of pluripotent cells, also renewing interest in the notion of "customized" therapeutic cloning. However, as already mentioned, considerable research remains to be conducted to verify their pluripotency, as well as to efficiently utilize the differentiation potential of these cells for the consistent derivation of specific cell lineages.

Recent reports suggest that in vitro–produced gametes are unable to complete meiosis (72). In addition, it remains unknown whether these cells establish the correct genomic imprint upon fertilization. It is therefore unlikely that OLCs will replace natural

oocytes for the development of reproductive therapies (i.e., to help women overcome reproductive defects). A more likely application of OLCs generated from somatic cells would be to serve as a model for studying the mechanisms governing germ cell specification, proliferation, survival, and differentiation in vitro compared to the events reported in vivo. In addition, these cells may provide a means to improve existing technologies for therapeutic applications. In each instance, somatic cell nuclear transfer (SCNT) could be applied. This approach is based on transplanting a patient-derived somatic cell nucleus into an enucleated oocyte, thereby generating a cloned embryo which, upon reaching the blastocyst stage, could be used as a source of ESCs. The plastic nature of these cells could then be exploited to induce differentiation into the desired lineage, either for studying a particular pathogenic phenotype or for the treatment of a disease following transplantation of the resulting cells into the damaged tissue. In theory, OLCs induced to differentiate from adult somatic stem cells could potentially be used for SCNT, as this procedure is currently highly inefficient, requiring a large supply of donor oocytes to generate each viable blastocyst. In addition, use of in vitro–differentiated OLCs or "synthetic" oocytes produced from a patient's own somatic cells, in combination with an appropriate nucleus, would result in an exact genetic match, including at the level of mitochondrial DNA. Genetically matched cells of the desired lineage subsequently differentiated from the in vitro–produced ESCs would lessen the chance of possible rejection following transplantation. As already mentioned, in order to successfully generate patient-tailored ESCs for therapeutic applications, it will be essential to improve the overall quality of synthetic oocytes, as well as the efficiency at which nuclear reprogramming can be achieved. Given that the quality of natural oocytes varies considerably, it is reasonable to assume that for the mere study of a given disease, synthetic oocytes would not need to be "perfect." Their main role would be to influence the developmental status of the somatic nucleus enough to support blastocyst formation without adversely affecting the differentiation potential of the resulting ESCs. However, in using SCNT in combination with synthetic oocytes for therapeutic purposes, the safety concerns related to substandard reprogramming are significantly higher. The synthetic ESCs not only would have to be able to fully support differentiation into the desired cell lineage, but more importantly, these differentiated cells would then need to appropriately integrate into the damaged tissue in vivo and function in their new microenvironment in a physiologically relevant manner. Consideration of the potential applications of somatic cell–derived synthetic oocytes to regenerative medicine should be approached with considerable caution, as it remains to be experimentally determined whether these cells are fully functional, and whether they would ever merit replacing natural oocytes in SCNT procedures.

As already mentioned in the current review, our observations indicate that most OLCs formed from skin stem cells in vitro are unable to support IVF, although some do spontaneously undergo parthenogenesis. Therefore, an alternative approach that bypasses SCNT would be to attempt isolating ESCs directly from blastocyst-like cells generated in vitro to produce patient-specific stem cells. In order for this to be feasible, the spontaneous formation of blastocyst-like cells would need to be confirmed by evaluating whether OLCs are able to support the early stages of embryonic development using more traditional activation techniques. We are currently investigating whether it is possible to activate our OLCs to induce blastocyst formation. One major challenge that remains to be overcome is the fragility of the skin-derived OLCs, which has also been observed in OLCs generated from ESCs (52). As mentioned, while the final size of a subset of OLCs is consistently greater than 100 μm, the zona pellucidas of these cells appear to be less developed than those of natural oocytes, which may account for their fragile nature. In both our work and studies conducted with ESCs, not all zona pellucida proteins are expressed in late-stage OLCs (19,52). It will be important to gain insights from analyzing the in vitro expression of these proteins and others related to the formation of this structure, such as FigLa, to improve the structural integrity of the OLCs. This would allow us to manipulate them more easily, opening up the possibility of using standard reproductive techniques such as IVF to further study their function and hopefully making them better donors for SCNT.

While the potential applications of OLCs discussed above may be applied in the future, current culture systems utilized for in vitro gamete differentiation rely on undefined

cocktails of growth factors to support formation of heterogeneous cell populations that also include germ cell–like cells. The ability to specifically direct differentiation solely toward gamete production has not yet been accomplished, and the process therefore remains inefficient. Identifying the critical factors that promote and support the induction of germ cell formation in vitro could lead to more efficient and consistent differentiations. For our system, variations in lots of FBS and poly-D-lysine, changes in porcine follicular fluid (PFF) content, as well as the stem cells themselves, which are isolated from different individuals for each differentiation, have resulted in considerable fluctuations in the overall number and quality of OLCs generated in vitro. Changes from one lot of FBS to another have resulted in a high level of variability in the differentiation cultures. Some FBS lots have completely failed to support germ cell formation, while others supported this process, but with only approximately 20% of the expected number of OLCs present at the final stages of differentiation. It is well known that the quality of FBS may vary from batch to batch, and stem cells are significantly more sensitive to even small changes in serum content than the average cell line. Without better knowledge of the factors present in FBS that are essential in supporting a successful differentiation, all lots must be extensively tested prior to use.

The constitution of PFF, which is also required for our gamete differentiation protocol, has proven equally variable. This variation may be attributed in part to the season in which the PFF is collected, with better results typically achieved in winter, and on the ratio of large to small follicles from which the fluid is aspirated. The consistency of this particular additive is difficult to control, as there is no hallmark factor that can be used to assess overall PFF quality. As is the case with FBS, it is likely that even small differences in the concentrations of essential factors from batch to batch may have a dramatic effect on gamete formation in vitro. We are currently attempting to standardize the collection procedure in order to optimize the process as much as possible.

Unlike researchers working on the in vitro formation of germ cells from ESCs who are able to ascertain the quality of the undifferentiated input cells, our work requires early-passage primary cells. Hubner et al. (73) stress that partially differentiated ESCs, or cultures that are contaminated even at a low level with feeder cells, may have a negative impact on the formation of OLCs, resulting in very low to no yields. We only use freely floating skin-derived sphere cells at passage two as the founder cells for initiating induced differentiation into the germ lineage. However, sphere cells themselves are not a homogenous population, and we estimate that only a very small percentage of the total cells plated are Oct4+. It is also difficult, from our perspective, to try to optimize a process that is largely unpredictable with regard to the timing of when OLCs first appear in the culture. Induced differentiation is by no means a synchronized event, even within a single experiment in which the same group of cells is seeded onto several identically coated tissue culture dishes.

It is clearly evident that a combination of factors is required to support efficient in vitro gamete formation from somatic stem cells, and determining the identity of this unique combination presents a considerable challenge. Much of the variability that we are currently attempting to reduce could be eliminated if high quality recombinant proteins could replace the use of FBS and PFF. In the process of determining the identity of these critical proteins, likely candidates will need to be screened, alone or using a combinatorial approach. It may also be possible to influence the outcome of the differentiation by including natural support cells (derived from a natural ovarian follicle) in the differentiation culture, as they are known to secrete specific factors required for the formation of an antral oocyte in the ovary. A defined system would be invaluable in better understanding the mechanisms underlying gamete formation in vitro, as it would provide a model to study the effects of specific factors on this complex process. Of course, it would be ideal to direct the in vitro differentiation of somatic stem cells into OLCs in a defined media, improving the overall efficiency of gamete formation and the quality of OLCs arising in the culture. Many unanswered questions remain to be addressed regarding how the culture system induces the differentiation of skin-derived stem cells into germ cell–like cells. One major question is whether the OLCs generated in vitro are meiotically competent and functional. The ultimate proof of course rests with the birth of live, normal offspring generated from one such gamete.

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8 The Male Gamete

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INTRODUCTION

Sperm production is defective in a significant proportion of males aiming fatherhood.

These defects can be divided into defects leading to an abnormally decreased sperm production, resulting from low to absent sperm in the ejaculate, or defects in sperm physiological characteristics, independent from sperm count but causing reproductive failure.

Men producing no spermatozoa are the main focus of sperm cell development from stem cells. Up to now, these couples were directed to the use of donor sperm.

Sperm donation has been an effective way to achieve pregnancies and healthy newborns safely when sperm cell count was dramatically decreased, until the intracytoplasmic sperm injection incorporation, and later, when no sperm cells are available. Obviously, the main concern to use donated sperm is the lack of biological paternity.

In these cases, the objective is to develop functional spermatozoa that can be employed in assisted reproduction treatments to achieve healthy newborns containing father's genetic features.

Initially, testicular stem cells can be identified in some males, but their use to create sperm is dubious, given their origin: malfunctioning testis. The ideal situation is to reach sperm creation by reprogramming their own adult stem cells obtained from other tissues, and reach this end in vitro.

Several concerns must be taken into account to avoid genetic problems to the offspring. These include epigenetic deregulation and the high risk of chromosomal abnormalities when immature spermatogenic cells have been employed in assisted reproduction.

Also, their success will depend on the achievement of certain physiological characteristics of spermatozoa, different to morphological features, such as sperm DNA status and other molecular features that have been recently related to correct fertilization of the ova, embryo development, and implantation.

Investigations are on the way to create sperm from adult stem cells, but actually, no clinical application in humans can be done.

With this chapter, our aim is to describe the current needs, requisites, and situation to create male gametes from stem cells.

MALE INFERTILITY PROBLEMS

Defective Sperm Production

Sperm production is defective in a significant proportion of males aiming fatherhood.

These defects can be divided into defects leading to an abnormally decreased sperm production, resulting from low to absent sperm in the ejaculate, or defects in sperm physiological characteristics, independent from sperm count but causing reproductive failure.

In the last two years we have seen the publication of reports that suggest a global decline in semen quality (1). Within western Europe, a four-center study on fertile couples (2) has shown substantial variations in semen parameters. However, the data on trends in semen quality are conflicting and sensitive to geographical variations (3). It has also been difficult to prove an association between deterioration in semen quality and male infertility. The quality of existing studies has been questioned on the basis of population and selection biases, variations in laboratory standards, and statistical methodologies used for the analysis.

Regarding the seminal patterns of the male partners of the infertile couples, submitted to an infertility unit for assisted reproduction, few data are published (4).

From our results we have observed that 15% of our patients had normal sperm parameters following WHO (World Health Organization) criteria, while 85% had abnormal

semen analysis, with morphological alterations (teratozoospermia) being the most common (27.8%) of the disorders observed.

Azoospermia, defined as the absence of spermatozoa in the ejaculate after assessment of centrifuged semen on at least two ejaculates, is observed in 1% of the general population and in 10% to 15% of infertile men (5). The introduction of intracytoplasmic sperm injection (ICSI) offered a novel opportunity for parenthood to these couples. The first pregnancies reported after fertilization by ICSI with frozen testicular sperm in men with obstructive azoospermia (OA) were published by our group in 1995 (6). Tournaye first reported the use of testicular sperm extraction (TESE) in nonobstructive azoospermia (NOA) (7). This problem affects 10% of infertile men and is diagnosed in 60% of azoospermic men (8). Etiologies for testicular failure include genetic disorders such as sexual chromosomal abnormalities, translocations and microdeletions of the Y chromosome, cryptorchidism, testicular torsion, radiation, and toxins (9). Testicular spermatozoa can be retrieved in some NOA men despite the absence of ejaculated spermatozoa in their semen, because of the existence of isolated foci of active spermatogenesis. However, lower recovery rates (around 50%) were observed in large series (10).

Current recommendations on the diagnosis of NOA dictate that it should only be based on histopathological findings since clinical and endocrine parameters cannot accurately distinguish between OA and NOA (11). The former represents an important issue since sperm can be retrieved in almost all cases of OA, but only in 50% of NOA when no preliminary selection of patients on the basis of histopathology has been performed (10).

Sperm Defects

Highly differentiated spermatozoa are generated through multiple cellular and molecular processes maintained by Sertoli cells. The cellular events associated with germ cells include proliferation, protein folding and transportation, as well as sequential changes in chromatin and cell organelles.

These processes are strictly controlled by the expression of specific genes, and its defects could induce structural and ultrastructural defects. These defects may affect their function and could be closely related to male infertility. These imperfections could compromise all the sections of the sperm cells and has been widely described in multiple works.

Changes have been described in the morphology of the acrosome membrane and head of the infertile sperm. The acrosome of infertiles could represent a bigger proportion of sperm head than in the fertile males. The acrosome membrane in the infertile men could be less intact and less smooth than in fertile men. More droplets were attached to the acrosome membrane in the infertile men than in the fertile (12).

The morphological analysis of the spermatozoa from fertile and infertile men by using light and electron microscopy can clarify the relationship between sperm morphology and fertility. Abnormalities in the spermatozoa can be classified into three types for the tails, two types for the midpieces, and six types for the heads, according to the criteria adapted from WHO guidelines, stated in the *WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction* (4th edition) in 1999. Approximately 14% of the spermatozoa from the fertile men have abnormal tails at the light microscopic level while approximately 44% had abnormal heads. Most abnormal cell types are encountered in semen from fertile men too, although the incidence of abnormalities is low (13).

Asthenozoospermia is a frequent cause of male infertility and could be explained by alterations of flagellum structure. These alterations may be nonspecific and acquired affecting a variable number of spermatozoa, or may be primary and specific, observed in most spermatozoa (14). Numerous abnormalities of the axoneme and the periaxonemal structures are known in humans. An absence of both dynein arms revealed that these structures are essential to flagellar motion. Several other axonemal abnormalities have been found in infertile men, which could involve any of the axonemal components (15). Abnormalities of the fibrous sheath, of the mitochondrial sheath, and of the attachment of the flagellum to the nucleus have also been found to be responsible for male infertility (16).

ICSI is considered as an efficient treatment to overcome male factor infertility. Initially proposed in severe oligozoospermia (17), ICSI has been applied with ejaculated spermatozoa in the presence of oligoasthenoteratozoospermia and with epididymal and testicular

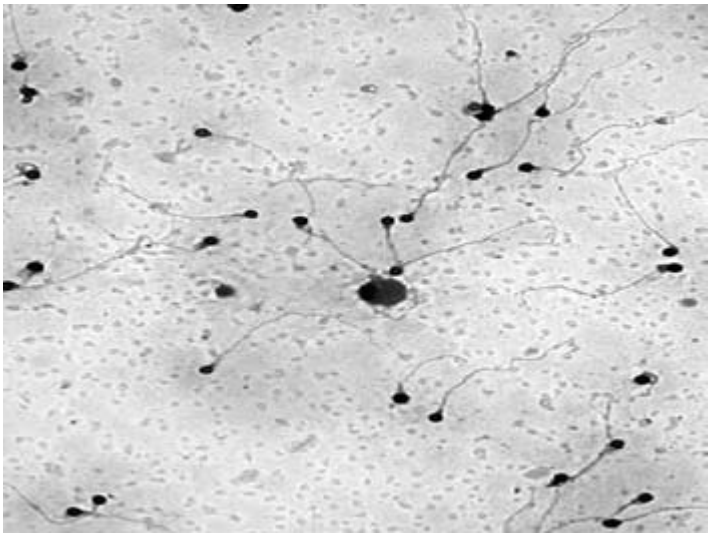

Figure 1 Hematoxylin-eosin staining of ejaculated spermatozoa from a patient suffering from globozoospermia. Magnification 400x.

spermatozoa in azoospermia, with fresh or frozen-thawed samples (18). Furthermore, fertilization and delivery were also obtained with ICSI using immotile but viable spermatozoa (19).

Until now, ICSI outcomes have only been reported in rare cases of specific flagellar abnormalities responsible for immotile spermatozoa, such as absent dynein arms (20), absent central microtubules (21), or dysplasia of the fibrous sheath (16). Most of these infertile patients with ultrastructural defects of their sperm flagella also exhibited clinical features, suggesting abnormalities of their respiratory cilia, i.e., Kartagener syndrome, or chronic airway infections (20).

Globozoospermia is a very rare condition observed in $<1\%$ of infertile patients, where the major morphological anomaly is the absence of an acrosomal cap in sperm (Fig. 1). The sperm head appears small and round due to the failure of the acrosome to develop (22). The pathogenesis of globozoospermia most probably originates in spermatogenesis, more specifically in acrosome formation and sperm head elongation. The absence of the acrosome renders globozoospermic spermatozoa unable to bind to the zona pellucida (ZP) or fuse with the oocyte oolemma. Therefore, men affected by globozoospermia are infertile and untreatable by artificial insemination or conventional in vitro fertilization (IVF). But when globozoospermic spermatozoa bypass the ZP and oocyte oolemma with ICSI, normal fertilization, embryo development, and pregnancy may occur. However, globozoospermia reduced ICSI fertilization rates compared with other forms of teratozoospermia. Fertilization failures have been attributed to a deficiency in oocyte activation capacity. Pregnancy and live birth can be achieved with chemical activation in a globozoospermic patient with a low fertilization previous cycle. Our group detected an improvement in the fertilization rate, development, and quality of the embryos generated with assisted oocyte activation (22).

Sperm Donation: Clinical Use

Men producing no spermatozoa are the main focus of sperm cells development from stem cells. Up to now, these couples were directed to the use of donor sperm.

Sperm donation has been an effective way to achieve pregnancies and healthy newborns safely when sperm cell count was dramatically decreased, until the ICSI incorporation and, later, when no sperm cells are available. Obviously, the main concern to use donated sperm is the lack of biological paternity.

Artificial insemination with the use of semen from anonymous donors (AID) has been a low-complexity assisted reproduction technique (ART) widely used for many years in the treatment of women wishing to conceive in specific situations (23). But ART also covers interventions like IVF and ICSI, all of which have the ultimate aim of assisting the infertile

patient to become pregnant and deliver a live infant. In both situations, use of donor sperm could be mandatory, although the indications for its employment could or should be different (24,25). Actually, these are severe male factors, both in patients with very low or absence of spermatogenesis and in males with genetic disorders that might be transmissible to the progeny, as well as repeated ICSI failure (IF) and in homosexual women and women without a male partner. The introduction of ICSI to the assisted reproduction laboratory has notably decreased the number of artificial insemination (AI) over recent years, mainly in patients showing severe alterations of the spermatogenesis. In other cases, we expect a reduction in the use of donor sperm as new techniques become developed or when other techniques become firmly established, for instance, preimplantation diagnosis of genetic diseases as well as sperm washing for HIV serodiscordant couples (26). Hence, it is essential to determine the relevant factors affecting the indications of donor sperm in AID and IVF/ICSI and how they influence the results of the programs, and in this way we can establish the adequate criteria to counsel, estimate success prognosis, and improve success rates. Different studies have described the influence of different parameters such as maternal age, male etiology, female etiology, ovarian stimulation protocol, and many others with controversial or rather diverging results. Nevertheless, some significant risk factors for low pregnancy and live birth rates have been identified, from them female infertility factor, older maternal age, low number of previous births, and lack of ovulatory stimulation are those with clear effects (27,28). A number of studies with adequate sample sizes were conducted from different clinics together in multicenter studies, where probably the selection criteria, control, and management of patients are slightly different, thus adding heterogeneity to the sample (29).

Clinical Indications and Reproductive Outcome

We have reviewed our experience spanning more than eight years with almost 2934 AID and IVF nonselected consecutive cycles and investigated the distribution of the clinical indications to use donated sperm, comparing between AID and IVF and studying their evolution during the period analyzed.

Approximately 57% of the cycles of ART using donor sperm were AID and 42.8 were IVF.

We compared the indications for sperm donation between patients undergoing intrauterine insemination (IUI) and IVF/ICSI. There were significant differences in the indications between them, while women without partner kept the same proportions in both groups; we observed an increased number of patients coming from ICSI failure in IVF and a decreased percentage with a previous diagnose of NOA (Fig. 2).

We studied the evolution in the indications for the use of donor sperm in the last years, comparing global distributions with the indications in the last year (2007), and there are significant increases in women without heterosexual couple from 26.4% to 34.9%; azoospermia (from 33.9% to 40.1%) and a decrease of IVF/ICSI failure (from 37.2% to 25.0%, respectively),

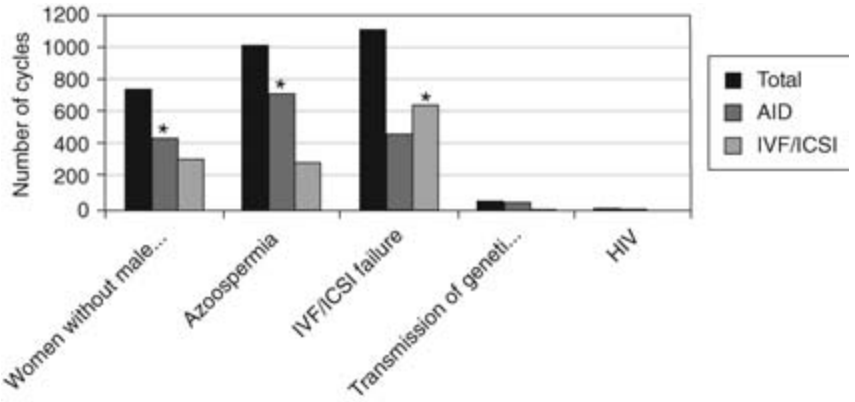

Figure 2 Number of cycles with donor sperm depending on the ART procedure (AID vs. IVF/ICSI) and clinical indications for donor sperm. The distribution of the proportions was compared by Chi-square analysis. (*) denotes statistically significant difference between proportions presented in AID versus IVF, $p < 0.0001$.

almost disappearing the indications to replace males' gamete because of a genetic disorder or the presence of a sexually transmitted disease (Fig. 3). We also represented the evolution in the number of cycles and the different indications from 2000 to 2007 in AID and IVF cycles (Fig. 4).

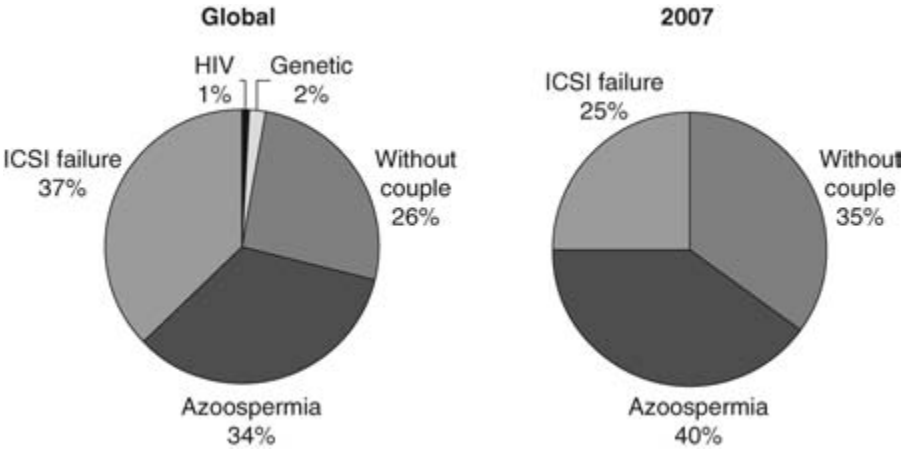

Figure 3 Distribution of the clinical indications for sperm donation. In the left graph, the global proportion of each indication is represented (from 1999 to 2007). In the right graph, the proportions observed in the last year are represented. The distribution of the proportions was compared by Chi-square analysis. A statistically significant difference in the proportions presented in the two distributions were observed, $p < 0.0001$.

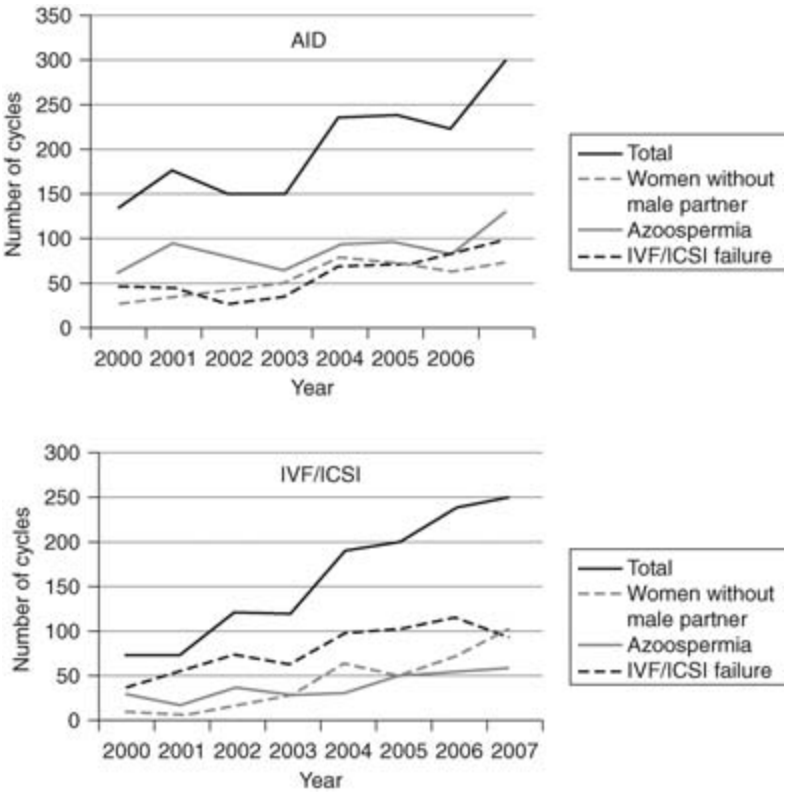

Figure 4 Number of cycles with donor sperm depending on the clinical indication in the ART procedure. These values are presented in linear fashion by the years from 2000 to 2007. Upper panel represents the AID cycles and the lower panel the IVF/ICSI cycles.

We observed that while in AID the indications kept the same proportions, in IVF the proportions tend to invert from the original distribution.

Regarding pregnancy rates (AID), NOA patients presented the highest (29.1%) in comparison with ICSI failure and single women (27.6% and 22.6%, respectively). Couples with previous ICSI failures presented the highest pregnancy rates in IVF cycles (48.7%) compared with azoospermia and single women groups (42.0% and 38.2%, respectively).

Help Wanted: Sperm Donation in the Era of ICSI

Over the last decade the clinical indication of sperm donation has been growing together with the rest of the assisted reproduction treatments in our unit. This could be due mainly to a change in our lifestyle linked to developed societies. The open distribution of all new advances and new technologies related to assisted reproduction in the media (newspapers, radio, TV, etc.) has permitted people a better understanding of their problems and therapeutic options. These include the growing resources of a sperm bank unit.

We have shown a higher use of sperm donation for artificial insemination than conventional IVF, and this difference is probably based on the favorable cost-benefit ratio that an AID cycle could offer to a patient, with acceptable pregnancy rates when the sperm bank is adequately managed.

In AID procedures we have the highest pregnancy rates in patients without sperm production, compared with IVF/ICSI failures and single women. This marked difference could be explained because those couples from azoospermic patients used to be fertile and have never been in contact with a male gamete when attempting pregnancy. Moreover, those coming from IVF failure could have some associated female factors, often linked to bad oocyte quality, and regarding single women, also frequently associated with advanced maternal age. In the last situation, the sperm bank choice used to be the last option before an unsuccessful couple searching being in consequence simultaneous to an advanced age.

In the IVF procedures, we found similar pregnancy rates in patients coming from IVF failure or unsuccessful sperm retrieval, and this rate is always higher than in single women, since in this last situation, advanced age pattern is commonly present.

Despite that sperm bank indication for assisted reproduction has increased in the last years, we have observed a significant reduction in the number of cycles related with IVF failure. The cause seems to be quite evident: new procedures have been developed and others improved in clinical embryology and this has been reflected in successful treatments and less IVF/ICSI fertilization or transference failures.

SPERMATOGONIAL STEM CELLS TO RESTORE THE MALE FERTILITY

The introduction of ICSI has played a significant role in the treatment of many severe male infertility problems (30). However, in extreme cases of male infertility, there is a complete absence of spermatozoa within the ejaculate due to absence of sperm production, the obstruction or the absence of some conducts. In these cases, testicular biopsy and ICSI is mandatory for proper diagnosis and treatment (31). Moreover, in approximately one half of the NOA, it is almost impossible to recover the sperm from the testis (10). Therefore, at the present time, the only solution is sperm donation, resignation or adoption (32).

Recently, the new avenue of research identifying the testicular stem cells has arisen as a potential new field to be explored to treat these males. Spermatogonial stem cells (SSCs) are the adult stem cells of the testis (33). They are located at the periphery of the seminiferous tubules flanked by the Sertoli cells, their niche cells. Like any other stem cell in the body, SSCs can either self-renew to maintain the SSC population or differentiate and give rise to cells from the germ cell line. The SSCs start to differentiate and will eventually produce mature spermatozoa in the continuous spermatogenesis process. This process of spermatogenesis occurs throughout a man's entire adult reproductive life and is preserved by the self-renewing capacity of the SSCs. Both proliferation and differentiation are well-regulated mechanisms, which are mainly directed by the Sertoli cells (34).

The importance of the testicular stem cells is reflected in a variety of research fields. The study of fundamental aspects of germ cell development has become possible, thanks to the introduction of the testicular stem cell transplantation technique (35,36) and germ cell culture

(37). In the future, these techniques may also have clinical applications. Indeed, the preservation of SSCs may be an important strategy in preserving fertility in young cancer patient (38).

These SSCs possess the capacity to repopulate nude mice treated with busulphan and to produce spermatozoa de novo (39). Currently, a typical assay for the presence of SSCs in a cell suspension is the SSC transplantation technique; in the future, this technique is intended to be used to restore the male fertility in patients with azoospermia. In experimental animals, azoospermia has been treated by the repopulation of the testes with SSCs obtained from different animals (40). However, in humans, the main clinical objective is to obtain SSCs from a subject to repopulate the same testis (41) after a sterilizing treatment (such as chemotherapy on prepubertal boys because the spermatogenesis has not been started in these patients). This presents the problem of reimplanting SSCs into destructured testis, and this problem leaded to think about another alternative option: to develop in vitro spermatogenesis and subsequent cryopreservation of the generated mature spermatozoa. Then, these in vitro–derived spermatozoa could be used in assisted reproduction techniques to obtain live offspring. This issue will be useful, for instance, in patients with maturation arrest (42) where the testicular niches are unable to produce the factors needed to support spermatogenesis.

On the other hand, the embryonic stem cells are pluripotent cells able to undergo meiosis and generate functional haploid male gametes in vitro (43), as shown by Nayernia et al., achieving fertilization after intracytoplasmic injection into mouse oocytes. Then, the resulting two-cell embryos were transferred into oviducts and live mice were born. However, the pups born were either too small or too large and died prematurely (44). So this technique is unable to be employed in the treatment of infertility in humans.

Maintaining the genetic integrity of testicular stem cells is important to preserve the quality and function of the differentiated cells, but also to guarantee a correct transmission of genetic information from one generation to the next (43). Adult testicular stem cells are thus essential for both the production of gametes and the continuation of the species.

Recent studies showed that murine SSCs have the ability to generate pluripotent cells and to differentiate spontaneously into derivates of the three embryonic germ layers (45). This is an important alternative to the use of embryonic stem cells for cell-based regenerative medicine therapy (34).

In conclusion, several research lines are on the way to create sperm cells from different origins, but we must keep in mind that spermatozoa are something else than mere "DNA bags," and they should be structurally and physiologically competent to succeed, as we will discuss in the next section.

MOLECULAR FEATURES OF THE FUNCTIONAL SPERMATOZOA

To develop sperm from reprogrammed or undifferentiated cells, there are some aspects that need to be considered before assisted reproduction with laboratory-created gametes is performed.

Until now, the only analysis to estimate the male potential to become a father is the spermiogram following WHO criteria (32), where sperm volume, concentration, and the percentage of spermatozoa with progressive motility and normal shape are the main parameters considered. When men's fertility is analyzed, these values have been considered the best indicators for decades.

Interestingly, there are a significant proportion of infertile males presenting normal sperm counts, thus diagnosed as having idiopathic infertility (46). This fact is clearly pointing to some underlying defects within their spermatozoa, confirming that male fertility is something else than having a high sperm output.

Several studies arisen in the last years about molecular factors implicated in sperm biology, which need to be considered when "creating" successful spermatozoa in the laboratory.

Chromosomal Content and Genetic Status

Obviously, sperm chromosomal content is a key issue to consider when deriving sperm cells from stem cells, mainly taking into account that a correct chromosomal content obtained from an adequate meiotic division from diploid cells is necessary.

Actually, except chromosomal aneuploidies (47,48) or Y chromosome microdeletions investigations (49), both linked to severe alterations in sperm count, no molecular features of sperm complementing the basic sperm analysis are available as a diagnostic tool.

The presence of chromosomal abnormalities in infertile males' ejaculates is relatively frequent, as the severity of the male factor increases, reaching almost 40% of males with sperm counts lower than 2 to 4 million (50), while the presence of microdeletions is confirmed in 14% of azoospermic males (49).

This clearly indicates a close relationship between testicular function and genetic problems that should be seriously considered when creating spermatozoa from stem cells.

Nevertheless, the fluorescent in situ hybridization techniques to confirm the absence of chromosomal aneuploidies in created sperm are widely employed and available in research laboratories, and these tests are mandatory before the sperm cells are employed, though the presence of microdeletions caused by the methodology of gamete creation are unlikely.

Imprinting

Epigenetic mechanisms, including DNA methylation, covalent histone modification, chromatin remodeling acting on mRNA transcription, and postranscriptional modifications by noncoding mRNAs, are genetically regulating the expression of one allelic copy depending on their parental origin.

These genes present a highly relevant role in embryo development.

Given that epigenetic regulation is done during gametogenesis, this is a fundamental issue that needs to be tackled when aiming to create sperm from stem cells.

On the germ line, the epigenetic reprogrammation begins with the complete deletion of all genes, to begin with the de novo methylation and chromatin remodeling of all the genome (51). This reprogrammation provides to the gametes the molecular programs to activate the oocyte, the zygote, and embryo development.

Given that the genetic imprinting establishment is produced during gametogenesis, it has been postulated that this process can be affected as a consequence of assisted reproduction treatments, mainly caused when immature sperm cells are employed, as a consequence of ovarian hyperstimulation protocols, in vitro oocyte maturation, germ cell cryopreservation, and the stressful mechanisms caused by IVF micromanipulation procedures (52).

This fact is especially relevant when we consider their consequences. Several diseases have been related to imprinting defects, such as neurological and developmental diseases (Beckwith-Wiedemann, Prader-Willi, and Angelman syndromes), metabolic diseases (neonatal transitory diabetes mellitus), psychiatric and behavioral diseases (autism, schizophrenia, and bipolar disease), and cancer (retinoblastoma, Wilm's tumor, osteosarcoma, and rhabdomyosarcoma).

Moreover, some authors found data from newborns obtained by means of assisted reproduction supporting these affirmations, although it is still a controversial issue.

The same happens with imprinting caused infertility: several authors found in their works results supporting a close link between imprinting defects and infertility, but this needs to be further confirmed (51,52).

Sperm DNA Integrity

To date, there are a lot of studies concerning DNA analysis of human spermatozoa suggesting that the determination of DNA fragmentation levels can be a parameter of semen quality, directly implicated in male fertility (53,54).

Sperm chromatin structure assay (SCSA) from the works of Evenson et al. (55) has been the initial methodology to determine sperm DNA alterations, and several works have found a negative predictive value in pregnancy outcome in ART (56,57). A strong correlation between the SCD and SCSA tests has been found (58), as expected, since both of these tests determine susceptibility of sperm DNA to acid denaturation in vitro.

Nevertheless, among all the studies presented, highly controversial results have been reported (59). A high percentage of the works is unable to find a significant relationship between sperm DNA fragmentation and the incapacity to reach pregnancy. Furthermore, among all the research work finding a positive relationship, different thresholds and cut-off values have been settled, limiting the clinical application of the test.

Moreover, some works are openly against the value of the measurement of the DNA fragmentation extent to predict fertility potential (60–63).

When we compared the results of the sperm DNA fragmentation on the same samples employed in IUI cycles achieving a pregnancy with those who failed, in a total number of 100 samples, there were no statistical differences (62).

In IVF procedures, we can obtain further data to determine the influence of sperm DNA fragmentation in male fertility, due to the close follow-up of embryo development (61). In this work, a negative correlation with fertilization, increased proportion of zygotes showing asynchrony between the nucleolar precursor bodies of zygote pronuclei (73.8% vs. 28.8%, $p = 0.001$), implantation rate, and slower embryo development together with worse morphology on day 6 was correlated with higher sperm DNA fragmentation samples was also found, and that this situation is not compromising pregnancy chances, probably as a consequence of the embryo selection before transfer (61).

Oxidative Stress Defense in Sperm

Reactive oxygen species (ROS) are by-products of the physiological metabolism of O_2 in cells under aerobic conditions, necessary for the normal cell function in organs or tissues but controlled by the antioxidants content (64,65).

In sperm, ROS in controlled levels are required for the achievement of fertilizing ability (66), fusion with the oocyte, and capacitation (67,68).

Under some circumstances, ROS are highly reactive against cellular structures and molecules, interfering with their biological functions and properties (69,70).

Therefore, oxidative stress (OS) in sperm is defined as the disequilibrium between pro-oxidative and antioxidative molecules in a complex biological system, where the oxidants prevail over the defensive systems, causing damage to DNA molecules, deregulating the acrosome reaction, and impeding the sperm/oocyte recognition and fusion (69,70).

It is known that human spermatozoa are extremely sensitive to ROS-induced damage due to their special plasma membrane composition (71). This sensitivity is related to their high content of polyunsaturated fatty acids. This results in the fact that sperm-egg fusion, acrosome reaction, and motility are compromised in OS situations.

Free radicals overload has been correlated with diminished fertilization rates and also with bad embryo quality (72).

GPX-4 and GPX-1 are differentially expressed in fertile and infertile males, and the protein formed from these mRNA exhibited altered parameters, thus suggesting a role of these enzymes in male fertility (73,74).

Moreover, when day 3 embryo parameters were evaluated, GPX4 mRNA expression in sperm cells was significantly lower when asymmetric embryos were observed, and poorer embryo development and morphology on day 5 was statistically related to lower sperm GPX1 activity (74).

The appropriate control of these systems done by the molecular content of sperm cells is essential for the reproductive function.

Other Molecular Keys of Sperm Physiology

Other molecules have been described to be relevant in sperm function, and then they should be considered to be important in "artificially created" sperm.

Some of them are involved in the apoptosis process. For instance, when selecting nonapoptotic sperm cells via immunomagnetic separation after cell labeling against caspase (75), sperm fertilization potential showed to be higher, as assessed using hamster oocyte penetration assay and hamster oocyte-ICSI.

Other example is platelet-activating factor (PAF), a signaling phospholipid found in sperm that has been studied and related to fertilizing potential (76).

Also heat shock protein 2A (Hsp2A) has been detected in sperm and present in higher levels in infertile males (77). The same group tested whether low sperm Hsp2A ratios and CK activity were predictive of IVF pregnancies, showing excellent results, and the selection of sperm to bind HA-coated slides causes a significant decrease in sperm chromosomal anomalies, therefore providing better results in ICSI treatments.

Ubiquitin forms covalently linked polyubiquitin chains on substrate proteins and targets forming "ubiquitinated" substrates for degradation to collaborate in the removal of defective sperm and debris.

Increased levels of sperm ubiquitination in men with idiopathic infertility were found (78). This could reveal the presence of a defect on the defective sperm removal, thus increasing the fertilization chances by a malfunctioning sperm cell. Moreover, some negative correlations between sperm ubiquitin and embryo development have been described (79).

In a recent work, we studied the implications of mucin MUC-1 (80) in sperm function, infertile patients showed less spermatozoa stained than fertile sperm donors.

This is the demonstration that many molecular features of sperm need to be considered before tagging spermatozoa as functional or not.

Information Obtained from the Application of Massive Analysis Techniques of Sperm

Finally, the massive analysis of sperm, by means of proteomics or genomics technology, reveals huge differences between the molecular features of sperm able to yield a healthy pregnancy or not.

The development of the microarray technology, derived from the results of the human genome project, made it possible to determine cell or tissues expression profiles of the whole genome, enabling the comparison between two different biological conditions, which is expected to be essential in the development of research, diagnostic, and therapeutic tools (81).

Sperm mRNA was first described in the late 1980s in different animals, and the mRNA detection as a potential infertility marker in human sperm was seriously considered several years later (82,83).

There are evidences about the need for an adequate embryo formation of sperm-delivered mRNAs, from experiments confirming the lack of success in the oocyte's parthenogenetic activation, the absence of certain mRNAs within the oocyte, and the implication of concrete sperm mRNAs to embryo development (84–86). In these works, the bases for future research were established, since several known and unknown protein-coding mRNAs were described.

A comparison between ejaculates was obtained from idiopathically infertile males, with normal sperm counts and partners without evident infertility problems, in comparison with healthy and previously fertile sperm donors. Numerous genes were found to be differentially expressed in both directions, when a cut-off value of 2 was settled (87), expressed at least twice in fertile versus infertile males or vice versa. Only considering those genes expressed at least 10 times in one of the groups in comparison with the other, a total number of 116 genes were found. These results were confirmed by random quantitative PCR.

Among them, some were involved in spermatogenesis, such as microseminoprotein-β, t-complex-associated-testis-expressed-1 like-1, sperm-associated antigen 5 (SPAG5), and spermatogenesis-associated gene 7 (SPATA7), which are overexpressed in fertile males in comparison with infertile males without a decreased sperm count and motility. Additionally, some genes with roles not yet described in spermatogenesis such as ornithine decarboxylase antizyme 3 (OAZ3), the suppressor of potassium transport defect (SKD), and ribosomal protein (RPS3A) were found with very different expression levels, being overexpressed in the fertile patients group (87).

Moreover, when applying functional analysis platforms to analyze genes grouped on the basis of their function blindly (the analyst unknowing the origin of the data), at different grouping levels of analysis, several processes implicated on gamete formation are found to be different between both groups. These analyses statistically test if the existence of a determined number of genes over- or underexpressed within each group is due to a real effect or chance.

As described in the Figure 2, several groups reveal a significant disparity between the proportions of genes over- or underexpressed in infertile males and fertile sperm donors. In a different analysis level, different biochemical pathways or processes are implicated. Interestingly, when over or underexpressed genes in infertile males were grouped by their function, those involving spermatozoa differentiation were present at all analysis levels. Also, a comparison between genes expressed in fertile males' sperm, not found in infertile males, and inversely, detected in infertile males while they are not within sperm of fertile males was performed.

We found 27 spermatogenesis- or testes-related genes expressed only in previously fertile sperm donors not expressed in infertile males with normal sperm count. Only one was expressed in infertile males, while it was not present in controls. Among those 27 genes, we can

remark, for example, ODFP2 (outer dense fiber protein 2) that may have a modulating influence on sperm motility and SPAT9 (spermatogenesis-associated protein 9) that is not expressed in testes of patients with Sertoli cell–only syndrome and shows variable expression in patients with spermatogenic arrest. Meanwhile, the unique gene in infertile males' group not present in fertile donors was H2BWT (H2B histone family member W testis specific), an atypical histone H2B that does not recruit chromosome condensation factors and does not participate in the assembly of mitotic chromosomes, if present (88). These findings are even more interesting when we consider that sperm samples did not differ in terms of sperm concentration and motility between fertile donors and infertile males, thus being focused on idiopathic male infertility.

Proteomic mapping of male infertility has also been recently attempted. This technology analyzes a different aspect of the sperm physiology that could potentially be employed as an independent fertility predictor. The information contained in the sperm mRNA will be translated into proteins that will be lately modified and will result in the functional biomolecules of spermatozoa. Both human seminal plasma and sperm have been tested by means of this technology to determine their protein profile (89,90). Lots of different spots can be found from the bidimensional electrophoresis analysis of sperm protein profile, representing each a single protein.

A list of proteins present in human spermatozoa has been recently reported from sperm lysates in both soluble and insoluble fractions.

No report considered yet the differences between fertile and infertile males, and this technology seems to be a step forward from the wide genomic analysis. Only the description of a case report of in vitro fertilization failure in comparison with fertile donors has been published (91).

Then, we can conclude that there are still several difficulties to confirm if derived sperm could behave like sperm from fertile males, before undergoing assisted reproduction with guarantees.

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9 Growth Factor Signaling in Germline Specification and Maintenance of Stem Cell Pluripotency

Hsu-Hsin Chen and Niels Geijsen

INTRODUCTION

The critical importance of germ cells in the propagation of a species has long inspired research into the origin of these cells. In many lower organisms, germ cell specification is controlled by specialized germ plasma; a maternally inherited complex of proteins and RNA that is localized to the posterior pole of the unfertilized egg (1–3). Following the first cleavage divisions, cells that receive the germ plasma are destined to become the germline stem cells. Such a predetermination model of germ cell specification assures that germ cells are set aside during the earliest steps of embryonic development, protecting them from the lineage specification and differentiation events that craft the somatic lineages of the embryo.

Unlike the rigid specification of germ cells in lower organisms, mammalian segregation of germline and somatic cells occurs later in embryonic development and is guided by extracellular cues (1,4). Growth factor signals converging in the proximal epiblast define a band of cells, which are protected from differentiation at the time when the body plan of the embryo is laid out. Recently, several marker genes have been described, which allow the early identification of the developing primordial germ cells (PGCs).

MOLECULAR MARKERS OF MAMMALIAN PGC SPECIFICATION

The first PGCs can be detected in the proximal epiblast at E6.5 of murine embryonic development (5,6) as a small cluster of 6 to 10 cells that express the molecular marker Blimp-1. These Blimp-1 positive cells migrate to the base of the allantois where at E7.5 they start expressing a second germ cell–specific marker gene, Stella (Dppa3) (4).

Blimp-1 knockout studies have demonstrated an essential role for this gene in germ cell determination (5,6). In the absence of Blimp-1 expression, early PGCs fail to suppress Hox gene expression, resulting in the activation of a somatic differentiation program and loss of germ cell identity. The germ cell–specific expression of Stella during the specification of these cells suggested a role in the cellular commitment to the germline, but germ cell development proceeds normally in Stella knockout mice (7,8). Stella-deficient animals are viable, and both sexes are fertile and demonstrate no defects in germ cell development. However, litters from Stella-deficient females are small, and further analysis of second-generation embryos revealed that early development is impaired, and most embryos fail to develop to the blastocyst stage. Impaired development could not be rescued by crossing the Stella-deficient females with wild-type males, demonstrating that Stella is an important maternal factor that plays a critical role during preimplantation development (8). While the molecular role of Stella is not fully understood, a recent report suggests that Stella may act to protect the maternal genome from demethylation shortly after fertilization (9). Regardless of its function, Stella remains the most faithful molecular marker for early germ cell fate commitment. Unlike Blimp-1, which is also expressed in the developing primitive endoderm, Stella expression is restricted to developing PGCs and thus can be used to track the development of these cells both in vitro and in vivo (10).

The early PGCs form and reside in the proximal epiblast at the boundary between embryonic and extraembryonic tissues. Several important morphogen signals intersect at this location and likely play an important role in orchestrating mammalian germ cell specification.

SIGNALS DETERMINING GERM CELL SPECIFICATION

The TGF-β family is a large group of cytokines playing pivotal roles in various aspects of embryonic development (reviewed in Ref. 11). The cytokines consist of homo- or heterodimers, which bind and activate specific type I and type II receptor serine/threonine kinases at the cell membrane. Upon ligand stimulation, type II receptor phosphorylates and thereby activates the type I receptor kinase, which in turn phosphorylates the downstream R-SMAD proteins. Phosphorylation of the R-SMADs by the receptor kinases promotes their nuclear localization and dimer/trimer formation with SMAD4 (reviewed in Ref. 12); the R-SMAD-SMAD4 oligomers then directly bind to DNA and other transcription factors.

TGF-β cytokines are divided into two subgroups: the TGF-β/Activin/Nodal subfamily and the BMP/GDF/MIS subfamily, each of which activates distinct combinations of receptors and downstream effectors (11). Members of both subfamilies are implicated in the establishment of germ cell fate in the mouse embryo, although the specific roles of these cytokines and the interaction among these signaling pathways are still unclear.

BMP4, BMP8b, and BMP2

Mouse embryos lacking BMP4 fail to form any PGCs, whereas BMP4 +/− embryos show a ~50% reduction in PGC number compared to wild type, regardless of the genetic background (13). BMP8b knockout embryos display a similar phenotype in the C57Bl/6 background but the phenotype is much less severe in other genetic backgrounds (14). Thus, although both BMP4 and BMP8b are essential for PGC development, the former appears to be the more dominant signal. In addition, BMP4+/− BMP8+/− double heterozygotes did not differ in PGC number from the BMP4 heterozygotes, suggesting that BMP4 and BMP8b may function as heterodimers as well as homodimers in vivo (14). BMP2 is highly homologous to BMP4 in primary sequence. BMP2-null embryos also showed a reduction in PGC number at early developmental stages; however, its effect is much smaller than BMP4 and BMP8b (15). It has been suggested that BMP2 homodimers may function in the same fashion as BMP4 homodimers, while the source and timing of these signals are different (see the following text).

BMP4 expression becomes detectable at E5.5 in the extraembryonic ectoderm adjacent to the epiblast (13). Its expression in this region continues throughout gastrulation, spanning the window of PGC lineage restriction (E6.5–E7.25, early midstreak stage). In situ hybridization showed that BMP8b transcript is also expressed in the extraembryonic ectoderm during this period (14). BMP2, on the other hand, is detected in the posterior visceral endoderm (VE) at E5.0 to E6.5 by in situ hybridization (15). The proximal epiblast cells that will soon become founders of the PGCs would then be receiving multiple BMP signals from two different directions. The multiple sources of signals in vivo may determine the confinement of PGCs in their specific location; however, the requirement of each BMP signal for the induction of PGC fate in vitro is still controversial. In one study, epiblasts deprived of the extraembryonic ectoderm at E5.5 to E5.75 could still form PGCs when recombinant BMP4 is supplied at high concentration (16), showing that BMP8b function may be replaceable by BMP4. However, when E6.0 to 6.25 epiblasts are grown on COS cells transfected with BMP expression vectors, the only feeder cells that could support PGC formation are the ones expressing both BMP4 and BMP8b (17). It has been proposed that BMP4-BMP8b dimers may be the most potent signal, whereas the higher concentration of the BMP4 homodimers could overcome the signaling threshold (18). The requirement for BMP2 in tissue culture environment is also likely to be dispensable.

Of the type I BMP receptors, only ALK3 expression has been detected in the early epiblast (19); that is, ALK3 is the only candidate receptor for the above-mentioned BMPs in the founder cells of PGC. ALK3-null embryos could only survive till E8.0 without developing any mesoderm; its effect on PGC formation has not been investigated in the ALK3+/− heterozygotes (20). The genetic study on ALK2 revealed another level of complexity of BMP signaling in PGC lineage restriction. ALK2 is not detected in the epiblast but is expressed in the VE surrounding the boundaries of the epiblast and the extraembryonic. Interestingly, deletion of ALK2 also resulted in total ablation of PGCs in the mouse embryos, and this effect is dose dependent as well (21). Furthermore, expression of constitutively active ALK2 in the VE could partially rescue PGC formation in the BMP4−/− embryo (21). Hence the effect of BMP4 on PGC formation is at least partially indirect, acting through the VE.

There are three type II receptors for BMPs: BMP type II receptor (BMPRII) and activin type II receptors A and B (ActRII, ActRIIB) (11). The effect of knocking out these receptors on PGC formation has not been studied. BMPRII mutant embryos displayed similar phenotypes as the ALK3 knockouts, and BMPRII transcript is expressed ubiquitously in the early embryo, suggesting it might be the relevant type II receptor for the BMPs in PGC formation (22). While the ActRII knockout mice are viable and fertile, and that the ActRIIB knockout also survive till birth (23), the ActRII/IIB double knockouts are arrested before gastrulation (24). Since the transcripts for both activin type II receptors are detected at E5.5 to E7.5, they may also play a role in mediating the BMP signaling in germ cell fate determination. However, the phenotypes associated with the disruption of activin type II receptor genes are likely due to inactivation of the Nodal signaling pathways, which we shall discuss in the following section.

The main downstream effectors of the BMP signaling pathways are SMAD1, SMAD5, and SMAD8 (12), all of which are expressed in the pregastrulation epiblast. Total phosphorylation of these three SMADs is upregulated in the epiblast upon recombinant BMP4 treatment (25). SMAD1−/− embryos could form virtually no PGCs as judged by alkaline phosphatase activity, evidently indicating the requirement of SMAD1 in the BMP pathways (25,26). SMAD5 deletion also resulted in a major reduction in PGC number, and the effect is dose dependent as in the case of the BMPs discussed in the preceding text (27). The role of SMAD8 in PGC formation has not yet been explored.

When the extraembryonic ectoderm is dissected away from the epiblast at E5.25, BMP4 treatment fails to induce PGC formation as it would in epiblast from embryos at E5.5 (16). The identity of this required signal from the extraembryonic ectoderm during this period is still unknown. In this time window, SMAD5 is upregulated in the proximal but downregulated in the distal epiblast, and its expression in the specific cell populations may account for the change in their responsiveness to BMP4 (16), Interestingly, in a separate study, the expression patterns of SMADs 1, 5, and 8 at E6.25 were found to be heterogeneous in single-cell RT-PCR from the proximal epiblast (26). It is thus possible that the earlier signals from the extraembryonic ectoderm determine the SMAD signature of individual epiblast cell, which in turn dictates the potential of each cell to commit to the PGC lineage.

Similar predetermination signals from other sources are also essential. Embryos deprived of the VE from E5.5 to E6.0 also failed to form PGCs in culture, regardless of the presence of BMP4 in the culture media (21). This unidentified signal may be dependent on ALK2 expression in the VE, since the time point and the resulting phenotype are similar between ALK2-null embryos and explants without VE, and that in both cases their phenotype can be partially rescued by coculture with STO feeder cells (21). The identity of this VE-derived signal remains unknown.

In summary, the PGC founder cells in the proximal epiblast require a combination of BMP signals that are both spatially and temporally regulated to attain germ cell fate. We have summarized current data and models in Figure 1. At E5.25 to E5.5, the extraembryonic ectoderm induces a change in responsiveness to BMP in the proximal epiblast, possibly by upregulating SMAD5. Later at E5.5 to E6.0, the VE releases another unknown signal to the proximal epiblast, possibly following the activation of BMP4-ALK2 pathway in the VE. During the window of PGC lineage restriction (E6.5–E7.25), BMP4 and BMP8b from the extraembryonic ectoderm and BMP2 from the VE activate the ALK3/BMPRII receptors in the PGC founders cells, which are primed for germ cell fate commitment by the earlier sequence of signals.

Nodal

Nodal is a chordate-specific cytokine that belongs to the same subfamily as TGF-β and activin (reviewed in Ref. 28). Nodal signaling establishes the anterior-posterior axis, induces mesoderm and endoderm formation, and sets up the left-right asymmetry in early embryogenesis. Homozygous deletion of Nodal results in the loss of primitive streak and early embryonic lethality (29–31). The role of Nodal signaling in PGC fate decision has not been looked into; however, there is sufficient circumstantial evidence to suggest that this pathway may also be involved in germline determination.

Nodal is distinct from other TGF-β family members in that it requires a coreceptor in order to activate the type I/type II receptor complex (reviewed in Ref. 32). There are two

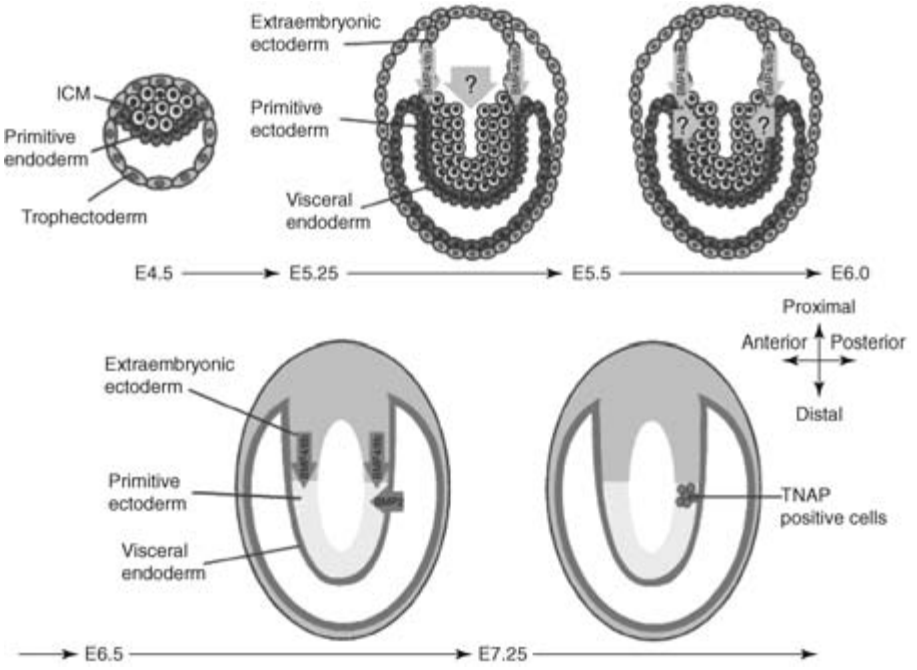

Figure 1 Signals required for PGC formation in mouse embryonic development. PGC specification requires multiple signals from different sources during mouse development. At E5.25 to E5.5, a yet unknown signal generated by the extraembryonic ectoderm is needed to prime the cells for later BMP signals, presumably by upregulating SMAD5 in selected cell populations. At E5.5 to E6.0, a second unknown signal from the VE is required. This signal is dependent on ALK2 signaling in the visceral endodermal cells, suggesting that the BMP4/8b from the extraembryonic ectoderm stimulate the production of this second unknown signal. The lineage restriction of PGCs occurs at E6.5 to E7.25. At this last stage of PGC specification, both BMP4/8b signals from the extraembryonic ectoderm and BMP2 signal from the VE are required; the BMP2 expression is the highest in the posterior region of the VE. After E7.25, PGCs are detectable by alkaline phosphatase activity. *Abbreviations*: ICM, inner cell mass; TNAP, tissue nonspecific alkaline phosphatase; PGC, primordial germ cell; VE, visceral endoderm.

coreceptor genes in human and mouse: Cripto and Cryptic. Cripto expression is required for gastrulation (33), whereas Cryptic is important in the later process of setting up the left-right axis (34). The known type I receptor for Nodal is ALK4, although ALK7 may also be a potential receptor, and the type II receptors are likely to be ActRIIB and ActRII (24). Activation by Nodal/Cripto induces phosphorylation of SMAD2 and possibly SMAD3 as well. Nevertheless, other SMAD-independent pathways are also involved, since deletion of either SMAD2 (35) or SMAD4 does not incur all the phenotypes observed in Nodal knockouts.

The expression of Nodal mRNA is detected throughout the primitive ectoderm and VE at E5.5. By E6.5, Nodal expression is lost from the VE and becomes localized in the proximal-posterior region of the epiblast (31). Cripto is also expressed uniformly in the primitive ectoderm, and its expression pattern in the epiblast follows that of Nodal in the pregastrulation and gastrulation stages (32). In fact, Cripto transcription in the epiblast is dependent on Nodal signaling (29). Thus, the posterior proximal epiblast, which contains the founder cells of PGCs, expresses the highest level of both Nodal and Cripto before and during the time window of germ cell fate decision.

In addition to the consistent expression profiles, studies of ES cell pluripotency also hint at a potential functional role of Nodal in PGC specification. Human ES cells and mouse inner cell mass outgrowth from blastocysts both require SMAD2/3 phosphorylation, which is downstream of nodal/activin, to maintain pluripotency (36,37). In the human ES cells, conditions that preserve pluripotency correlate with activation of SMAD2/3 and inhibition of SMAD1/5 (36), suggesting an antagonistic relationship of the Nodal pathway and the BMP

pathways. Interestingly, SMAD2/3 activation is not required for the maintenance of mouse ES cell pluripotency; moreover, it has been suggested that the human ES cells resemble the early ICM cells, whereas the mouse ES cells may be more similar to the primitive germ cells. The different growth factor requirements between the human and mouse ES cells may thus reflect their putative difference in origin, alluding to an involvement of Nodal in segregating the germline from somatic cells.

As mentioned earlier, TGF-β family members could form both homodimers and heterodimers. Nodal has been found to form heterodimers with BMP4, and this interaction inhibits both Nodal and BMP4 signaling (38). It is not yet clear whether such an interaction exists in vivo and whether the heterodimerization could occur extracellularly, since Nodal and BMP4 are produced in different cell types. Nevertheless, the potential of competition between TGF-β pathways in determining germ cell fate is intriguing.

FIBROBLAST GROWTH FACTORS

Of the 22 fibroblast growth factors (FGFs) in the mammalian genome, FGF2 (bFGF) and FGF7 have been shown to be important in the post-gastrulae migration of the PGCs to the genital ridges (39). FGF2 has also been shown to promote PGC proliferation in vitro (reviewed in Ref. 40). However, mice lacking FGF2 are viable and fertile (41–43), suggesting possible compensation signals from other FGFs. Whether the FGFs are also involved in the early events that determine PGC fate is not yet clear; nevertheless several FGF pathways have been shown to be essential for early embryonic development (44,45).

In human ES cells, FGF signaling pathways have been found to cooperate with Nodal signaling (37) and to suppress BMP signaling (46) in maintaining pluripotency. A recent report demonstrated that activation of the Nodal signaling pathway in human ES cells increases the expression of FGF4 and bFGF, while the expression of FGF receptors is downregulated (47). Given the importance of TGF-β signals in PGC development, it is probable that the FGFs also play a role in specifying the germline.

At the egg-cylinder stage, FGF4 is expressed in the epiblast and its receptor FGFR2 is expressed in the epiblast and at a higher level in the extraembryonic ectoderm. Both FGF4- and FGFR2-null embryos do not survive past the egg-cylinder stage, due to defects in ICM proliferation and trophoblast proliferation (44,48). The temporal and spatial expression patterns of FGF4 and FGFR2 thus support a putative role in PGC specification.

Furthermore, FGF4 is required, along with FGF8, for cell movement from the primitive streak during gastrulation (49), and the expression pattern of FGF8 is intriguing from the viewpoint of germline development. Just before gastrulation, FGF8 is first detected in a group of cells in the posterior epiblast, the same area of PGC formation, and also in the VE. It would be interesting to test whether the FGF8-activated signaling pathways at this stage would also affect germ cell fate decisions in the embryo.

In other developmental systems, changes in the level of FGF4 or FGF8 have been shown to be important for retinoic acid–mediated processes (50–52). As we speculated earlier, retinoic acid may be the extraembryonic signal required for potentiating the proximal epiblast. It is possible that FGF4 and FGF8 are also involved in changing the responsiveness to BMP4 at E5.25 to E5.5.

GERM CELLS AND PLURIPOTENT STEM CELLS

The above signaling pathways have been implicated to play an important role in germ cell specification and differentiation, but their functional role in these processes remains to be determined. Experimental evidence, both in vivo and in vitro, points toward a role in suppressing somatic differentiation and maintaining a so-called pluripotent state. Stem cell pluripotency is the ability of cells to generate derivatives of all embryonic germ layers. Somatic differentiation and commitment to a cellular lineage are associated with loss of pluripotent potential. In contrast, germ cells retain full pluripotent potential up to the initiation of meiosis. When removed from the developmental restraints imposed by the somatic microenvironment, in vitro cultures of PGCs isolated from embryonic gonads give rise to embryonic germ cells lines (EG cell lines) which, similar to embryonic stem cells (ES cells) can be propagated indefinitely in vitro while maintaining the ability to give rise to derivatives of all embryonic

germ layers (53–55). In similar fashion, spermatogonial stem cells from newborn and adult testes were recently shown to yield pluripotent stem cell lines, upon brief in vitro culture (56–58). Thus, in vitro, germ cells reveal their *ability* to convert to a pluripotent state. It is important however to note that when left in the context of the gonadal microenvironment, germline stem cells are fated to do just one thing, generate oocytes or sperm. This stresses the importance of the somatic microenvironment in repressing the germ cell pluripotent conversion and preventing the emergence of teratomas, tumors of pluripotent origin. Much less clear is the role of growth factor signaling during the in vitro derivation and maintenance of pluripotent stem cell lines. Similar to their role in suppressing differentiation during PGC specification in the early embryo, growth factors present in the tissue culture media may merely act to prevent somatic differentiation. Indeed, growth factor withdrawal from the tissue culture media generally induces stem cell differentiation. Yet, signals in the tissue culture environment may play an additional role in defining the pluripotent stem cell state itself. The recent derivation of pluripotent stem cell lines from murine epiblast embryos (59,60), and our derivation of a novel type of stem cell from murine blastocyst embryos (61) demonstrates that in the mouse, entirely different growth factor combinations are able to support the stem cell pluripotent state. Moreover, pluripotent stem cells cultured under different growth factor combinations, while all capable of multi-germ layer differentiation, demonstrate remarkable differences in their morphology, gene expression profile, and developmental capabilities, demonstrating that growth factor signaling is a critical determinant of pluripotent stem cell identity.

The functional role of growth factor signaling in mammalian germ cell specification may be difficult to dissect in vivo. Redundancy in signaling between the various members of the FGF family, and possible cross talk with other signaling pathways including BMP and Nodal signaling, make it difficult to dissect the possible roles of individual members of this large family of growth factors in PGC specification and migration. Further analysis of the role of growth factor signaling in defining different pluripotent stem cell states may, in this case, provide a tractable tool for future analyses.

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10 Stem Cell–Based Therapeutic Approaches for Treatment of Male Infertility

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INTRODUCTION

Stem cells have the capacity for self-renewal and the ability to differentiate to various cell lineages. Thus, they represent an important building block for regenerative medicine and tissue engineering. Current research focuses on the possible exploitation of stem cells in medicine and their potential to offer a range of effective treatments for various diseases. A variety of stem cells, ranging from embryonic, bone marrow, endogenous, and amniotic fluid have been investigated and may prove useful as novel alternatives for organ regeneration both in vitro and in vivo. ESCs are pluripotent cells derived from the inner cell mass of the early mammalian embryo. Because of their plasticity and potentially unlimited capacity for self-renewal, ESCs have generated tremendous interest both as models for developmental biology and as possible tools for regenerative medicine. This excitement has been attenuated, however, by scientific, political, and ethical considerations. To exploit this potential, it is essential to be able to control ESC differentiation and to direct the development of these cells along specific pathways. Embryology has offered important insights into key pathways regulating ESC differentiation, resulting in advances in modeling gastrulation in vitro and in the efficient induction of endoderm, mesoderm, and ectoderm, and many of their downstream derivatives. This has led to the identification of new multipotential progenitors for the hematopoietic, neural, and cardiovascular lineages and to the development of protocols for the efficient generation of a broad spectrum of cell types including hematopoietic cells, cardiomyocytes, oligodendrocytes, dopamine neurons, and immature pancreatic β- cells. The next challenge will be to demonstrate the functional utility of these cells, both in vitro and in preclinical models of human disease.

The germline stem cells in the mammalian testis form the basis of male fertility. Aberrant germ cell development can result in abnormal gonadal function, incomplete embryogenesis and infertility, or germ cell tumors. Our understanding of the molecular regulation of normal germ cell development in mammals has progressed significantly because of the utility of the mouse as a genetic model system. However, the molecular regulation of human germ cell development is almost completely unknown due to the historical lack of a malleable model. IVF has been an efficient medical treatment for infertility in the past decades. However, conventional IVF approaches may be insufficient when gametes are lacking or nonviable, thus precluding a significant number of patients from treatment. The use of donor gametes may bring legal, ethical, and even social problems of acceptance that can discourage infertile couples from the donor route. Fortunately, emerging reproductive and stem cell technologies, and preliminary results from animal experiments provide some hope for alternative sources of gametes through which these infertile patients can finally conceive their own genetic child.

DEVELOPMENT OF MALE GERM CELLS

Studies in previous years have enormously advanced our understanding of the process of germ cell development. In the mouse, primordial germ cells (PGCs), the progenitor cells of the germline are apparent in the embryo at embryonic day E7.25 (2). They arise from the epiblast, early in gastrulation, and then as they start to proliferate, they migrate from the allantoic base along the hindgut to finally arrive at the genital ridge (3). Formation of the primary testis cords occurs as the somatic-origin Sertoli cells migrate inward and envelope the PGCs. During the formation of the seminiferous tubule, PGCs continue to proliferate and differentiate into gonocytes, and then remain mitotically quiescent until birth. A few days after birth, the gonocytes resume proliferation, move to the basal membrane of the seminiferous tubules, and differentiate into SSCs also known as undifferentiated type-A spermatogonia (4). Upon

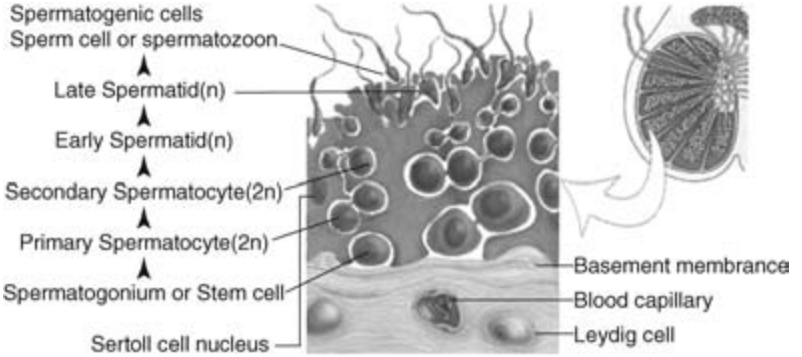

Figure 1 Different stages of development of male germ cells in testis.

division, the SSCs can give rise to two daughter cells which can either separate and lead to stem cell renewal, or can remain connected as a pair of so-called A_{pr} spermatogonia. A_{pr} formation initiates differentiation that ultimately, after a series of maturation steps, leads to the production of spermatozoa (Fig. 1).

DERIVATION OF MALE GERM CELLS FROM ES CELLS

Germline cells are unique in their ability to carry the genetic information onto the next generation. Many studies have focused on the cellular and molecular mechanisms underlying the proliferation and differentiation of germline cells. However, germ cell development in humans has not been elucidated in great detail due to practical and ethical difficulties. A cell-based model that could recapitulate the in vivo conditions of spermatogenesis and provide a readily accessible system to examine the genetic and epigenetic mechanisms of germ cell formation would be extremely valuable. Recent advances have shown that ESCs can potentially fill this need. Studies in the past few years have reported that successful differentiation of mouse ESCs into PGCs as well as into mature male and female gametes can be achieved in vitro (5–7).

On the basis of ESCs carrying an Oct4 promoter-driven GFP reporter gene construct, Hubner et al. (5) were able to visualize the initial steps of germ cell formation in vitro. To restrict the expression of the Oct4-GFP gene only in germ cells, upstream regulatory sequences that drive the expression of Oct4 during blastocyst- and epiblast-stage embryo were deleted. Mouse ESCs transfected with this reporter gene were then allowed to differentiate, and PGCs developed in vitro. Further culture showed that these cells were able to give rise to oocytes, form follicle-like structures with adjacent cells, and subsequently, develop into blastocysts, presumably by parthenogenesis. In contrast with the two-dimensional differentiation approach mentioned above, a three-dimensional method was used by Daley and colleagues (7). In their study, PGCs were also tracked by an Oct4 promoter-driven GFP reporter gene and by immunomagnetic sorting of surface antigen SSEA1-positive cells. It was demonstrated that in vitro differentiation of ESCs into embryoid bodies (EBs) could give rise to PGCs, which in the presence of retinoic acid (RA) proliferate and form embryonic germ cell–like clones that show germline epigenetic modifications. Using FE-J1 antibody that specifically recognizes male meiotic germ cells and round spermatids, EB-derived haploid cells were isolated by flow cytometry. These cells were then injected into oocytes to investigate their biological function. About 20% of the injected oocytes progressed to blastocyst stage. Early evidence that ESCs can produce functional germ cells in vitro was shown by a study from Toyooka et al. (8). Mouse ESCs harboring a gene construct with germ cell–specific mouse vasa homolog (*Mvh*) gene and *LacZ* reporter were used to generate germ cells in vitro. Transplantation of the ES-derived MVH-positive cells into reconstituted testicular tubules demonstrated that they could integrate into a somatic epithelium, undergo meiosis, and develop to some extent into early sperm cells in vivo. However, significant unanswered questions about the functionality of these cells remained to be addressed. But recently, it was shown that mouse ESCs in culture can give rise to SSCs, which are capable of undergoing meiosis and forming functional sperm-like cells that can generate viable transgenic offsprings (9).

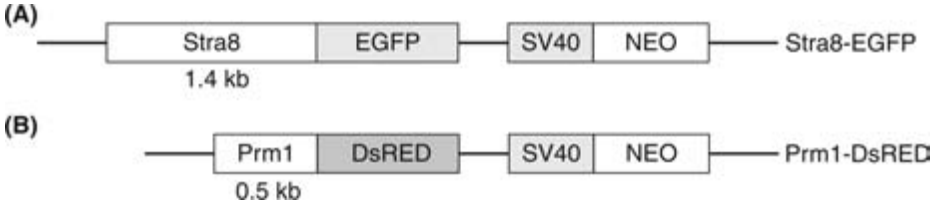

Figure 2 Schematic representation of (**A**) Stra8-EGFP and (**B**) Prm1-DsRED reporter genes harboring a 1.4 and 0.5 kb promoter region of *Stra8* and the mouse protamine 1 (*Prm1*) gene, respectively. These regions are able to direct reporter gene expression specifically in premeiotic and haploid male germ cells, respectively. Both reporter genes contain neomycin phosphotransferase II gene (NEO), which is driven by the SV40 early promoter and enhancer (SV40).

The culture system used by Nayernia and coworkers (9), to demonstrate that ESCs are able to differentiate to mature sperm can fertilize oocytes and produce live animals, involved two stages.

In the first stage, mouse ESCs were transfected with a fusion gene harboring the coding region of enhanced green fluorescent protein (EGFP) under the control of mouse *Stra8* promoter region (10). *Stra8* is an RA-responsive gene that is expressed in premeiotic germ cells and had been shown, in previous studies, that *Stra8* promoter can direct the expression of a reporter gene (Stra8-EGFP) specifically in these cells (11). Transfected mouse Embryonic stem cells (mESCs) were then positively selected using the neomycin phosphotransferase II gene (NEO), which was driven by the SV40 early promoter in the reporter gene (Fig. 2).

Having established mESCs harboring the Stra8-EGFP construct, cells were then cultured in the presence of RA. RA is known to sustain the survival and self-renewal of mouse germ cells in the absence of somatic cell support and also to promote the developmental progression of spermatocytes through early stages of meiosis. After RA induction, EGFP positive cells (later shown by immunohistochemistry and Reverse Transcription - Polymerase Chain Reaction (RT-PCR) to express premeitotic germ cell markers) were isolated by fluorescent activated flow cytometry (FACS).

In the second stage, EGFP-expressing cells were transfected with a second gene construct, which contained the promoter region of protamine 1 gene fused to red fluorescent protein gene (Prm1-DsRED) (Fig. 2). Protamine 1 promoter region is known to be exclusively active in postmeiotic male germ cells (12). Following transfection with Prm1-DsRED construct, positive colonies were then selected by PCR using primers specific for the DsRED-coding region. After a further RA induction for 72 hours, red positive cells that arose from EGFP-expressing cells were released in the medium and could be collected from the supernatant. Isolation and injection of red expressing postmeiotic cells into mouse oocytes gave rise to full-term development of embryos and subsequently to viable animals that carried the transgenic allele.

However, all the animals born were infertile, suffered from severe breathing or walking difficulties, were abnormally large, or had stunted growth. All died within five days to five months of being born, compared with the normal life span of two years for healthy mice. One of the explanations given for the phenotypic abnormalities observed in live animals and the immotile sperm was the impaired establishment of male germline-specific methylation imprints during in vitro ESC-derived gamete formation. This notion was supported by imprinting analysis in the transgenic offspring that showed methylation abnormalities in specific loci. The capacity of the Stra8-EGFP, Prm1-DsRED transfected cells for further development was also investigated in vivo. Cells were transplanted into one of the testes of germ cell–depleted recipient mice. Histological analysis of testes after four months showed the appearance of spermatogenesis-like structures and sperm in the lumen. However, all sperm were immotile or showed reduced motility.

DERIVATION OF MALE GERM CELLS FROM SOMATIC STEM CELLS

Accumulated evidence suggests that in addition to hematopoietic stem cells, bone marrow also harbors endothelial stem cells (EnSCs), mesenchymal stem cells (MSCs), and multipotent adult progenitor cells (MAPCs). Recently, it has also been shown that bone marrow contains a population of stem cells that express early developmental markers such as SSEA and Oct4 (13). These are markers of embryonic stem cells, epiblast stem cells, and PGCs. The presence of these

cells in the bone marrow supports the concept that a population of pluripotent stem cells resides in the adult bone marrow, these pluripotent cells are probably deposited there at the early gastrulation stage during embryo development (13). (During embryonic development, some of the stem cells from germ lineage may go astray on their way to the genital ridges and colonize at fetal level, and subsequently, by the end of the second trimester of gestation, together with fetal liver-derived hematopoietic stem cells, move to bone marrow tissue.) These cells were named very small embryonic-like (VSEL) stem cells (13).

Recently, it has been discovered that bone marrow grafts to female mice, and possibly humans, can produce new follicles and oocytes in the recipient's ovary (14). It has also been reported that these tissues share genes typical of germ cells and proposed that bone marrow stem cells can migrate and colonize the ovaries to maintain a plentiful stock for reproduction (14). Later, Drusenheimer et al. (15) managed to isolate a fraction of bone marrow cells that were able to differentiate to male germ cells. These cells exhibited expression of germ cell and male germ cell–specific markers such as *Oct4, Fragilis, Stell, Vasa, c-Kit, cyclinA2,* and *Piwil2.* However, Drusenheimer et al. (15) failed to determine whether these male germ cells could undergo meiosis and form functional spermatozoa.

Later, Nayernia et al. (9) used the transgenic mouse line Stra8-EGFP to isolate for the first time male germline stem cells from bone marrow MSCs. Bone marrow stem (BMS) cell-derived germ cells expressed the known molecular markers of PGCs such as Fragilis, Stell, Rnf17, Mvh, and Oct4, as well as molecular markers of SSC and spermatogonia including Rbm, c-Kit, Tex18, Stra-8, Piwil2, Dazl, Hsp90α, β1-, and α6-inegrins (9). Their finding addressed one of the critically important questions in the field of stem cell plasticity: Can adult somatic cells differentiate to germ cells? By showing that BMS cells can differentiate to early germ cells, Nayernia et al. (9) demonstrated that the earlier question is possible. However, a major important and unresolved problem was observed in their study: BMS-GCs stop differentiating at the premeiotic stage of germ cell development, which is an indication of failure in the meiotic programming of BMS-GCs. No EB was observed in their differentiated adherent bone marrow (BM) cell culture, which was present in differentiated ESC cultures suggesting supportive roles of other cell types (Sertoli cells) for differentiation of BMS-derived germ cells.

THERAPEUTIC APPROACHES

Different approaches of stem cell–based therapy of male infertility are shown in Figure 3. These approaches are based on the following strategies:

- Derivation of gametes from ESCs
- Maturation of germline stem cells in vitro following testicular transplantation
- Heterologous transplantation of germline stem cells
- Derivation of gametes from somatic (stem) cells
- Reconstruction of germline stem cell niche

ESCs to Germ Cells—In Vitro Cell-Based Model

The differentiation of ESCs into germ cells of various stages appears to be a spontaneous and quick process, probably due to the nature of ESCs themselves and the microenvironment of the culture conditions that favor this process. However, to be able to apply this technique to humans, we need to improve this method. A detailed analysis of the in vitro differentiation process and comparison with the in vivo germ cell differentiation will enhance our understanding for the molecular, cellular, and morphological events underlying both processes. Furthermore, the derivation of germ cells from ESCs in vitro provides an invaluable assay both for the genetic dissection of germ cell development and for epigenetic reprogramming. To gain a better comprehension of the molecular mechanisms behind the in vitro formation of germ cells from ESCs, we need a robust cell-based model that will allow step-by-step monitoring of the differentiation process during spermatogenesis.

Formation of the male gamete occurs in sequential mitotic, meiotic, and postmeiotic phases. Many germ cell–specific transcripts are produced during this process. Their expression is developmentally regulated and stage specific (16).

To analyze regulated gene expression of ESC-derived germ cells, a microarray-based kinetic comparison of parental ESCs with ESC-derived germ cells can be performed. In addition,

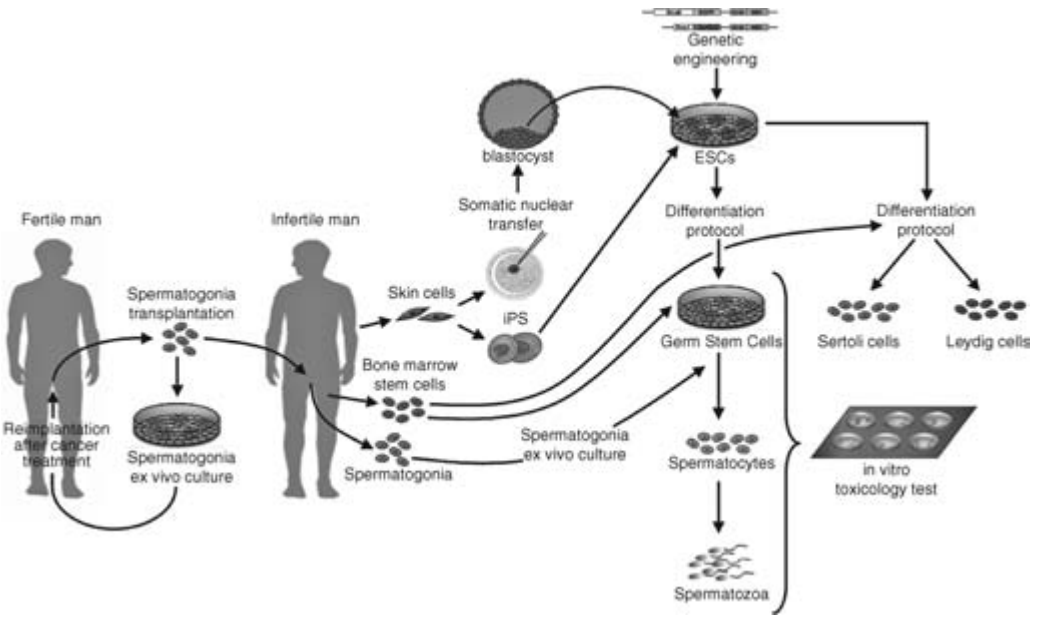

Figure 3 Schematic representation of different stem cell–based approaches for treatment of male infertility.

studying the similarities and differences in gene expression between ESC- and testis-derived germ cells can provide very useful information about the in vitro germ cell differentiation.

Genomic imprinting is the parent-of-origin-specific gene expression, which is a vital mechanism through both development and adult life. One of the key elements of the imprinting mechanism is DNA methylation, controlled by DNA methyltransferase enzymes. Germ cells undergo reprogramming to ensure that sex-specific genomic imprinting is initiated, thus allowing normal embryo development to progress after fertilization. The setting of male-specific epigenetic information is a complex process, which involves a major global reorganization as well as localized changes of the nucleus structure during the premeiotic, meiotic, and postmeiotic stages of the male germ cell differentiation (17). How and exactly when these changes interact, how they affect the epigenetic information, and how the paternal epigenetic marks contribute to the future genome are indeed major issues remaining to be explored. An experimental model that allows the isolation of germ cells at different stages during spermatogenesis (premeiotic, meiotic and postmeiotic) will provide a significant advantage in order to perform a detailed analysis of the imprinting pattern changes exclusively for each developmental stage.

Spermatogenesis is a developmental process that implies drastic changes in cellular morphology and metabolism of germ cells. Many of the changes that occur during this time are essential for the production of fertile sperm. Spermiogenesis includes modifications of the nucleus and perinuclear organelles, formation of the acrosomic system, assembly of the tail structures, topographical arrangement of the cell surface, and cytoplasmic reorganization, the final phase of which results in release of spermatozoa into the lumen of seminiferous tubules (18). An in vitro cell-based model will enable us to study this complex process and understand the sequential changes in the structure and associated molecules of sperm formation.

Taking advantage of the access, at different developmental stages during ESC differentiation to germ cells, provided by a cell-based model like this, the questions that can be addressed are unlimited. Revealing the mechanisms of gamete development can have major implications for treating infertility. ESC differentiation into germ cells can improve our knowledge of the genetic basis of mammalian infertility and can enable analysis of many more genes when an infertile couple enters the clinic. At the moment, the only genetic tests commonly offered to infertile patients are karyotype analysis, sequence analysis of the cystic fibrosis transmembrane conductance regulator gene, and Y chromosome deletion analysis (19).

Although the technological procedures and advances in the fertility clinic have vastly developed, still a lot of controversies and dilemmas remain. One of those with particular importance is that ART for severe male and female factor infertility serves to not only

overcome sterility but also bypasses natural barriers to the inheritance of defective genes. This results in considerable concern that genetic defects will be transmitted to the next generation. In the future, improved protocols for differentiation of ESCs to functional mature male germ cells (sperm) in culture might be used to provide the ability to parents, who are carriers or affected by inherited diseases, to have healthy offsprings without any risk. This can be accomplished by genetic modification of ESCs and subsequent differentiation into germ cells in vitro. However, this prospective application presents many social and ethical challenges, as germline genetic modification could also potentially allow parents to produce a designer baby.

Recent studies have shown that human skin fibroblasts can be directly reprogrammed to pluripotent state cells, so-called human-induced pluripotent stem (iPS) cells, by over-expression of only four proteins (20). These advances provide the potential in the future for treating cases of arrested spermatogenesis by stem cell–based therapeutic approach using cells obtained from skin biopsies.

Ex Vivo Culture of Germ Cells

Isolation of male germ cells from testicular tissue of nonobstructed azoospermic men was recently reported in the literature (21). In this study, it was shown that after six weeks of culturing the male germ cells obtained from the biopsy, haploid cells were generated. These in vitro–produced haploid cells (spermatids) were able to activate human oocytes and induce the initial cleavage. Although the culture system of this study gave rise to haploid cells, these cells were not able to complete the final step of the spermatogenic maturation, that is, spermiogenesis, as sperm was not observed. Investigation of ESC differentiation into germ cells might allow us to define the appropriate culture conditions. Thereafter, having established the culture conditions required for germ cell differentiation in vitro, we could isolate SSCs from patients exhibiting maturation arrest at a specific stage of spermatogenesis, and allowing them to develop in vitro, followed by reimplantation. Providing an ex vivo environment that mimics the normal in vivo conditions present in healthy testis offers a very attractive alternative solution for the therapy of patients who are positive for premeiotic or meiotic germ cells but negative for post-meiotic cells in the seminiferous tubules.

Germline stem cells can be transplanted from the testis of a fertile donor animal to the testis of an infertile recipient (22). These cells can then reestablish spermatogenesis in the recipient testis; however, the sperm produced will transmit the genotype of the donor to the offspring of the recipient. Many patients undergo treatment for cancer by chemotherapy or irradiation. Prior to treatment, a patient undergoes a testicular biopsy to recover stem cells. These cells are then cryopreserved and reimplanted later to restore fertility (23). However, the successful rates for this method are appreciably low.

Development of methods that will allow in vitro differentiation of stem cells to provide mature spermatozoa would be enormously valuable in understanding the complex process of spermatogenesis. It will also allow characterizing factors and signals that support self-renewal and initiate differentiation of human SSCs. This will advance the development of the appropriate culture conditions for human SSCs and will facilitate ex vivo culture, which can potentially improve transplantation and restoration of fertility in patients who underwent cytotoxic treatments for cancer.

Reconstruction of Germline Stem Cell Niche

Cross-sectionally, the testicle is mainly built up of seminiferous tubules where spermatogenic process takes place in normal condition, producing sperm as a sign of fertility (Fig. 1). Within the seminiferous of the testis, developing germ cells and Sertoli cells are in close association with each other. Through the entire spermatogenesis process, to allow synchronous development of cell stages in each cell division from a spermatogonium to spermatids, the cells remain connected by cytoplasmic bridges. As the spermatogenic cells divide and differentiate, they interact closely and extensively with the adjacent Sertoli cells. These associations between developing male gametes and the Sertoli cells are essential for spermatogenesis. Without physical and metabolic support of Sertoli cells, it is impossible for germ cells' differentiation, meiosis, and transformation into spermatozoa to occur. Impaired development or proliferation of Sertoli cells in early fetal life has been associated with various testicular disorders. Failure of Sertoli cells to mature will result in inability to express functions essential for supporting spermatogenesis.

In a similar way, insufficient spermatozoa production in adulthood is caused by failure of Sertoli cells to proliferate normally at appropriate period in life. Different strategies can be developed to reconstitute defective germline stem cell niche and treat male infertility. One of these approaches is derivation of Sertoli cells from stem cells and transplantation in testis of infertile men.

In Vitro Toxicology Test

Treatment with cytotoxic chemotherapy is associated with significant gonadal damage in men (24), and alkylating agents are the most common agents implicated, which all act to cause damage to the genome. The vast majority of men receiving anticancer regimens for the treatment of lymphomas are rendered permanently infertile. Establishment of stem cell–based assays as innovative platforms for drug screening is a nascent field compared with parallel studies on stem cells for regenerative medicine (25). Preclinical efficacy and toxicity testing are conducted largely in animal models as a means to validate the mechanism of action and predict adverse effects of compounds in human subjects. For developmental toxicity, for example, the most prevalent models for drug investigation are whole animal studies in rabbits and rats. In vivo studies rely on administration of compounds to pregnant animals at different stages of pregnancy and embryonic/fetal development. However, these and other in vivo animal models are limited by a lack of robustness between animal and human responses to chemical compounds. Thus, a human stem cell-oriented predictive in vitro model, such as the in vitro cell-based mode described in this chapter, provides the ability to investigate the effect of various anticancer drugs in the germline. Screening of drugs in in vitro isolated germine cells allows us to examine the pharmacological effects of a drug and to determine the germ cell population where the possible action occurs, as each isolated germ cell population represents a different developmental stage during spermatogenesis.

CONCLUSION AND SUMMARY

ESCs hold a great promise for treating male infertility following recent advances showing differentiation of ESCs to germ cell lineage. Exploiting this potential could increase our understanding of how germ stem cells differentiate and also allow us to dissect and recapitulate the complex process of spermatogenesis in vitro. Furthermore, taking advantage of the ability to generate germ cells from ESCs can provide the starting point for the development of a number of stem cell–based approaches to help patients with problematic spermatogenesis either by genetic and environmental causes or as a result of cytotoxic cancer therapies. These stem cell–based strategies can involve in vitro generation and maturation of germ cells from ESCs or somatic stem cells and ex vivo maturation of germ cells followed by reimplantation in the testis. These models can also support further platforms for investigation of imprinting patterns, chromatin modification, drug toxicity, and molecular profile of germ cells. The increased knowledge about the derivation of gametes in vitro can have a great practical potential in the future for therapeutic approaches to male infertility.

Of the 15% of couples who experience difficulty in conceiving, approximately half involve some degree of male factor infertility, and for 30% to 50% of these men, no cause is identified for the poor sperm characteristics. Although assisted reproductive technologies (ARTs) such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have dramatically improved the prospects for infertile couples, there are some types of male factor infertility that remain untreatable. These include arrested spermatogenesis that may be due to defective germ cells, an abnormal testicular environment, or aberrations in endocrine pathways regulating testis function (1). One of the essential bases for treatment of male infertility is the understanding of the human spermatogenesis by modeling human germ cell development. There has previously been no robust cell-based model for examining the genetic and epigenetic mechanisms of germ cell formation. Embryonic stem cells (ESCs) could potentially fill this need, as all cell types analyzed to date (including mature germ cells) can be identified by marker analysis during ESC differentiation. Furthermore, ESCs could also be used to differentiate mature male germ cells (sperm) in culture as an alternate reprogramming cell for somatic cell nuclear transfer. Another approach is isolating spermatogonial stem cells (SSCs) and allowing them to develop in a more “normal” environment ex vivo, followed by reimplantation. Establishment of human SSCs and investigation of their differentiation to

sperm in vitro might lead to new ways for treating male factor infertility. These techniques could be proven as powerful tools for undertaking new types of reproductive studies and particularly might support the development of new approaches and novel technology in assisted reproductive treatment of male infertility.

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11 | Adult Stem Cell Population in the Testis

Herman Tournaye and Ellen Goossens

INTRODUCTION

The preservation of spermatogenesis throughout a human's adult reproductive life depends on one cell type only: the adult spermatogonial stem cell (SSC). SSCs are undifferentiated cells that give rise to the spermatogenic cells and, finally, the spermatozoa. Even though there is a continual loss of differentiated cells, the spermatogenic cell lineage maintains its cell number thanks to the adult SSCs, which produce both new stem cells (self-renewal) and differentiating cells.

Maintaining the genetic integrity of SSCs is important not only to preserve the quality and function of the differentiated cells but also to guarantee a correct transmission of genetic information from one generation to the next.

Adult SSCs are thus essential for both the production of gametes and the continuation of the species.

This chapter gives an overview of the theoretical aspects of SSCs as well as of the current research on potential clinical applications.

THEORETICAL ASPECTS OF SPERMATOGONIAL STEM CELLS

Where Do Spermatogonial Stem Cells Come From?

SSCs originate from the primordial germ cells, which derive from pluripotent cells of the epiblast (1). The mechanisms responsible for the differentiation of germ cells in the epiblast involve several factors, including bone morphogenetic protein 2 (BMP2), BMP4, and BMP8B. Germ cells move, at a very early stage of embryonic life, through the primitive streak into the extraembryonic region at the base of the allantois. In the fourth week, some cells present in the yolk sac, near the base of the allantois, differentiate into primordial germ cells, which can be identified by their expression of alkaline phosphatase, Oct4, VASA, SSEA1, EMA1, F9, and the tyrosine kinase receptor c-kit. In the fifth week, primordial germ cells become embedded in the wall of the hindgut and migrate through the dorsal mesentery to reach the gonadal ridges where they proliferate and increase in number (2). The migration, proliferation, and survival of primordial germ cells is driven by signaling between stem cell factor (SCF), which is expressed in somatic cells along the pathway of primordial germ cell migration, and its receptor c-kit, expressed in germ cells (3). During migration, primordial germ cells proliferate actively, but do not differentiate. The differentiation into gonocytes starts when the primordial germ cells become enclosed by their niche cells, i.e., Sertoli cells. These germ cells differ morphologically from the migratory primordial germ cells and are therefore called gonocytes (4). The gonocytes are positioned in the center of the seminiferous tubule. Gonocytes proliferate for a few days and then become quiescent in the G_0/G_1 phase of the cell cycle. In mice and rats, these cells resume proliferation about two days after birth. By day 6, all gonocytes have migrated to the basement membrane and become A_s (A_{single})-spermatogonia (5,6). This event marks the initiation of spermatogenesis and is mainly directed by the Sertoli cells. In mice, Sertoli cells express only a soluble SCF until day 7 after birth. Between the 7th and the 11th day, the Sertoli cells switch their production from a soluble into a membrane-bound SCF. This switch is accompanied by the start of SSC differentiation, assuming that the membrane-bound form is more important in spermatogenesis than the soluble form (7). C-kit-negative SSCs acquire sensitivity for SCF thanks to another factor that is secreted by Sertoli cells very early in postnatal life and downregulated in adult life, i.e., BMP4. Its receptor Alk3 is expressed in postnatal spermatogonia. Stimulation of the Alk3 receptor with BMP4 triggers a cascade of actions that results in the expression of the c-kit receptor (8).

How to Identify Spermatogonial Stem Cells?

SSCs are single triangle-shaped cells, located on the basement membrane of the seminiferous tubules, in close contact with the Sertoli cells. They have an ovoid nucleus and a dense cytoplasm. The nucleoli, which lack heterochromatin, are positioned close to the nuclear membrane. The cytoplasm contains a small Golgi apparatus, few mitochondria, and many free ribosomes (9).

SSCs can be distinguished from pluripotent stem cells by their expression of Oct4 (Table 1). This transcription factor is downregulated in multipotent and unipotent stem cells. Adult SSCs can also be identified by the absence of the tyrosine kinase receptor c-kit. Pluripotent stem cells, primordial germ cells, and neonatal SSCs express this receptor, whereas in the adult testis, the c-kit-receptor is expressed in all differentiating spermatogonia, but not in the undifferentiated spermatogonia (10). A reciprocal relationship exists between c-kit and Thy1 expression; when c-kit expression is established, Thy1 expression is downregulated. Thy1 is a differentiation marker for thymocytes, T cells, and some neuronal cells, but it is also found to be expressed on hematopoietic stem cells. C-kit-negative SSCs were found to express β_1- and α_6-integrins on their surface (11). These integrins are also found on stem cells from other tissues. Kanatsu-Shinohara et al. found that CD9, a β_1 integrin-associated transmembrane protein, which is involved in cell adhesion and cell motility events and which is expressed on stem cells of other tissues, is also expressed in SSCs. Expression of CD9, however, is not specific to SSCs. The CD9-selected cells showed a significant amount of c-kit, which is expressed on differentiating spermatogonia (12). Other markers to identify spermatogonia in a testicular cell suspension are the major histocompatibility complex (MHC)-I molecules. Although MHC-I molecules are expressed on virtually all nucleated somatic cells, the expression is very limited in spermatogonia. It was shown that undifferentiated spermatogonia express the receptor for glial cell line–derived neurotrophic factor (GDNF), the GDNF family receptor α1 (GFRα1) and the associated tyrosine kinase receptor [(c-Ret) REarranged during Transfection]. GDNF, which is produced by the Sertoli cells, is required for SSC renewal and spermatogenesis (13). Another family of markers for undifferentiated spermatogonia is the Daz (deleted in azoospermia) family. Daz is a Y-chromosome-linked protein in humans, and Dazl (Daz-like) is an autosomal homolog expressed in humans and mice. These proteins were found to be located in the nucleus of spermatogonia. During male meiosis, however, the proteins are transferred from the nucleus to the cytoplasm (14). A factor that is required for self-renewal and maintenance of the SSC population is the promyelocytic leukemia zinc finger (Plzf). Plzf is coexpressed with Oct4 in undifferentiated spermatogonia and functions as a transcriptional repressor regulating the epigenetic state of undifferentiated cells (15,16). Another protein that is expressed exclusively in the premeiotic germ cells is the cytoplasmic protein stimulated by retinoic acid-protein (Stra8) (17). The expression of

Table 1 Differential Expression of Spermatogonial Stem Cell Markers

Marker	Expression site	Cell types that express the marker
Oct4	Nucleus	ESCs (high expression) Undifferentiated spg (moderate expression)
c-kit	Surface	ESCs, PGCs, neonatal SSCs, differentiated spg
Thy1	Surface	SSCs
α_6-integrin	Surface	Undifferentiated spg, ESCs
β_1-integrin	Surface	Undifferentiated spg
CD9	Surface	spg, ESCs
MHC-I	Surface	Nucleated cells, except SSCs and ESCs
GFR α1	Surface	Type A spg
c-Ret	Transmembrane	SSCs
Dazl	Nucleus	ESCs, PGCs, spg
	Cytoplasm	Postmeiotic germ cells
Plzf	Nucleus	SSCs
Stra8	Cytoplasm	Premeiotic germ cells
TAF4b	Nucleus	spg

Abbreviations: ESC, embryonic stem cell; PGC, primordial germ cell; spg, spermatogonia; SSC, spermatogonial stem cell.

stem cell-specific molecules, such as Plzf, c-Ret, and Stra8, requires the expression of the gonad-specific TAF4b component of TFIID, a transcriptional regulator expressed in mouse testes (18). Other genes that are expressed in spermatogonia, but not in somatic cells, are Mage, Ube1y, Usp9y, Rbmy, Ott, Ddx4, Tex14, Usp26, Piwil2, and Pramel1.

Although, at present, there is no specific marker to exclusively select SSCs, the use of a combination of differentially expressed markers can result in a highly enriched stem cell population. Such a combination of factors was used by Kubota et al. They found an enrichment of stem cells after flow cytometric cell sorting in the fraction containing (MHC-I)$^-$, Thy1$^+$, and c-kit$^-$ cells. The (MHC-I)$^-$, Thy1$^+$, and c-kit$^-$ cells also showed α_6-integrin and CD24 expression, but were negative for α_V-integrin, Sca1, and CD34 (19).

Spermatogonial Stem Cell Lines

Two groups have reported the establishment of SSC lines (20,21). In both studies, type A spermatogonia were immortalized by using the Simian virus large T antigen. The cell lines were characterized and found to express Oct4, a marker for undifferentiated spermatogonia. Expression of c-kit, normally expressed in type A spermatogonia from late A_{al} ($A_{aligned}$) spermatogonia onward, could not be detected. Detectable levels of some more protein markers specific for germ cells such as Dazl, GFRα1, Piwil2, and Pramel1 were found.

The establishment of these cell lines is a great step forward in the study of spermatogonial gene expression and regulation.

Spermatogonial Stem Cell Renewal

SSCs have, like stem cells in other tissues, the capacity to self-renew or to differentiate. Two models exist for stem cell renewal and spermatogonial proliferation in nonprimate mammals: the A_s model and the slightly different A_0/A_1 model.

The mechanism of proliferation for primates is somewhat different. Since most of the research focuses on rodent spermatogenesis, the proliferation schemes of both nonprimate and primate mammals will be presented.

Two Models for Nonprimate Mammalian SSC Proliferation

The A_s model was originally proposed by Huckins (1971) and Oakberg (1971) and is still the most widely accepted (22,23). This model is a one-compartment stem cell model. At the onset of spermatogenesis, a compartment of undifferentiated type A spermatogonia is subdivided into A_s, A_{paired} (A_{pr}), and A_{al} spermatogonia. These type A spermatogonia have the same morphology and can only be distinguished according to their topographical arrangement on the basement membrane of the seminiferous tubules. The A_s spermatogonia are considered to be the true population of SSCs. It was found that A_s spermatogonia have a longer cell cycle than A_{pr} and A_{al} spermatogonia, resulting in a lower proliferation rate and thus less accumulation of replication errors (24). When A_s spermatogonia divide, they produce either two A_s daughter cells or two type A_{pr} or paired spermatogonia. When A_s spermatogonia divide into two A_s cells, they usually migrate separately. If cytokinesis is incomplete, the daughter cells stay together and become A_{pr} spermatogonia, connected to each other by an intercellular cytoplasmic bridge. The production of type A_{pr} spermatogonia is the first step toward differentiation. Normally, about one-half of the SSCs divides to form A_{pr} spermatogonia, while the other half goes through self-renewing divisions, thereby maintaining the size of the SSC population. The type A_{pr} spermatogonia that are formed divide once again to produce groups of four type A_{al} spermatogonia, which are also connected to one another. The A_{al} cells proliferate, resulting in chains of 8, 16, and occasionally, 32 A_{al} cells, all interconnected by cytoplasmic bridges. Most of the A_{al} spermatogonia will undergo a morphological change and transform into type A_1 spermatogonia, which are the first generation of differentiated spermatogonia (Fig. 1). These differentiated spermatogonia subsequently divide in A_2, A_3, A_4, In, and B spermatogonia to form the primary spermatocytes.

In mice, spermatogenesis occurs in a cyclic manner and can be divided into 12 stages (I–XII). At stage VIII, A_s, A_{pr}, and a few A_{al} spermatogonia are present. These cells start to proliferate when stage X is reached. The proliferation stops at stage II–III, and cells become

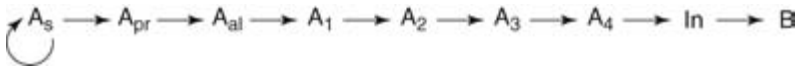

Figure 1 The A_s model for nonprimate spermatogonial differentiation.

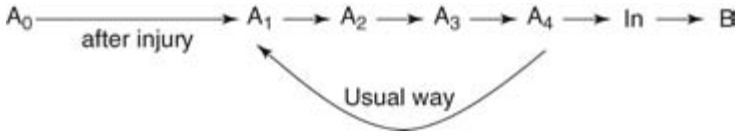

Figure 2 The A_0/A_1 model for nonprimate spermatogonial differentiation.

arrested in G_1/G_0 phase. At stage VII–VIII, A_{al} cells undergo morphological changes and become A_1 spermatogonia, which enter S phase. At stage IX, these cells divide into A_2 spermatogonia. At stage XI, A_3 spermatogonia are formed, and A_4 spermatogonia first appear at stage I. A_4 cells divide into In spermatogonia at stage II, and these form B spermatogonia at stage IV. The final spermatogonial division occurs at stage VI with the formation of primary spermatocytes.

The A_0/A_1 model by Clermont and Bustos-Obregon (1968) and Clermont and Hermo (1975) is based on a slightly different identification scheme for type A spermatogonia (25,26). This is a two-compartment stem cell model. In the normal adult testis, the A_s and the A_{pr} spermatogonia are quiescent and are called A_0 spermatogonia in this model. These cells will only divide after cell loss and are therefore also called "reserve stem cells" (27). The differentiating spermatogonia A_1, A_2, A_3, and A_4 of the A_s model are considered cells with stem cell properties. A_4 spermatogonia can divide into A_1 or In spermatogonia. The proliferating A_{al} cells are not considered a distinct class and are called A_3 and A_4 cells, which can retransform into type A_1 spermatogonia. Thus, according to this alternative model, two stem cell compartments exist, i.e., the type A_0 compartment or "reserve stem cells" and the compartment consisting of type A_1 through A_4 spermatogonia, also referred to as "renewing stem cells" (Fig. 2).

This model has been the subject of controversy because of some experimental findings. First, the A_s and A_{pr} spermatogonia are not quiescent in the adult testis. Second, A_{al} cell cycles bear more resemblance to the cell cycles from A_s and A_{pr} spermatogonia than to those from A_1–A_4 spermatogonia. Third, in the absence of A_1 spermatogonia, A_{al} spermatogonia can still be formed. Fourth, A_{al} and A_1–A_4 spermatogonia do not have the same radiosensitivity, suggesting that A_{al} and A_1–A_4 spermatogonia are different cell types. And finally, the length of A_1–A_4 chains is longer than that of A_{al} spermatogonia.

The A_p/A_d Model for Primates

In primates, two morphologically different classes of type A spermatogonia are observed: the dark A_d and the pale A_p spermatogonia, named after their staining intensity with hematoxylin. It is generally accepted that in primates there is a dual stem cell compartment as in the A_0/A_1 model. Clermont applied his rodent theory to the primates with the A_d in the role of the A_0 (reserve stem cells) and the A_p as the A_1 through A_4 (renewing stem cells) (28). In general, there are equal numbers of A_d and A_p spermatogonia. A_p spermatogonia divide once every cycle, whereas A_d spermatogonia normally do not divide. The self-renewal of the type A_p spermatogonia is analogous to that in the A_s model, because most of the type A_p spermatogonia appear in clones of two, four, and eight cells, but single A_p cells may also exist. Furthermore, the A_d and A_p cells can transform into each other. A_d spermatogonia are often found in clusters. Such a cluster of A_d cells could be a result of a transformation of A_p into A_d at low renewal frequency. Conversely, after cytotoxic injury, the A_d spermatogonia may become active again and transform into A_p spermatogonia, which can start to proliferate and form type B spermatogonia. In monkeys, there are four generations of B spermatogonia (B_1–B_4), whereas in humans, there is only one.

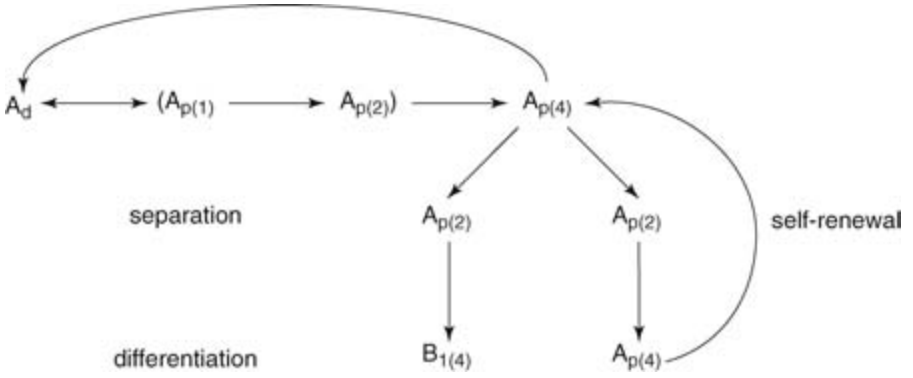

Figure 3 The A_d/A_p model for primate spermatogonial differentiation.

New information about this model was reported by Ehmcke et al. They concluded that the population of A_p spermatogonia cycle continuously and initiate spermatogenesis by an equal spermatogonial self-renewing division at stage VII. Subsequently, the clones of A_p spermatogonia will separate and initiate a second division at stage IX, which leads to clones of B spermatogonia as well as pairs or quadruplets of A_p cells. These latter cells are responsible for the maintenance of the original size of the A population. Because the A_p, which are found in clones of two or four cells, cycle continuously, the true stem cells are probably the seldom-dividing single A_p and A_d spermatogonia (29) (Fig. 3).

The process of spermatogenesis results in a large amount of sperm cells. It is estimated that a mouse testis contains about 35,000 A_s spermatogonia. One single A_s cell is theoretically able to give rise to 4096 spermatids. In the human, this number is lower because of fewer mitotic divisions, apoptosis, and the low effectiveness of the process (30,31).

Regulation of the Size of the Stem Cell Population

SSCs can develop in three different ways: they can renew themselves, they can differentiate, or they can go into apoptosis. The mechanism determining which pathway will be followed is the subject of a great deal of investigation. The only cells in direct contact with the stem cells are the Sertoli cells. Hence, the regulation of the germ cell pool will probably occur through the Sertoli cell. Sertoli cells have large contact areas with the germ cells through desmosome–gap junction complexes. However, these intercellular contacts are rarely seen on type A spermatogonia. Therefore, it is assumed that Sertoli cells regulate spermatogonial proliferation by secreting paracrine factors. In addition, Sertoli cells limit the expansion of SSCs, since it is assumed that each Sertoli cell can only support a defined number of SSCs.

The spermatogonia are also in contact with the basement membrane of the seminiferous tubules and may therefore also respond to diffusing paracrine factors secreted by Leydig cells or myoid cells in the interstitial space.

Regulation of Stem Cell Renewal

In normal seminiferous epithelium, the ratio between self-renewal and differentiation should be about 1. More self-renewal than differentiation would reduce the seminiferous epithelium to only SSCs, and a tumor might be formed. In the other case, if there is more differentiation than self-renewal, the testis would become depleted of SSCs. However, in rodents, it was observed that during a period of active proliferation of the SSCs, the number of these cells slowly decreases, indicating that more than half the daughter cells become A_{pr} spermatogonia. The cell density in the seminiferous tubules is kept constant by density-dependent degeneration of differentiating spermatogonia (32).

On the other hand, when the seminiferous epithelium is depleted by cytotoxic agents or irradiation, the restoration of spermatogenesis must occur from SSCs. Though SSCs are less sensitive than differentiating spermatogonia, they can still be lost. Surviving SSCs may

start repopulating the seminiferous tubules by forming colonies that grow along the length of the tubules. Moreover, in this situation, the percentage of A_{pr} daughter cells was much lower than that in the normal testis, which indicates that SSCs prefer self-renewal above differentiation (33).

These last few years, a number of factors have been characterized that influence SSC survival and proliferation in vitro. SSC self-renewal and differentiation depend on GDNF, a factor produced by Sertoli cells (34). Loss of GDNF expression results in depletion of spermtagonial stem cell reserves, and overexpression leads to an accumulation of undifferentiated spermatogonia. Stem cell self-renewal is also regulated by the pituitary gland. In neonatal testes, gonadotropins stimulate self-renewal, but in the adult, they have the opposite effect.

Regulation of Differentiation

A crucial phase during nonprimate spermatogenesis is thought to be the transition from A_{al} to A_1 spermatogonia. Different factors and/or mechanisms have been reported that cause an arrest in spermatogonial differentiation and therefore an increase in the stem cell population in the seminiferous tubules (Table 2).

A first mechanism is cryptorchidism, developed by Nishimune et al. The higher temperature in the mouse testis causes infertility due to the disruption of spermatogenesis, including the reduction of germ cells. Sertoli cells also appear to be temperature sensitive, though the mechanisms involved are not clear. In mice that were made cryptorchid, only type A spermatogonia remained after two months. Despite the fact that these cells were still proliferating, they were unable to give rise to more differentiated cells. However, when the testis was transferred back into the scrotum, spermatogenesis could be reestablished (35).

Second, as mentioned before, c-kit receptor and its ligand stem cell factor play an important role in the differentiation of type A spermatogonia. This can be illustrated by the following two mutant mouse models: the dominant white-spotting (W) mutation that hampers the expression of the c-kit receptor and the Steel (Sl) mutation that blocks the transcription of SCF. Males that are heterozygous for mutations in one of the genes, encoding for these proteins, show an arrest of spermatogenesis on the spermatogonial level (36,37).

A third mechanism is vitamin A deficiency by which only type A spermatogonia, which are unable to differentiate, will remain (38). However, after vitamin A supplementation or continuous administration of retinoic acid, spermatogenesis can be restored (39).

A fourth mechanism has been described in adult male mice, homozygous for the mutant gene juvenile spermatogonial depletion (jsd). These animals are sterile and have small testes (40). Even though type A spermatogonia are found, they are not able to differentiate. In a study by Mizunuma et al., it was concluded that the defect must be intratubular, since the intertubular environment of jsd/jsd-mice was normal (41).

In rats, a fifth mechanism has been described. When they were given 2.5-hexanedione, a Sertoli cell toxicant, spermatogonial differentiation was found irreversibly impaired. The only cell type that remained were A spermatogonia, which were able to actively proliferate (42).

A final mechanism has been reported in LBNF1 rats. These rats were x-irradiated with doses of 3.5 to 6 Gy, which caused a depletion of the seminiferous epithelium (43). Yet, four to six weeks after irradiation, the seminiferous epithelium recovered. Unexpectedly, spermatogenesis

Table 2 Mechanisms Causing an Arrest in Spermatogonial Differentiation

Model	Deficiency
Cryptorchid	Temperature too high
Steel or W mutation	No SCF or c-kit expression
Vitamin A deficiency	No retinoic acid production
Jsd mutation	Intratubular defect
2,5-Hexanedione	Sertoli cell damage
LBNF1 rats	Irradiation

Abbreviations: Jsd, juvenile spermatogonial depletion; SCF, stem cell factor.

subsequently slowed down until only undifferentiated spermatogonia were present. These cells were incapable of differentiating, unless the rats were treated with either gonadotropin-releasing hormone agonist or testosterone (44). Since no hormone receptors are as yet known to exist on spermatogonia, this finding suggests that the Sertoli cells, rather than the spermatogonia, were damaged by irradiation and that these hormones could induce the Sertoli cells to regain their function.

At present, it is still not understood how the differentiation of undifferentiated spermatogonia is regulated. Sertoli cells probably play the most important role. In all mechanisms of arrested differentiation described above, Sertoli cell function is impaired, which results in deficient spermatogenesis. Only in the W/W mice, which lack the c-kit receptor, is the problem located within the spermatogonia, which are unable to respond to SCF secreted by the Sertoli cells.

Regulation of Apoptosis

One of the proteins involved in stem cell apoptosis is p53, which has important functions in cell growth and differentiation. Although p53 is not expressed in normal spermatogonia, high levels of p53 have been observed after an irradiation dose of 4 Gy in mice, resulting in apoptosis of the spermatogonia (45).

The B-cell CLL/lymphoma 2 (Bcl2) protein family is another group of apoptosis regulators. During normal spermatogenesis, large numbers of differentiated spermatogonia go into apoptosis as a result of the density regulation. When Bcl2 or Bcl-x_L is overexpressed, apoptosis is suppressed, and this density regulation does not occur. As a consequence, spermatogonia will accumulate, and finally, both the spermatogonia and spermatocytes will enter into apoptosis (46). Bcl-w is another pro-survival factor that participates in the regulation of apoptosis by binding the pro-apoptotic factors Bax and Bak. It is suggested that the ratios of Bax/Bcl-w and Bak/Bcl-w are decisive for the survival of spermatogonia (47).

CURRENT RESEARCH ON POTENTIAL CLINICAL APPLICATIONS

The importance of the SSC is reflected in a variety of research fields. The study of fundamental aspects of germ cell development has become possible thanks to the introduction of the SSC transplantation technique (31,48) and germ cell culture (49). These techniques may also have clinical applications in the future. Indeed, the preservation of SSCs may prove to be an important strategy in preserving fertility in young cancer patients (50). Another field of research with clinical potential is the introduction of transgenes into the germ line by the transfection of spermatogonia (51). Although ethically very challenging, this application might one day pave the way for transgenerational gene therapy. We shall focus on some of the recent scientific breakthroughs and potential future use of spermatogonia.

Spermatogonial Stem Cell Transplantation

The technique of SSC transplantation, introduced by Brinster's group in 1994, involves the introduction of a germ cell suspension from a fertile donor testis into the seminiferous tubules of an infertile recipient mouse (31). SSCs were able to relocate onto the basement membrane and colonize the tubules in the first month after transplantation. From that moment on, stem cells started to proliferate and initiated spermatogenesis. Spermatozoa obtained by this method were capable of fertilization. Currently, two research models for stem cell transplantation are in use. A first model involves the transplantation of testicular cell suspensions from a fertile donor into the tubules of a sterile W/W^V mutant mouse. A mutation in the dominant white-spotting gene hampers the expression of the c-kit receptor, resulting in testes with the appearance of a Sertoli cell–only syndrome (germ cell aplasia). A second model uses busulfan-treated recipient mice. Busulfan is a cytotoxic drug, which eliminates most germ cells from the testis. Since some endogenous spermatogenesis may remain, it is necessary to use testicular cells from a donor with a genetic marker in order to distinguish donor spermatogenesis from endogenous (recipient) spermatogenesis. One popular marker is lac-Z, a gene transcribing for the β-galactosidase protein. Transgenic cells can be visualized after staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Another popular marker is green fluorescent protein (GFP), which enables cells to be visualized under UV light.

Although healthy live offspring could be obtained from transplanted mice, it has recently been shown that the fertilizing capacity of posttransplantation spermatozoa may be diminished (52). Also, development of blastocysts (53) and postimplantation development (54) were hampered.

A few years after the introduction of the SSC transplantation technique, it was shown that the testis of one species can provide support for spermatogenesis from another species. França et al. found that it is the transplanted stem cells themselves that impose the duration of the cell cycle and not the Sertoli cells. A cell suspension from a rat testis was transplanted into an immunodeficient mouse, which eventually resulted in rat spermatozoa being produced. The duration of spermatogenesis was comparable to that of the rat and not to that of the mouse (55). Xenogeneic transplantations to the mouse testis were also performed with testicular cell suspensions from hamster, rabbit, dog, primate, and even human. Hamster cells were able to colonize and to produce spermatozoa. However, these spermatozoa developed abnormally and were lower in number compared with mouse-to-mouse and rat-to-mouse transplants (56). Rabbit and dog cell suspensions could colonize seminiferous tubules in which they formed chains of cells; however, they failed to produce mature spermatozoa (30). Primate cell suspensions were also able to colonize, but not to proliferate in the recipient's tubules (57). Finally, human cell suspensions were not able to colonize the murine seminiferous tubules, probably because of noncompatible cellular interactions and immunological rejection (58). Two years later, proliferation of human stem cells was reported after transplantation into mouse seminiferous tubules. These stem cells could survive for six months in the murine testis (59). These observations demonstrate the relationship of the transplantation success with the phylogenetical distance between donor and recipient species.

Selection of Spermatogonial Stem Cells

It has been estimated that, in the adult mouse, a testis contains only one stem cell in 5000 testicular cells. Since the number of stem cells is crucial for the success of SSC transplantation, enriching the proportion of stem cells may improve colonization efficiency and bring clinical applications closer. Shinohara et al. selected SSCs on the basis of the ability of these cells to adhere to laminin, a component of the basement membrane of the seminiferous tubules. It was found that SSCs could be enriched 5- to 10-fold with magnetic beads and antibodies against β_1- and α_6-integrins, which are expressed on the surface of the SSCs (11). In addition, stem cells have little or no c-kit and α_V-expression, and can therefore be negatively selected by fluorescence-activating cell sorting (FACS). By combining these two selection methods, a 166-fold enrichment of SSCs was obtained (60). Highly enriched stem cell populations were also obtained with FACS using the following selection criteria: $(\text{MHC-I})^-$, Thy1^+, and c-kit^{-19}.

Magnetic-activated cell sorting (MACS) is another technique that may be used to enrich spermatogonia. Von Schonfeldt and colleagues reported the isolation of c-kit-positive spermatogonia via MACS. A total of 25% to 55% of the isolated magnetically labeled cells was c-kit-positive (61).

Sedimentation velocity and differential adhesion were used by Dirami et al. to obtain a population of 95% to 98% porcine type A spermatogonia (62). Using adhesion to laminin, the isolation of SSCs has been found to increase three- to fourfold (11). Morena et al. used sedimentation velocity in conjunction with differential adhesion to achieve a suspension, with 85% of the cells being type A, c-kit-positive spermatogonia (63).

Apart from selecting SSCs by the presence or absence of cell surface molecules, specific culture systems can also be used for enriching the stem cell fraction.

Culture of Spermatogonial Stem Cells

The development of SSC transplantation has provided a functional assay for the evaluation of SSC culture. Nagano et al. reported the first long-term survival of SSCs on SIM mouse embryo derived thioguanine and ouabain resistant (STO)-cell feeder layers for approximately four months (49). In addition, suppressing the differentiation of SSCs improves their in vitro maintenance (64). SSCs were kept in culture for more than five months, when GDNF, epithelial-derived growth factor, basic fibroblast growth factor, and leukemia inhibitory factor were added to the culture medium (65). The same group has recently shown that in the absence of

any feeder layers or serum, stem cells could survive for more than six months (66) and expanded approximately 1.4×10^{13}-fold from the initiation of culture to 149 days (67). However, it was found that culture outcome is dependent on the age of the isolated tissue. Prepubertal cells were shown to be twice as viable as adult cells in culture conditions. Therefore, it should be borne in mind that cultures of adult tissue are not representative for prepubertal tissue (68).

Not only has the maintenance of SSCs in culture been investigated, but in vitro differentiation has also been studied. Izadyar et al. reported differentiation from bovine type A spermatogonia into spermatocytes and spermatids (69). Culture at 37°C in minimal essential medium containing 2.5% fetal calf serum provided an optimal condition for survival, proliferation, and differentiation of bovine type A spermatogonia.

In vitro tissue explants represent an alternative strategy to culture testicular cells. Bovine testicular tissue pieces were maintained in culture for two weeks, and an increase in colony numbers indicated that SSC proliferation had taken place (70).

Recent studies showed that neonatal as well as adult SSCs can retain the ability to generate pluripotent cells and are able to differentiate into derivatives of the three embryonic germ cell layers in vitro (71,72).

Testicular Tissue Grafting

Testicular tissue containing stem cells can also be grafted. Attempts have been made to graft immature and adult testicular tissue in both ectopic locations and homotopic locations. In all studies of mouse-to-mouse grafts, mouse testicular tissue derived from newborn mice completed spermatogenesis. Grafting to the mouse was also successful using tissue from hamster, pig, goat, calf, rabbit, and monkey. Grafting with frozen-thawed testicular tissue was also found to be efficient (73–75). However, results obtained from adult tissue grafting were less promising. While it was shown that prepubertal murine tissue can be grafted successfully, with spermatogenesis in almost all the grafts, adult murine and adult human grafts are generally lost because of sclerosis or atrophy, although spermatogonia may survive (76,77). Recently, the authors studied the possibility of preserving the SSC niche and inducing spermatogenesis in human prepubertal testicular tissue xenografts. The tissue was grafted under the dorsal skin for nine months. At the time of evaluation all the grafts were well preserved. In the majority of the seminiferous tubules, only Sertoli cells could be observed, but some surviving spermatogonia could still be detected (78). In a study by Wyns et al., immature human cryptorchid testis tissue was used to evaluate the feasibility of xenografting to the scrotum after cryopreservation (79). After a short grafting period (three weeks), they observed good preservation of the seminiferous tubules, but already a substantial reduction in the number of spermatogonia.

In 2002, it was reported by Shinohara et al. that (xeno)grafting mouse and rabbit testis tissue to the testis of sterile mice, by making an incision in the tunica albuginea, resulted in growth of the graft and spermatogenesis (75). An analogous study was performed in our laboratory with similar results (80).

Cryopreservation of Spermatogonial Stem Cells

Cryopreservation of SSCs would be a valuable tool for any clinical application. But cryopreservation could also be valuable for the conservation of the genetic material of endangered species or valuable laboratory animals.

In 1996, Avarbock and coworkers were the first to report on successful cryopreservation of SSCs. Although frozen-thawed suspensions of murine testicular cells were able to colonize recipient testes and initiate spermatogenesis, the search for an optimal freezing protocol is still ongoing (81). In 2002, a noncontrolled rate-freezing protocol was described using minimal essential medium; it contained 10% fetal bovine serum (FBS), 10% Dulbecco's modified minimal essential medium (DMEM), and 0.07 M sucrose to yield the highest number (70%) of surviving cells after freeze-thawing (82). Interestingly, the rate of survival of stem cells was higher compared with other testicular cells, which resulted in an enrichment of stem cells in the final suspension. The authors were not able to confirm this. With their best freezing protocol (uncontrolled, 1.5 M DMEM), they observed spermatogenesis only in 22.7% of the tubules after transplantation, whereas in fresh, unfrozen controls, spermatogenesis was obtained in 90% of the tubules, which indicated an important loss of functional stem cells after freezing (83).

An alternative way of preserving stem cells is to freeze the whole testicular tissue. A protocol has recently been proposed using 0.7 M DMEM as a cryoprotectant. The structure of the tissue was well preserved with especially the spermatogonia as surviving cells (9).

Very recently, testicular tissue collected from five prepubertal boys undergoing gonadotoxic treatment was cryopreserved using two freezing protocols, originally developed for fetal and adult human testicular tissue. In both the methods, DMEM was used as a cryoprotectant. No clear structural changes were observed in the cryopreserved testicular tissue after using the protocol developed for adult testicular tissue (84). Testicular tissue, frozen with DMEM, showed less fluid accumulation and/or detached cells in the spermatozoa-containing tubules than fresh tissue or tissue frozen with ethylene glycol (EG) (85).

Transfection of Spermatogonial Stem Cells

SSCs are the only stem cells in the humans that can transmit parental genetic information to the offspring, making them an attractive target cell population for transgenesis.

Mouse SSCs have been successfully transfected with the use of a retroviral vector. Germ cells were incubated with (*i*) retroviral-producing cells alone, (*ii*) an STO feeder layer and retroviral-producing cells, and (*iii*) an STO feeder layer with periodic exposure to retroviral particles. All methods generated stably transfected stem cells that colonized recipient testes, with the third protocol yielding the highest level of infection. In addition, coinjection of retroviral particles and germ cells into recipient testes also resulted in incorporation of the reporter gene. It is now possible to transfect both adult and immature stem cells by retroviral-mediated gene delivery in vitro (64) and in vivo (86) by using a retrovirus vector.

This method has multiple applications, such as cell therapy, the introduction of genes into certain animal species, and the genetic modification of stem cells to address fundamental questions about the self-renewal and differentiation of stem cells.

ASSOCIATED PATHOLOGIES

Male Infertility

Infertility occurs in 13% to 18% of the couples seeking to have children. In at least 20% of these couples, the cause of infertility is of paternal origin. Some specific male factor disorders can be treated, but for nonspecific male factor infertility, treatment is difficult. In order to understand, and eventually treat these defects, it is critical to identify the regulating mechanisms at the various stages of spermatogenesis. The culture system as well as the transplantation technique together with transfection of SSCs may prove very useful methods in achieving more knowledge about SSCs and their proliferation. Studies using these methods may not only reveal more insight into the disease but also help to identify markers for diagnostic purposes or serve as a model in pharmacological studies.

Contraceptive Strategy

Since condoms and vasectomy are currently the only available methods for male contraception, new contraceptive strategies for the male are more than welcome. There are several approaches to male contraception, but research and development of male hormonal contraceptives is the only one under clinical investigation.

The use of SSC culture systems might provide better insights into factors regulating spermatogenesis and may eventually lead to alternative methods of male contraception.

Testicular Germinal Cancers

Testicular cancer is the most frequent tumor affecting the young adult (20–35 years). Although most of these cancers can be cured, they still represent a significant public health problem. The current understanding is that tumors progress from a carcinoma in situ, which are malignant cells derived from gonocytes (87). Because there is still no animal model representing the characteristics of the human germinal tumors, studies carried out on SSCs in culture or in an animal host will be of great significance.

Preservation of Fertility for Oncological Patients

Oncological diseases such as leukemia and Hodgkin disease occur with an incidence of about 1 in 600 children before the age of 15 years. In recent years, remarkable progress has been made in

the treatment of childhood cancers, and up to 75% of the patients can now be cured. At present, 1 in 1000 adults in the age group of 20 to 30 years is a childhood cancer survivor (88). From these figures it is evident that the prevention of sterility in childhood cancer survivors will become a major challenge in reproductive medicine. When an adult man undergoes a sterilizing cytotoxic treatment, sperms can be frozen to circumvent sterility after his treatment. However, no such prevention is possible before puberty since no active spermatogenesis is present. The introduction of the SSC transplantation technique in a clinical setting would create new prospects for the preservation of fertility in prepubertal cancer patients (50). Since current cancer treatments may destroy all dividing cells, including SSCs, and since prepubertal boys do not as yet produce spermatozoa, it is not possible for them to preserve their fertility. However, testis tissue could be removed before any cancer treatment and cryopreserved. After the patient has been cured, the testicular cells could be transplanted back to the testis. Before such an application can be approved, safety needs to be evaluated. In the mouse, it was shown that sperm cells obtained after SSC transplantation were able to fertilize and produce normal embryos after assisted reproduction. However, the motility of the sperm cells after transplantation was reduced compared with that in fertile mice (89). It remains to be shown whether this technique is clinically efficient as well, especially when frozen-thawed cells are to be transplanted.

An important part of the treatment is the storage of the testicular tissue. Cryopreservation of testicular tissue or cell suspensions is one option. The long-term organ or cell culture is the other one.

It is obvious that the reintroduction of malignant cells into a cured patient must be avoided. A selection of spermatogonia or an elimination of cancer cells before transplantation should be carried out. Elimination of cancer cells can be achieved by decontaminating the testis sample or by grafting a tissue piece onto a host. The first approach makes use of MACS or FACS to select out cancer cells and/or positively select spermatogonia on the basis of certain membrane molecules (e.g., cancer cells:HLA^+; spermatogonia:HLA^-). This approach was used by Fujita et al. and was found to overcome malignant contamination by depleting the cell suspension from leukemic cells by FACS prior to transplantation (90). However, in the experiments by the authors, MACS and FACS were found not efficient enough to deplete testicular cell suspensions from malignant cells (91).

Xenografting can be used as an assay for testing the risk of malignant contamination of the testicular tissue (92). If in due course no malignant cells are detected in the xenograft, autotranplantation or autografting of the patient's own tissue after oncological remission could be considered.

Although all aforementioned methods may have clinical potential, the technique of SSC transplantation is considered the most promising tool for fertility preservation.

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12 Human Embryonic Stem Cells: A Model for Trophoblast Differentiation and Placental Morphogenesis

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OVERVIEW

The isolation of human (1) and nonhuman primate (2) embryonic stem cell lines from blastocysts has provided a useful platform to model the very earliest events in human embryological development and in turn for the study of a multitude of tissues and diseases. Although derived from the inner cell mass (ICM) of IVF-derived human blastocysts, a unique aspect of human embryonic stem cells (hESC) is their ability to differentiate into trophoblast cells (1), a characteristic not shared by mouse ESC. Until recently, trophoblast differentiation has been studied with cell lines derived from choriocarcinoma cells and with primary placental trophoblast cultures. A key disadvantage of these model systems is that the trophoblast cells have already undergone differentiation and are committed trophoblasts; therefore, the earliest lineage decisions cannot be identified. Thus, hESC have been used to model the embryonic events that lead to selection of the trophoblast lineage and subsequent early development of the placenta. hESC not only undergo spontaneous differentiation into trophoblast cells (1) but can be directed toward trophoblast differentiation by the formation of three-dimensional (3D) embryoid bodies (EBs) (3), by treatment with bone morphogenic protein-4 (BMP4) (4), and by knockdown of the pluripotency markers Oct-4 (5) and Nanog (6). In this chapter these paradigms will be discussed along with the specification of trophoblast stem cells (TSC) in the primate model and the divergence of the trophoblast lineage into distinct placental compartments.

TROPHOBLAST LINEAGE DEVELOPMENT

The first differentiation event during embryonic development begins within a week postfertilization with the compaction of the morula-stage embryo. The development of a specialized epithelium, termed the "trophectoderm" (TE), supports fluid transport to create the blastocoel cavity of the blastocyst-stage embryo. Upon entry into the uterine cavity, the expanded blastocyst will "hatch" or escape from the zona pellucida, and the TE will be the key player in the formation of the placenta. The development of the placenta varies widely among mammals, and the genetic components controlling its development are poorly understood outside of the mouse (7). Therefore, this chapter will primarily focus on human hemochorial placentation.

IMPLANTATION AND THE FORMATION OF THE HUMAN PLACENTA

There are three main stages that occur during human implantation. The first is the apposition stage where the blastocyst will come into very close proximity to the maternal uterine epithelium. Next, at the adhesion stage, the TE of the embryo will have formed microvilli that will bind a variety of integrins on the receptive uterus (8,9). Finally, the embryo will actively invade into the uterine endometrium so deeply that the maternal tissue will temporarily close over the entire embryo. From the developing embryo, the TE will form two distinct placental components: the terminally differentiated syncytiotrophoblasts (STB) and proliferative cytotrophoblasts (CTB).

Early human implantation has been examined morphologically (10); however, immunohistochemical (IHC) or molecular studies are not possible at this early time point. These studies have revealed that fetal mesenchyme begins to appear within the cords of the proliferating CTB, termed "trabeculae," during the first week of implantation. Pillars of these

trabeculae are covered with a layer of STB and are separated by lacunae or areas where the maternal blood percolates through fetal tissue, therefore allowing nutrient and gas exchange between the embryo and maternal blood. Collectively, these trabecular pillars will give rise to the anchoring and chorionic villi that attach to the maternal decidua. Mesodermal progenitors from the embryo will initiate vasculogenesis by forming endothelial tubes within the mesenchyme during the first month of development. This fetal vasculature within the placental component will coalesce and become contiguous with the vascular component within the umbilical cord and collectively will form the fetal vasculature proper (11,12). Another differentiation event that occurs at the tips of the CTB columns is the formation of the extravillous trophoblasts (EVTB). The EVTB undergo an epithelial to mesenchymal transition (EMT) and migrate away from the CTB cell columns and into the maternal decidua (13,14). EVTB are found in interstitial, perivascular, and endovascular locations within the stroma and maternal spiral arteries (15). The decision that EVTB make to detach from the cell-cell contacts within the cell column is unique in that they move away from their niche and lose their proliferative capacity.

Early pregnancy loss in addition to preeclampsia and intrauterine growth restriction have all been associated with improper placental morphogenesis. Thus, these earliest stages in human placental development are important areas for investigation and are extremely relevant to maternal and fetal health. Nevertheless, studies are limited by the fact that obtaining implantation sites from the first weeks of pregnancy is difficult, and due to obvious ethical limitations, the study of human embryos is problematic. Therefore, an alternative experimental model has been developed by our laboratory and others to study early implantation. Although the use of choriocarcinoma cell lines and primary cell cultures derived from placentas has provided many insights into placental function, the main weakness of these models is that the cells have already undergone differentiation and have committed to the trophoblast lineage in vivo; thus, the opportunity for exploration of early lineage commitment does not exist.

hESC DIFFERENTIATION TO TROPHOBLASTS

The isolation of hESC (1) from the totipotent ICM of the human embryo has opened new avenues of studying human embryonic development (16,17). hESC fulfill the criteria set for ESC derived from other nonhuman primate embryos in that they (1) are derived from preimplantation or peri-implantation embryos, (2) proliferate in vitro in an undifferentiated state, and (3) can form all three embryonic germ layers, thus demonstrating the characteristic of pluripotency (1,2,18,19). A novel attribute of human and nonhuman primate ESC is their capability to spontaneously differentiate into trophoblasts, something that is not seen with mouse ESC. Trophoblast differentiation was evidenced by the consistent detection of chorionic gonadotrophin (CG) by radioimmunoassay and by the transcription of genes for both the α and β CG subunits upon spontaneous differentiation of rhesus monkey (2), common marmoset (18), and human ESC (1,16). Also, if withdrawal of media conditioned on mouse embryonic fibroblast (MEF) feeder layers and fibroblast growth factor 2 (FGF2) (1) is sufficient to allow hESC to form trophoblasts at some discrete frequency, then we anticipated that it would be also possible to demonstrate the formation of trophoblasts in vivo upon teratoma formation using IHC and molecular approaches. Surprisingly, although low levels of expression of CG subunit mRNAs are detectable, trophoblast differentiation in teratomas is not widespread from either rhesus monkey or human hESC, as determined from IHC analysis (20).

BMP SIGNALING IN TROPHOBLAST DIFFERENTIATION

An alternative method to differentiate trophoblasts from hESC was identified when scientists attempted to direct the formation of mesodermal tissues with BMP4, and a population of cells with an epithelial morphology was obtained (4). Subsequent microarray analysis revealed a number of genes associated with trophoblast differentiation. Furthermore, treatment with BMP2, BMP7, and growth differentiation factor 5 (GDF5) all showed similar differentiation patterns (4), and these results were confirmed by our lab (21) and others (22) with different hESC lines (H1 and H7, respectively). Later studies showed that exogenous basic FGF (bFGF) and the endogenous Noggin protein found in the conditioned media suppress BMP signaling to sustain the undifferentiated proliferative state of hESC (23). More recently, studies utilizing

BMP4 treatment under low oxygen (4%) and high oxygen (20%) have shown that BMP4 drives trophoblast differentiation exclusively, that is, without the formation of other differentiated cell types (24). In addition, the patterns of differentiation occur from the periphery of the colony toward the interior, and differentiation is accelerated in cultures at 20% oxygen (24). Thus, there appear to be fine balances within the ESCs microenvironment to maintain ESC pluripotency, and subtle changes within these balances may skew differentiation to a particular lineage.

TROPHOBLAST DIFFERENTIATION FROM EMBRYOID BODIES

When ESC are resuspended into aggregates under conditions that do not promote pluripotency (i.e., without bFGF for hESC), ESC will form spherical structures called EBs. Cells within EBs will differentiate into all three germ layers: endoderm, mesoderm, and ectoderm (25). This method of differentiation was first described in mouse, but has been adapted for rhesus and human ESC. Although the EB paradigm does not recapitulate embryogenesis precisely, it seemed an appropriate model to understand differentiation in a 3D system. Formation of EBs from rhesus and human ESC is associated with an increase in human chorionic gonadotrophin (hCG) secretion, although in a transient manner compared with differentiated human trophoblast cultures (3). This may be due to the fact that trophoblast cultures come from terminally differentiated placental tissues, and EB-derived trophoblast cells might represent trophoblasts from an earlier developmental time point or a different trophoblast subtype. hESC-derived EBs also form a cytokeratin-positive epithelium on the surface of EBs that coexpress hCG (3). More recently, trophoblasts were differentiated from cystic EBs made from the hESC (3) line from ES Cell International (ESI) while simultaneously differentiating endothelial cells (26). This further establishes the EB model for in vitro trophoblast studies regardless of the source of the hESC line used.

TROPHOBLASTS AND THE EXTRACELLULAR MATRIX

Traditional cell culture techniques fail to mimic the dynamics spatially and physically that are seen in vivo. In vivo, cells exist in 3D contact with other cells and with the surrounding extracellular matrix (ECM). Therefore, it was reasoned that the ECM might possibly provide the external signals to drive advanced trophoblast differentiation that occurs during early implantation. Thus, EBs were manually introduced into 3D "rafts" made of Matrigel after two to eight days of suspension culture (3). Matrigel is a basement membrane matrix produced by EHS mouse tumor cells and has been widely used for culturing various cell and tissue explants (27,28). In placental studies, Matrigel is often used to induce the EVTB phenotype from villous explants. Culturing the EBs in suspension for eight days provided enough time for trophoblast differentiation to occur on the EB surface. Following several weeks of culture in the Matrigel rafts, the EBs secreted high and sustained levels of hCG, progesterone, and estradiol-17β into the culture media and did so at greater levels when the media was changed daily (3). Trophoblast differentiation was not limited to the 3D Matrigel paradigm but was also seen when EBs were plated on Matrigel-coated culture dishes [two-dimensional (2D) paradigm]. The EBs in 2D also secreted hCG, estradiol-17β, progesterone, made outgrowths, and proliferated, but were not able to sustain the high levels of hormone secretion found in the 3D paradigm (3). This highlights the importance of cell matrix interactions as well as the dimensional environment on trophoblast differentiation.

The next question to be addressed was, "which component of Matrigel might be promoting this enhanced trophoblast differentiation?" Studies in our lab-tested matrix components found in Matrigel separately. Results show that when EBs are plated in 2D on either Matrigel or collagen IV, no difference in hCG secretion is seen between the two ECM surfaces (Fig. 1) (Gerami-Naini B et al., unpublished data). This indicates that in 2D, collagen IV can support similar hCG secretion as Matrigel, and soluble factors within Matrigel are not responsible for hCG secretion under 2D conditions. It remains possible that in 3D factors secreted by the EBs themselves are evoking the trophoblast cells in the EBs to either secrete more hCG or produce more trophoblast cells, resulting in sustained amounts of hCG secretion. Alternatively, 3D mechanosensing mechanisms are at play in the Matrigel raft paradigm. With this information at hand, the question arises as to what other extrinsic cues could be

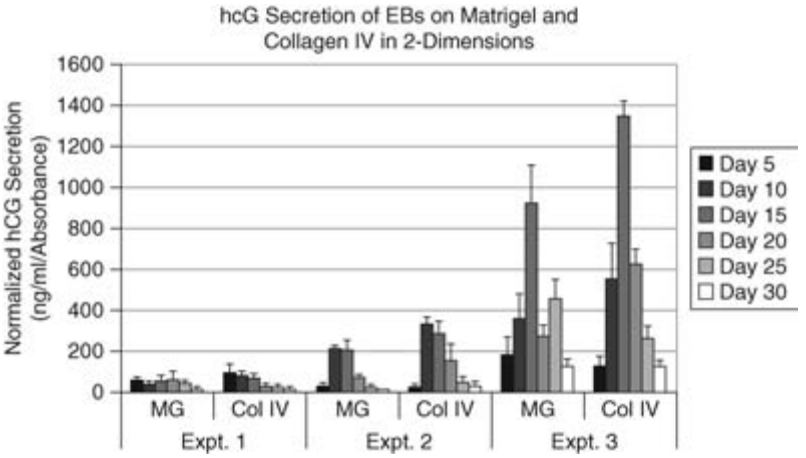

Figure 1 A Comparison of EBs grown in 2D on MG and ColIV for days 5, 10, 15, 20, 25, and 30 of culture. Secretion of hCG is shown and is normalized to the amount of metabolic activity of EBs in three independent experiments (Expt. 1, Expt. 2, Expt. 3). *Abbreviations*: MG, Matrigel; ColIV, collagen IV; hCG, human chorionic gonadotrophin; EB, embryoid body.

participating in early placental morphogenesis in vivo that can be applied to the in vitro culture system?

CELL-TO-CELL CONTACT AND SOLUBLE FACTORS IN PLACENTAL DEVELOPMENT

We approached further study of the 3D paradigm by reconsidering early placental morphogenesis. Upon the formation of the placental villi, fetally derived mesenchymal and endothelial cells begin to migrate in and are dispersed within the newly formed villi. It was therefore thought that EBs might require cell-cell contact from mesenchymal cells or endothelial cells to further trophoblast differentiation. A coculture system was thus devised where hESC were grown on feeder layers of fibroblasts derived from term human placentas to test if soluble factors from the fibroblasts could induce trophoblast differentiation. This was not the case for this paradigm: when hESC were grown on placental feeder layers, they clearly maintained an undifferentiated colony phenotype, in addition to maintaining expression of the pluripotency marker Oct-4 (Fig. 2). Similar results were obtained by Miyamoto et al. (29) when they cultured cynomolgus monkey ESC on human amniotic epithelial feeder cells and on human chorionic plate cells and found that the cells maintained ESC pluripotency characteristics. In addition, Genbacev et al. (30) found that hESC could be derived and maintained on human placental fibroblast lines, thus establishing hESC lines that are entirely human derived.

Thus, a new coculture system was developed where "combination EBs" were prepared by dissociating enhanced green flurorescet protein (ECFP)-labeled hESC from the culture dish and combining them with placental fibroblasts that were labeled with cell tracker red in suspension (Fig. 3). Both cells were placed in a nontissue culture dish to prevent adhesion and placed on a rocker at 37°C for 24 to 48 hours. The two cell types made spherical aggregates or combination EBs, where the EGFP-labeled hESC incorporated the red-labeled placental fibroblasts into the EB (Fig. 4). Combination EBs were sorted by size and by passage through a 100- and then a 70-μm cell strainer and examined by fluorescence microscopy. All combination EBs were kept in culture for 30 days, and media were collected daily. Combination EBs made with term placental fibroblasts (TPFs) showed a maximum fold change in hCG secretion on day 20 of culture compared with control suspension EBs that were made without any fibroblasts (Fig. 5) (Giakoumopoulos M et al., unpublished data). This indicated that cell-cell contact in 3D was beneficial in the induction of trophoblast differentiation. Subsequent experiments were performed to determine if the source of the fibroblast used as an "effector cell" would be a determining factor in trophoblast lineage development. These experiments entailed using dermal fibroblasts (CI2F), that is, utilizing cells from a nonplacental source as

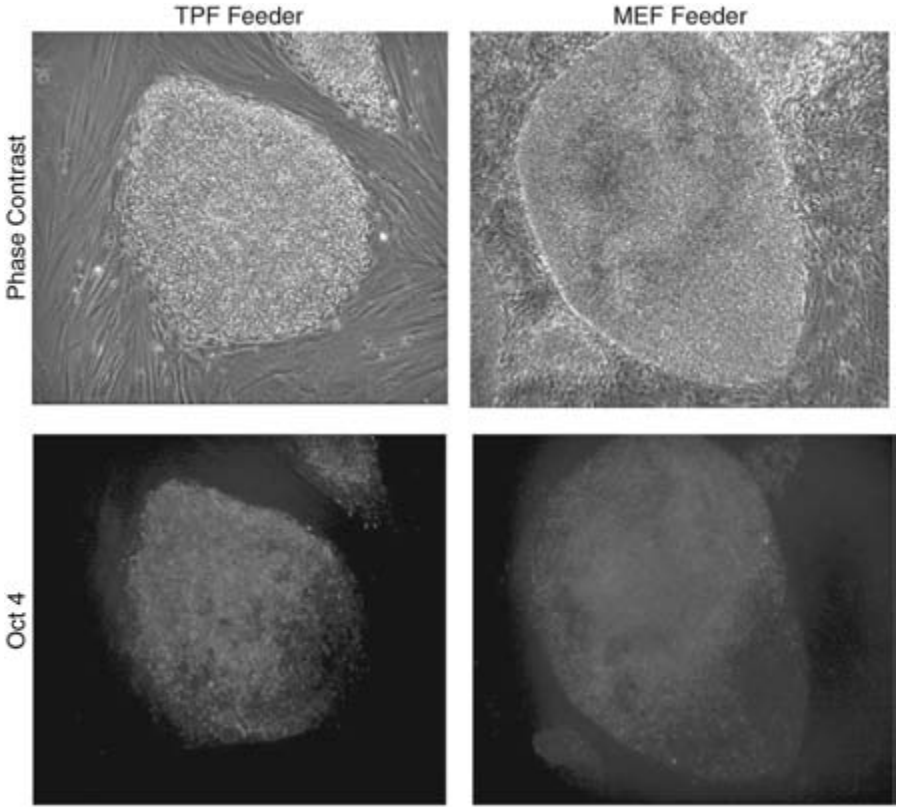

Figure 2 hESC grown in 2D on TPF and MEF feeder layers. hESC remain Oct-4 positive when grown on TPF and MEF feeder layers. Phase contrast images are shown in the upper panels, and Oct-4 immunofluorescent images are shown in the bottom panels. *Abbreviations*: TPF, term placental fibroblast; MEF, mouse embryonic fibroblast; hESC, human embryonic stem cells.

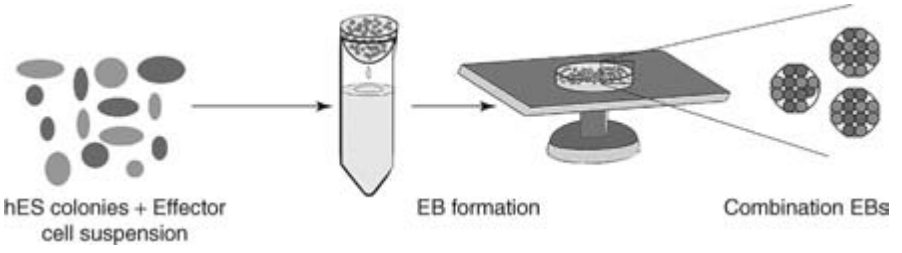

Figure 3 Schematic diagram depicting combination EB preparation. *Abbreviation*: EB, embryoid body.

the effector cell type. Although the trend in hCG secretion was lower than in TPF combination EBs, combination EBs made with dermal fibroblasts (CI2F) resulted in a surprising fourfold increase in hCG secretion compared with control suspension EBs made without effector cells on day 20 of culture (Fig. 5) (Giakoumopoulos Met al., unpublished data). Collectively, these experiments establish the idea that soluble factors from fibroblasts alone cannot support early trophoblast lineage differentiation, but rather cell-cell contact in a 3D environment supports trophoblast lineage differentiation. The data also suggest that this effect may not be tissue specific with regard to the source of the fibroblast type used as an effector cell. A possible interpretation could be that the fibroblasts might be secreting their own ECM within the combination EB and producing short-ranged signals that are only produced by cell-cell contact in 3D for the induction of trophoblast differentiation events not found in 2D monolayer coculture.

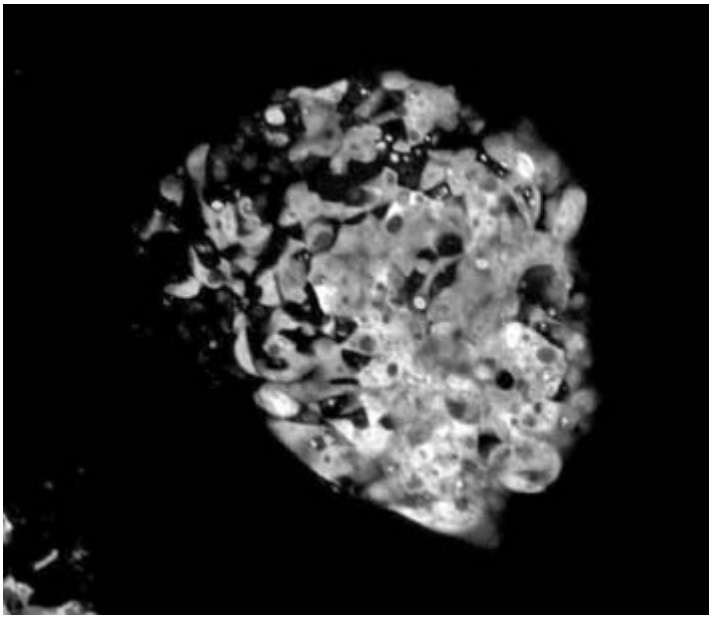

Figure 4 Confocal image of a TPF combination EB. Green cells are representative of EGFP-expressing hESC, and red cells dispersed within are the cell tracker red-labeled TPFs. *Abbreviations*: TPF, term placental fibroblast; EB, embryoid body; hESC, human embryonic stem cells.

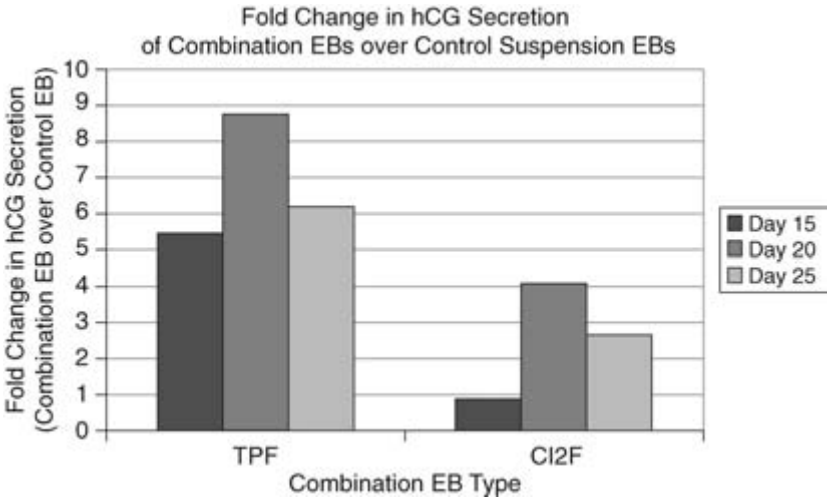

Figure 5 Fold change in hCG secretion of TPF or dermal fibroblast (CI2F) combination EBs over control suspension EBs on days 15, 20, and 25 of culture. *Abbreviations*: TPF, term placental fibroblast; EB, embryoid body.

Studies utilizing rhesus monkey ESC in 3D collagen culture systems demonstrated differential lineage commitment dependent on soluble factors and topologically different collagen matrices (31). Cells grown on collagen matrix and cocultured with fibroblasts showed preferential differentiation to the neural lineage, whereas cells grown on collagen sponges and cocultured with keratinocytes differentiated into endothelial cells, therefore demonstrating the importance of the ECM topography but also soluble factors that are involved in lineage development (31). Another example of an organotypic in vitro system resulting in the simultaneous differentiation of two distinct cell types was devised by Peiffer et al. (26). This two-step system includes the formation of cystic EBs in suspension in semisolid

Table 1 Culture Systems Resulting in Varying Lineage Differentiation

System	Surface	Cell line	Dimensions	Reference
Coculture	Collagen sponge Collagen I matrix	R366.4 rhESC	3D	Chen SS et al., 2003 (31)
Cystic EBs	Semisolid methylcellulose	hES3	3D	Peiffer I et al., 2007 (26)
EBs	Suspension culture Matrigel	H1	2D + 3D	Gerami-Naini B et al., 2004 (3)
EBs	Collagen I	H1EGFP	2D + 3D	Giakoumopoulos M, unpublished data

methylcellulose medium within which a platelet/endothelial cell adhesion molecule-1 (PECAM-1+) network develops, directing the differentiation of the endothelial component of the system (26). Then, subsequent growth of the EBs on the bottom on nontreated, polystyrene culture dishes produces a monolayer of trophoblast cell outgrowths, which secrete hCG (26). This again highlights the importance of both the spatial and physical aspects of the microenvironment that should be considered when designing studies to drive specific differentiation events in vitro (summary of experimental designs, Table 1).

INTEGRIN INTERACTIONS AND CELL SIGNALING

An important aspect of cellular differentiation is the integrin-ECM interactions that are not only relevant to anchoring or moving cells within a tissue but also for producing signaling events that influence cell fate. For example, studies with breast epithelial cells have established that ECM interactions with integrins demonstrate differential signaling when the mechanics applied to the matrix are different (flexible collagen I environment vs. rigid collagen I environment) (32). This model has been recently applied to our studies with EB-derived trophoblasts. Preliminary data suggest that the collagen I environment supports EVTB differentiation, but further analysis is necessary to identify the potential signaling events that occur in the rigid environment compared with the flexible one (Table 1). One could envision a model where the EB is representative of an early embryo coming into contact with the maternal endometrium and adhering to it in 2D and being exposed to the soluble factors in the surrounding environment, thus triggering the initial signaling events necessary for trophoblast proliferation. With time, the embryo embeds into the ECM, forming different integrin-ECM interactions and inducing more nonproliferative, terminally differentiated structures. Elucidating the signaling events that occur during this type of culture will help further define the model and its potential applications for studying early implantation. For example, EBs may be able to provide a manageable and more readily accessible model to extend previous studies implicating L-selectin as an adhesion molecule for human implantation (15).

TROPHOBLAST STEM CELLS

Differential lineages have been shown to be sustained by tissue-specific stem cell populations occupying defined niches in many tissues (33,34). Studies over the past decade have established the presence and role of TSC in the mouse placenta (7,35–37). Mouse TSC are isolated from blastocyst outgrowths or by culturing the ectoplacental cone of implanting embryos (35,38). Maintenance of the TSC population in culture or within the extraembryonic ectoderm depends on FGF4, which is expressed by the epiblast and binds to FGFR2 to drive downstream ERK1/2-activated pathways (39). Expression of key transcription factors maintains the TSC phenotype and is dependent on FGF4 expression. These factors include the homeobox gene Cdx2, the nuclear hormone receptor superfamily member ERRβ, and the T-box gene eomesodermin (Eomes) (37). Deletion of these genes by homologous recombination results in developmental defects and further supports their role in TSC maintenance. Therefore, TSC are central to mouse placental development.

Isolation and maintenance of cytotrophoblast stem (CTBS) cells from human blastocysts has proven to be more challenging (37). Recently, isolation of cells designated as CTBS cells from hESC was reported in feeder-free conditions (40). Initially, EBs generated from hESC and trophoblast cells were enriched by subsequent rounds of cellular aggregation and

disaggregation (40). Similar to the mouse conditions, medium containing FGF4 and heparin was used to maintain the human CTBS cells. After CTBS cell line isolation, spheroid trophoblast bodies were formed and placed on endometrial stromal cells where they displayed an invasive phenotype and some expression of HLA-G, an EVTB marker; however, if left unpassaged, cells would fuse and take on a syncytial morphology (40). Although it is not yet known if the conditions for directed differentiation to villous or extravillous lineages are consistent across additional cell lines, the isolation of CTBS cell lines may offer additional opportunities to study trophoblast differentiation and early human placental events.

Nonhuman primate opportunities for TSC derivation have recently been reported from rhesus monkey blastocyst outgrowths (41). In distinct contrast to mouse TSC, these rhesus TSC grow in the absence of feeder layers and bFGF and show invasive capabilities displayed by EVTB in addition to the ability to form giant cells in response to estradiol-17β (41). Surprisingly, RT-PCR analysis showed the expression of the pluripotency marker Oct-4, suggesting that the TSC maintain their potency potential. This also is in contrast to mouse TSC, which rapidly extinguish Oct-4 (35) as well as Nanog (42) and the transcription factor Sox2 (43) expression upon differentiation (37). On the other hand, TSC also express the mRNAs for syncytin-2, an STB marker, and mamu-AG, an EVTB marker (41). Although the homeobox transcription factor Cdx2 is a known trophoblast marker in mouse TSC, varying results have been obtained with trophoblast cells isolated from hESC (Garthwaite and Golos, unpublished data) and rhesus embryos (41), illustrating likely differences between mouse and primate placental development. Further analysis including evaluation of signaling events due to integrin and ECM as well as transcription factor expression will provide the appropriate information to determine if ESC-derived trophoblasts can truly fulfill their TSC potential.

FURTHER AREAS OF INVESTIGATION

hESC provide a promising model for regenerative medicine but also for developing new systems to study the basic aspects of human development. This basic knowledge would provide an excellent basis to study human disease. Some areas that would benefit from further investigation include the following:

- Determine the "dimensional role" of the ECM in the in vitro growth of cells in 2D compared to 3D in providing spatial cues for tissue differentiation.
- Elucidate the cell signaling events that are initiated upon differential mechanical pressures in a common ECM.
- Combine the biochemical, cell contact, and spatial cues necessary to mimic early extraembryonic development.
- Identify the potential of diverse ESC lines for trophoblast differentiation.
- Develop artificial scaffolding approaches to identify particular biochemical and spatial cues that transduce signaling pathways via the ECM.
- Compare paradigms using human ESC with nonhuman primate ESC.

The greatest promise of the nonhuman primate model is the potential of nonhuman primate ESC to not only define the in vivo differentiation potential of TSC but the pluripotency capacity of ESC and induced pluripotent stem cells as well (44,45).

ACKNOWLEDGMENTS

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13 Reproductive Stem Cells of Embryonic Origin: Comparative Properties and Potential Benefits of Human Embryonic Stem Cells and Wharton's Jelly Stem Cells

Chui-Yee Fong, Kalamegam Gauthaman, and Ariff Bongso

INTRODUCTION

Stem cell biology is moving forward with rapid progress, and many exciting scientific and clinical developments have taken place recently in this field. Stem cells have been isolated from a variety of sources including preimplantation embryos, germ cells, fetuses, umbilical cords, and adult organs. They have been classified as hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and embryonic stem cells (ESCs) based on characterization tests and plasticity. ESCs are generally pluripotent, while MSCs are multipotent and HSCs unipotent. The plasticity of all these types varies, decreasing as the human embryo develops into a fetus and then the adult. For example, human embryonic stem cells (hESCs) isolated from blastocysts are pluripotent and the most versatile, while the stem cells in adult organs are unipotent or multipotent. Since hESCs require the destruction of human embryos, hESC biology is charged with ethical controversies, emotion, hype, and frenzy and reinforced with political and commercial agendas and religious concerns. hESC research has also unjustly been associated with reproductive cloning. However, given the fact that hESCs can theoretically be differentiated into almost all 210 tissues of the human body, they hold the greatest potential to affect the lives of millions of people worldwide for the better by producing effective hESC-derived tissue therapy for several debilitating incurable diseases plaguing mankind.

Besides hESCs, other stem cell types in the human reproductive system have been reported. These novel reproductive stem cell types fall into a very unique category as some of their anatomical sources have an embryonic origin, and as such they may be very plastic, being either pluripotent or widely multipotent, thus serving as a complementary versatile source of stem cells to hESCs. For example, stem cells from the amnion, umbilical cord, testis (spermatogonia), ovary (oogonia), and uterus (endometrium) have all been demonstrated in the human reproductive system, and they have tremendous differentiation potential in the production of desirable tissues for transplantation therapy of many diseases, but can also help in the understanding of the pathogenesis of reproductive diseases and providing better treatment modalities for specific reproductive problems such as endometriosis; endometrial, ovarian and testicular cancers; and infertility. This full spectrum of stem cells of the reproductive system therefore opens a new field of "reproductive stem cell sciences" (Fig. 1).

Several differences exist in the differentiation potential of the stem cells of the various reproductive sources. This chapter will focus on two such embryonic sources of stem cells of the human reproductive system, viz., hESCs and stem cells from the Wharton's jelly (WJSCs) of the human umbilical cord. Comparisons in their isolation, growth behavior, plasticity, characterization, differentiation, and potential benefits will be made to illustrate how one can complement the other in terms of taking this branch of stem cell science to the clinic.

EMBRYOLOGICAL ORIGINS OF BLASTOCYSTS AND UMBILICAL CORDS IN THE HUMAN

The advent of human in vitro fertilization (IVF) has enabled the study of fertilization and early embryogenesis up to five to six days in vitro. Fertilization is brought about by mixing specific numbers of motile sperm with oocyte-cumulus complexes or microinjection of cumulus-free oocytes with single sperm by intracytoplasmic sperm injection (ICSI). Pronuclear formation, which is the first sign of successful fertilization, occurs within 20 to 24 hours followed by the

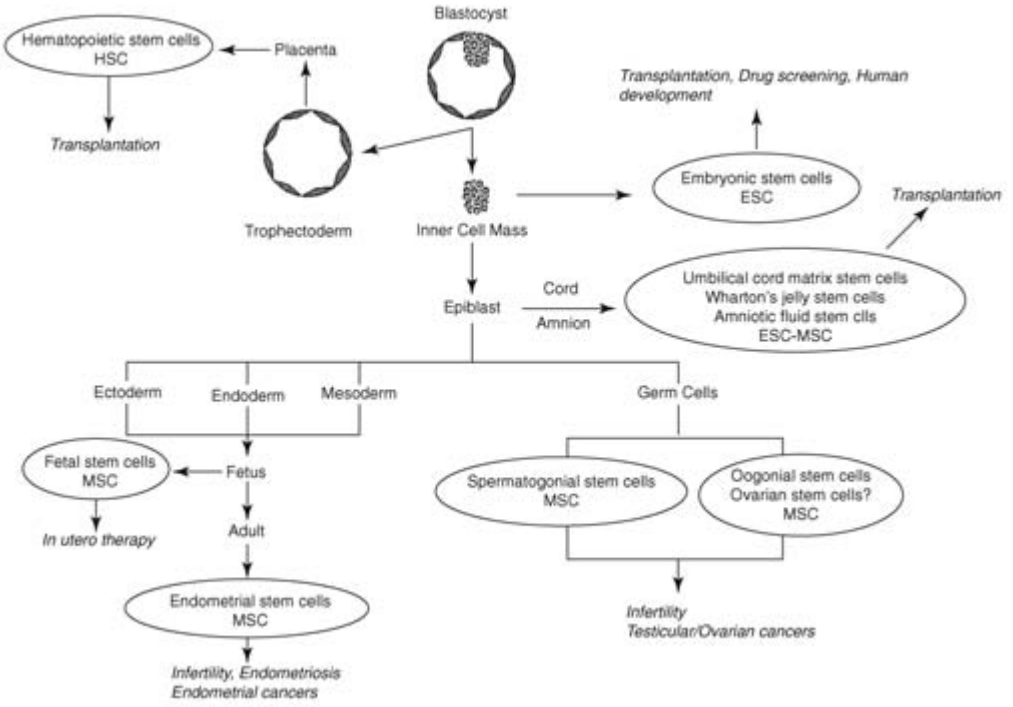

Figure 1 Schematic diagram showing the various stem cells of the human reproductive system reported to date and their potential applications.

first cleavage to the two-cell stage at around 25 to 26 hours postinsemination (1). Thereafter the early human embryo cleaves to the four- to six-cell stage at 48 hours, the eight-cell or compacting stage at 72 hours, the fully compacted or early cavitating stage at 96 hours, and the formation of early and fully expanded blastocysts at 120 hours and 144 hours (on days 5 and 6 postinsemination), respectively (1). Surplus blastocysts left over from such IVF clinics are usually donated with informed patient consent and institutional review board (IRB) approval for the derivation of hESC lines.

The blastocyst hatches out of the zona pellucida on around the seventh or eighth day postinsemination to implant into the endometrium of the uterus. With continuous embedding of the zona-free blastocyst deep into the endometrium, the approximately 40 to 50 cells of the inner cell mass (ICM) begin to rearrange themselves to form a bilaminar structure referred to as the epiblast and hypoblast. The hypoblast forms the yolk sac that degenerates during late embryogenesis in the human. The epiblast divides to form the three primordial germ layers (ectoderm, mesoderm, and endoderm), the amnion, allantois, and the constituents of the umbilical cord, viz., the umbilical blood vessels, Wharton's jelly, and other intervascular connective tissue. The amnion forms the wall of the umbilical cord. The part of the umbilical cord closest to the fetus also contains remnants of the allantois and yolk sac, both of which degenerate in late embryogenesis. Thus, the umbilical cord and its contents are truly embryonic in origin and theoretically therefore may contain some or all of the ESC markers seen in hESCs. The umbilical cord matrix cells were confirmed to be true stem cells that were mesenchymal in nature when tested for at least MSC markers (2). A cross section of the mid-region of the umbilical cord contains a single umbilical artery and two umbilical veins held together by intravascular and perivascular compartments. The Wharton's jelly, which is a gelatinous substance, is the connective tissue surrounding the blood vessels and includes the perivascular, intervascular, and subamnion regions (3).

DERIVATION AND PROPAGATION OF hESCs AND WJSCs

hESCs are traditionally derived from the ICM of five- to six-day-old blastocysts. The first report on the growth of ICMs and the isolation and partial characterization of hESCs was carried out by our group (4). Leftover IVF embryos donated by consenting patients were cocultured from the pronuclear to blastocyst stage on human fallopian tubal epithelial feeder cells. The zona pellucida was removed with pronase and zona-free blastocysts cultured on fresh irradiated human fallopian tubal epithelial fibroblasts as a "whole embryo culture" in the presence of Chang's medium supplemented with 1000 units/mL of human leukemia inhibitory factor (hLIF). Healthy ICM clumps that grew on the feeders were then mechanically separated, trypsinized, and passaged further on fresh human oviductal fibroblasts. Nest-like colonies were produced, which were mechanically separated again with sharp pointed hypodermic needles, disaggregated into single cells with trypsin-EDTA, and seeded onto fresh irradiated oviductal feeders. It was possible to retain the hESC characteristics of high nuclear-cytoplasmic ratios, prominent nucleoli, alkaline phosphatase positiveness, and normal karyotype for two passages in most of the embryos.

Later in 1998, Thomson et al. (5) were successful in generating hESC lines from preimplantation embryos that could be propagated for many passages. These workers used immunosurgery to isolate the ICM and grew these ICMs on irradiated murine embryonic fibroblasts (MEFs) instead of human fibroblasts.

hESC lines were also developed from single or groups of blastomeres extracted from poor-quality embryos using micromanipulation followed by coculture of such blastomeres with donor hESCs from already established hESC lines. The green fluorescence protein (GFP)-tagged donor hESCs coaxed the blastomeres to establish into hESC lines. This approach was considered ethically acceptable by some workers as it did not require the destruction of good-quality embryos since the biopsied embryo survived and could be used to establish pregnancy for the respective patient (6). However, Fong et al. (2006) (7) at the same time demonstrated that single or sister blastomeres from human embryos could not develop into hESC lines when plated directly on mitomycin-C-treated MEFs and kept in culture for as long as even 40 days. Some cells with typical hESC-like morphology were formed but degenerated soon in prolonged culture. Very recently, it was demonstrated that pluripotent stem cells [induced pluripotent stem cells (iPSCs)] could be produced by retroviral transfection of pluripotent genes (SOX2, NANOG, c-MYC, LIN28, KFP) into adult differentiated somatic cells. This approach not only had the advantage of bypassing the ethically sensitive need of using human embryos to derive panels of hESC lines but also helped to personalize the iPSCs and their derived tissues to specific patients to prevent immunorejection. The approach also overcame the need to use human or animal oocytes to personalize hESC-derived patient-specific tissues by nuclear transfer (NT) (8,9). Primate ESCs were produced by NT in the female rhesus macaque, using adult skin fibroblasts from male rhesus macaques for reprogramming (10). The same protocol was successfully repeated in the human to derive cloned human embryos, but these embryos were later destroyed, and development of cloned hESC lines not pursued (11).

Both feeder and feeder-free protocols have been developed to maintain and propagate hESCs once they are derived. Both murine and human feeders have been successfully used (12,13). They are either irradiated or treated with mitomycin-C to arrest their own development when used as feeders. A novel humanized system without animal-based xenosupports and xenoproteins was also reported to derive a xeno-free hESC line using human fetal skin fibroblasts, human serum, and human-based culture ingredients (14). This culture system prevents the transmission of animal prions and adventitious agents' crossing over from animal feeder cells to the hESCs and also avoids any changes in immunogenecity of the hESCs induced by the animal feeders. hESCs could also be propagated on noncellular matrices such as matrigel and collagen in the presence of hESC culture medium or with conditioned medium (medium previously grown on human or mouse feeders) (14,15).

Stem cells derived and propagated from the human umbilical cord matrix also have tremendous potential similar to hESCs as they are embryonic and originate from the epiblast (Fig. 1). The actual plasticity and true potential of such stem cells have been documented only recently, and their derivation and uses are less controversial as umbilical cords are discarded at birth.

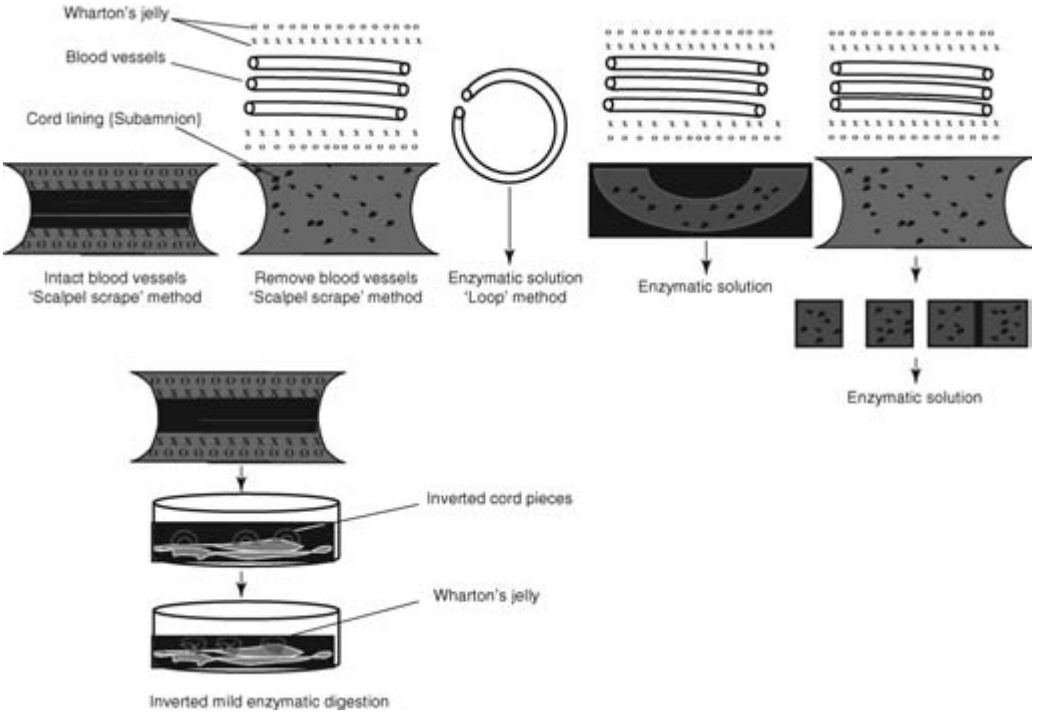

Figure 2 Schematic diagram showing six different methods of isolation of human UCMSCs. *Abbreviation*: UCMSCs, umbilical cord matrix stem cells.

Several authors have used different methods of isolating such stem cells from various areas within the umbilical cord (Fig. 2). Six different approaches of harvesting stem cells from the human umbilical cord matrix have been reported. The methods can be broadly classified on the basis of whether the umbilical cord vessels are removed or kept intact. In three of the six approaches, the blood vessels together with the perivascular matrix were removed, and the remaining inner surface of the cord piece scraped with a scalpel to retrieve stem cells, or the cord piece (without the vessels) was cut into small pieces or placed as a whole, in enzymatic solutions to digest the cells (2,16–18). It is difficult to confirm whether the stem cells originated from the subamnion, Wharton's jelly, or connective tissue fibroblasts around the cord walls. Another approach has been to remove the blood vessels and tie them into loops, which are then immersed into enzymatic solutions (19). In this method, stem cells may be recovered from the perivascular matrix and Wharton's jelly and probably not the subamnion. In the fifth approach the blood vessels were kept intact, and the inner cord matrix was scraped off with scalpel blades to isolate stem cells from the scrapings (20). It has been claimed that the most immature cells that have the greater competence to resume proliferation were located in the subamniotic and intervascular areas, while cells from the perivascular areas comprised mainly highly differentiated myofibroblasts (21). More recently, our group made a careful attempt to isolate stem cells specifically from the Wharton's jelly (22). In this method, the cord was cut into 2 cm pieces, washed in culture medium, and cut open lengthwise, and the blood vessels gently teased to remove any blood cells from within. The inner surface of the cord piece was then inverted directly into an enzymatic solution so that only the Wharton's jelly came into contact with the enzymes (Fig. 2). The enzymatic solution was a mixture of collagenase and hyaluronidase. Enzymatic digestion was only for 45 minutes at 37°C to loosen up the Wharton's jelly. After digestion the cord pieces were transferred to fresh culture medium faceup this time, and using the curved surface of watchmaker forceps instead of scalpel blades, the surface Wharton's jelly was gently separated and placed in centrifuge tubes. Utmost care was taken to gently detach only the Wharton's jelly. The gelatinous masses were resuspended with gentle pipetting in the

tubes, centrifuged at 300 g for 5 minutes, supernatant discarded, cell pellet resuspended in fresh medium, and the cells then plated on plastic or mitomycin-C-inactivated MEFs in the presence of hESC medium or a conventional umbilical cord matrix stem cell (UCMSC) medium used by other workers (23). The UCMSC medium was a basal DMEM (high glucose) medium supplemented with 15% fetal bovine serum (FBS), glutamine, and antibiotic-antimycotic solution, while the hESC medium was also DMEM (high glucose) but had 20% FBS, glutamine, antibiotic-antimycotic solution, insulin-transferrin-selenium (ITS), and bovine fibroblast growth factor (bFGF). We aimed to study comparatively the behavior of WJSCs in the presence of conventional UCMSC and hESC conditions to evaluate whether culture conditions had any influence on cell phenotype and behavior.

The growth behavior of hESCs and WJSCs appeared to be different even if given the same culture conditions. hESCs traditionally form nice circular colonies on feeders or feeder-free matrices with slight differentiation in the periphery and center of each colony. The undifferentiated areas are usually carefully cut out mechanically into small pieces and used for passaging ("Cut and paste" method). hESCs could also be enzymatically digested and seeded as a whole on feeder cells or matrigel-coated plastic dishes ("bulk culture") (24). WJSCs on MEFs form atypical colonies different from hESCs that contain a few hESC-like cells (22) (Fig. 3). The colonies degenerate with serial passaging. On plastic, the WJSCs form epithelioid islands in primary culture in hESC culture conditions, which transform into fibroblasts from the first passage onward. Under UCMSC conditions with or without feeders, the WJSCs produce fibroblasts from primary culture onward (22). The fact that the WJSCs started off as epithelioid cells in primary culture confirms that such harvested cells were not contaminated with normal connective tissue cells, which usually grow as fibroblasts in culture (22). It thus appears that culture environment influences the morphological characteristics of WJSCs. A very significant

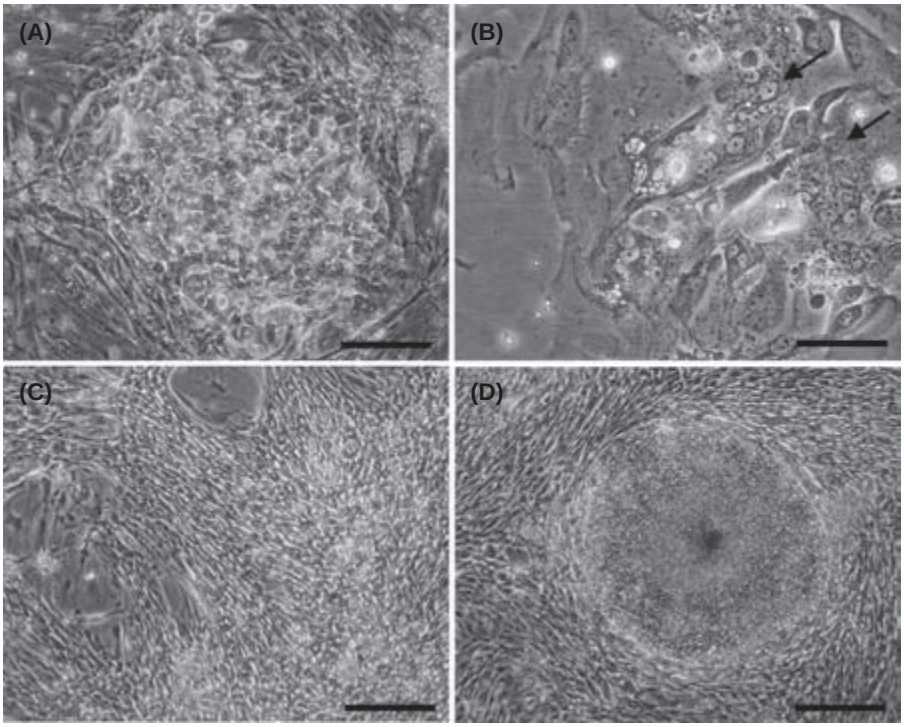

Figure 3 Morphological behavior of WJSCs grown on MEFs in the presence of hESC medium. (**A**) WJSC showing colony formation, which is not typically circular like hESC grown on MEFs in hESC medium. (**B**) Higher magnification of WJSC colony grown on MEFs in hESC medium, showing some cells similar to hESCs with large clear nuclei and prominent nucleoli (*arrows*). (**C**) WJSC colonies remaining as fibroblasts on MEFs in UCMSC medium. (**D**) Control typical hESC colony growing on MEFs in hESC medium. *Abbreviations*: hESC, human embryonic stem cell; WJSCs, Wharton's jelly stem cells; MEFs, mouse embryonic fibroblasts. *Source*: From Ref. 22 with permission from Reproductive Healthcare Ltd.

characteristic of WJSCs over hESCs is the short population-doubling time, thus making it possible to scale up WJSCs in a short time. Like hESCs, WJSCs also freeze well using snap-freezing or vitrification methods.

hESCs can be propagated for well over 200 passages without spontaneous differentiation and changes in characterization (unpublished data), while WJSCs retain their characterization and fibroblastic morphology only until about 50 to 55 passages and then take on a broad band-like morphology with senescence and cell death thereafter (Fig. 4). WJSCs have a habit to pile up like mounds in later passages, which some workers refer to as colonies. Such cell mounds do not exceed more than 0.4 mm in diameter (Fig. 5).

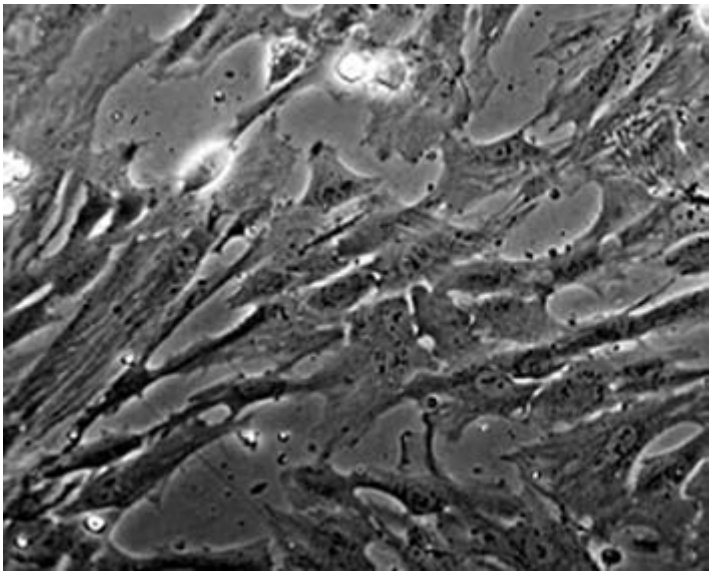

Figure 4 WJSCs at 50th passage changing to broad band-like morphology and undergoing senescence and cell death. *Abbreviation*: WJSCs, Wharton's jelly stem cells.

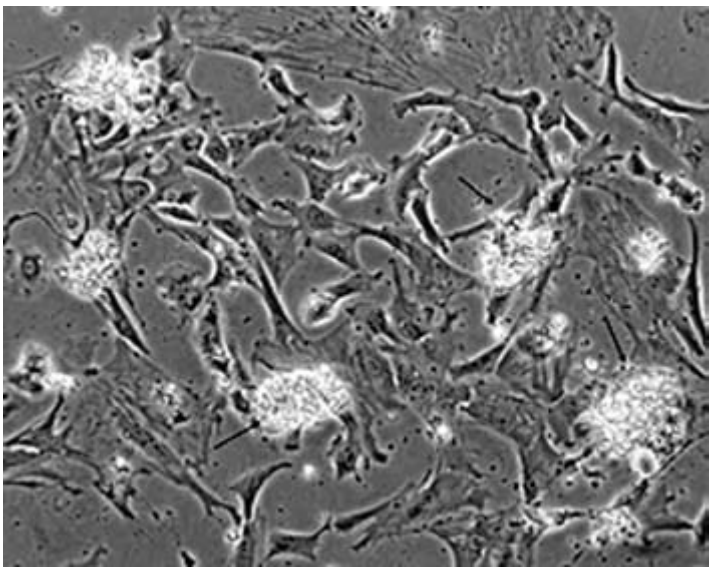

Figure 5 WJSC at 10th passage showing confluent fibroblast-like cells with piling up of cells ("mounds") (*arrows*), which is sometimes mistaken for as colony formation. *Abbreviation*: WJSC, Wharton's jelly stem cell.

Comparative Characterization of hESC and WJSC

Stem cells are generally classified as hematopoietic (HSC), mesenchymal (MSC), and embryonic (ESC) based on specific characterization markers. hESCs are positive for the typical ESC markers. These include the surface marker antigens (SSEA-1, -3, -4 and Tra-1 series), Oct 3 and 4, alkaline phosphatase, karyotype stability, embryoid body (EB) formation, telomerase production, genomic markers for the three primordial germ layers, and the SCID mouse teratoma assay (24). MSCs are usually negative for these markers but are positive for a battery of CD markers.

Most of the characterization studies on UCMSCs have used CD markers for MSCs, and it turned out that such stem cells are positive for most of the CD markers, which hESCs are negative for, viz., CD105, CD44, CD34, CD30, and MHC antigens. However, we exposed WJSCs to both MSC and ESC markers to find out whether WJSCs possess ESC markers under hESC culture conditions given the fact that the umbilical cord also originates like hESCs from the epiblast. Using immunofluoresence we showed that under hESC conditions (MEFs and hESC medium) as well as on plastic, WJSCs were positive for Tra-1-60, Tra-1-81, SSEA-1, and SSEA-4 but were negative for SSEA-3 (22) (Table 1). Interestingly, hESCs are positive for all these markers including SSEA-3 (Table 1). Thus SSEA-3 becomes an ideal differential marker to detect and separate WJSCs from hESCs and possibly other UCMSCs. Flow cytometric plots for WJSCs showed that they were positive for CD105, CD90, CD44 and negative for CD45, CD34, HLA-DR/DP, DQ–/CD30–, HLA-DR/DP, DQ+/CD30+, HLA-DR/DP, DQ+/CD30–, HLA-DR/DP, DQ–/CD30+ (22) (Table 2). Interestingly, Hoynowski et al. (2007) (25) demonstrated from flow cytometric studies that a subpopulation of WJSCs in horse umbilical cords was positive for the embryonic surface marker antigens SSEA-4 and Tra-1-60.

Table 1 Surface Marker Antigens and Teratoma Formation in SCID Mice for WJSCs and hESCs

			Surface marker antigens				Teratomas/SCID mice		
Cell type	No. of donors	Tra-1-60 ± (%)	Tra-1-81 ± (%)	SSEA-1 ± (%)	SSEA-3 ± (%)	SSEA-4 ± (%)	No. of mice	Weeks	Teratoma, *n* (%)
WJSC	3	+(100)	+(100)	+(100)	–(0)	+(100)	12[a]	12	0
hESC	2	+(100)	+(100)	+(100)	+(100)	+(100)	6	12	6

[a]Three mice each for WJSCs grown on plastic and MEFs with hESC and UCMSC medium.
Abbreviations: WJSCs, Wharton's jelly stem cells; hESCs, human embryonic stem cells; MEF, murine embryonic fibroblasts; UCMSC, umbilical cord matrix stem cell.
Source: From Ref. 22 with permission from Reproductive Healthcare Ltd.

Table 2 Immunohistochemical and Flow Cytometric Analysis in WJSCs and hESCs for CD Markers

Cell type	No. of patients	Passage	CD105[a]	CD90[b]	CD44[b]	CD34[b]	HLA^{-b} DR, DP, DQ^{-} $CD30^{-}$	HLA^{-b} DR, DP, DQ^{+} $CD30^{+}$	HLA^{-b} DR, DP, DQ^{+} $CD30^{-}$	HLA^{-b} DR, DP, DQ^{-} $CD30^{+}$
WJSC	3	2–4	+	+	+	−	−	−	−	−
WJSC	3	5–10	+	+	+	−	−	−	−	−
hESC[c]	2	3	−	+	−	−	−	−	−	−
hESC[d]	2	8–10	−	+	−	−	−	−	−	−

[a]Immunohistochemistry.
[b]Flow cytometry.
[c]Cut and paste method.
[d]Bulk culture method.
Abbreviations: WJSCs, Wharton's jelly stem cells; hESCs, human embryonic stem cells.
Source: From Ref. 22 with permission from Reproductive Healthcare Ltd.

WJSCs showed normal Giemsa-banded karyotypes at P1, P5, P10, and P50 passages and were also alkaline phosphatase positive using both immunohistochemistry as well as RT-PCR (22). When WJSCs were exposed to the conventional EB protocols used for hESCs, they produced pseudo-EBs that were circular but turned dark brown with time in culture and underwent degeneration. Histological and ultrathin examination of these pseudo-EBs did not reveal the presence of cells from the three primordial germ derivatives. They were thus considered negative for proper EB formation (22). Similar-looking pseudo-EBs growing as tight adherent clusters were also observed in umbilical cord blood stem cells by McGuckin et al. (2005) (26). When RT-PCR for the full battery of pluripotent genomic markers was carried out on WJSCs, they expressed 9 of 10 such markers including Oct 4, NANOG, SOX2, TERT, REX1, and ISL1 (Fig. 6). However, all these pluripotent markers were expressed at low levels in WJSCs compared with hESCs. Interestingly, Vimentin expression was much higher for WJSCs compared with hESCs, while nestin and AFP were lower in WJSCs compared with hESCs (22) (Fig. 6).

A salient characteristic of WJSCs is that when grown on plastic or MEFs in the presence of hESC or UCMSC medium, they did not produce teratomas in SCID mice when compared with hESC controls. Of 12 SCID mice injected with WJSCs, none produced teratomas at 12 weeks, while hESCs injected into six control mice produced teratomas in all of them (22) (Table 1). This feature is consistent with the inability of WJSCs to form EBs in vitro.

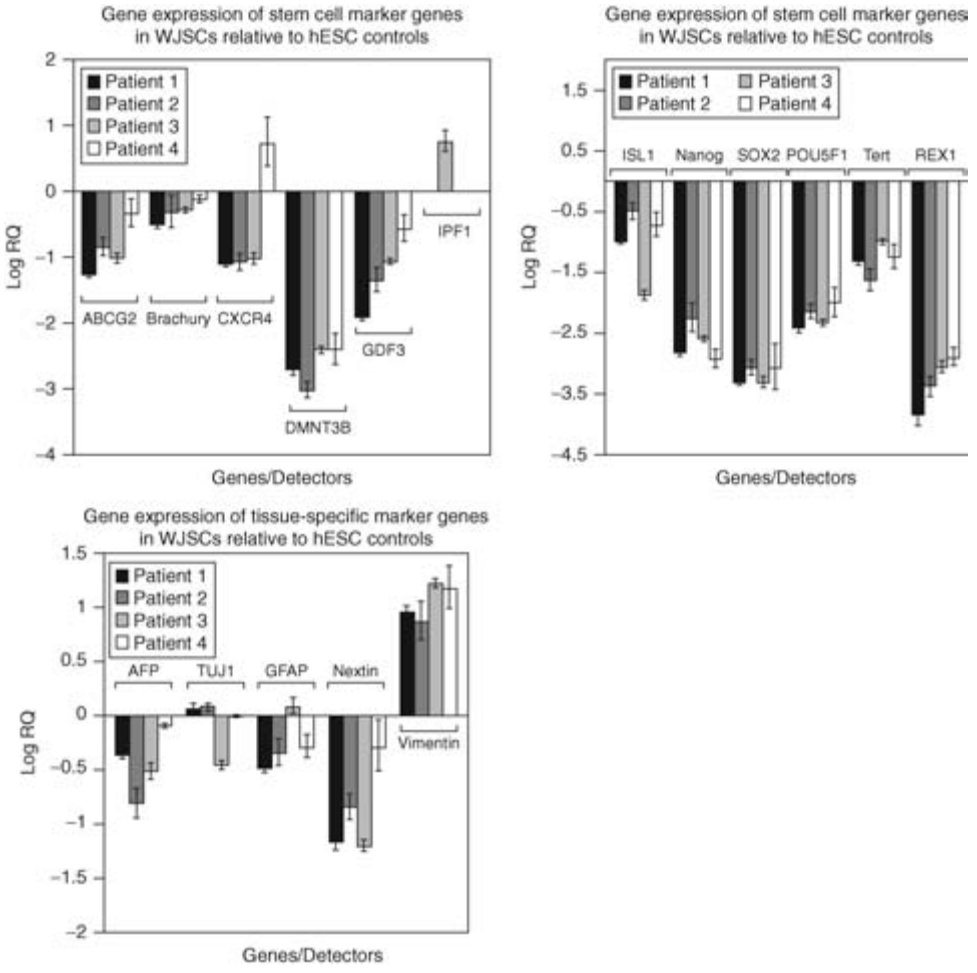

Figure 6 Quantitative real-time polymerase chain reaction gene expression of stem cell and tissue markers in WJSCs relative to undifferentiated hESCs. WJSC grown under hESC conditions. Actin-β endogenous control was used to normalize gene expression data. Undifferentiated HES-3 cells were used as the calibrator samples. *Abbreviations*: hESC, human embryonic stem cell; WJSCs, Wharton's jelly stem cells. *Source*: From Ref. 22 with permission from Reproductive Healthcare Ltd.

Differentiation and Plasticity of hESC and WJSCs

Stem cells are generally classified as totipotent, pluripotent, multipotent, and unipotent. Early mammalian embryos contain clusters of totipotent cells that have the potential to form the entire organism. hESCs are considered pluripotent as theoretically they could generate via the three primordial germ layers all 210 tissues of the human body. Multipotency is the ability to produce a limited range of tissue types and unipotent stem cells such as bone marrow stem cells produce tissues of one specific lineage such as blood cells.

hESCs appear to be the most versatile having been shown to differentiate to date into pancreatic islets, cardiomyocytes, neurons, bone, cartilage, hepatocytes, keratinocytes, and several other lineages. Differentiation into such lineages is either through spontaneous or controlled methods (27). In the spontaneous approach, the hESCs are made to form EBs, which if left alone, will produce the primordial germ layers within them. The desired tissue from a specific germ layer is then isolated, expanded, and purified in large numbers. Spontaneous somatic differentiation into neurons from hESCs was reported by Reubinoff et al. (2000) (28). Controlled differentiation can be successfully carried out by (*i*) coculture of hESCs with companion fetal or adult cells of the desired lineage, (*ii*) transfection of hESCs with specific genes, and (*iii*) the use of specific proteins or growth factors in the hESC culture environment. Confirmation of the desired tissue that is differentiated is by in vitro characterization tests or demonstration of functional competence of that specific tissue after transplantation into animal models.

When WJSCs were cultured with rat brain neuronal-conditioned medium, they could generate neuronal proteins, and the conditioned medium upregulated the astrocyte protein GFAP. The same authors were also able to increase CD11b microglial cells by twofold by subjecting the WJSCs to the same neuronal-conditioned medium (29). WJSCs treated with salvia miltiorrhiza (a shade growing perennial flowering plant) expressed the neuronal marker β-tubulin 111, neurofilament, and GFAP (30). We showed that WJSCs can be successfully differentiated into neurons using a standard neuronal differentiation protocol (22). The WJSCs were incubated overnight with bFGF, and the cells later exposed to DMSO and butylated hydroxyanisole (BHA). After five to six hours the culture environment was changed to a mixture of potassium chloride, valproic acid, forskolin, hydrocortisone, and insulin for long-term induction and maintenance of neuronal differentiation. This was also consistent with the findings of Mitchell et al. (2003) (16) and Wang et al. (2004) (17) who also demonstrated the differentiation of WJSCs into neurons.

Additionally, WJSCs could be induced into bone, cartilage, and adipose cells (17–19,31). WJSCs also expressed cardiac troponin 1, connexin 43, and desmin, which were the standard markers for cardiomyocyte differentiation and also showed cardiomyocyte phenotypes when exposed to three weeks of 5-azacytidine treatment (17). WJSCs also differentiated into endothelial cells in vivo after initial exposure to vascular endothelial growth factor (VEGF) and bFGF in vitro and then injected into mouse ischemic hearts (32). When WJSCs were exposed to myogenic culture medium, they expressed the Myf-5 marker for skeletal muscle on day 7 and Myo-D on day 11 and produced long multinucleated cells. When the WJSCs were injected into damaged rat muscles, these elongated cells produced the sarcomeric tropomyosin protein (31).

ENGRAFTMENT OF WJSC- AND hESc-DERIVED TISSUES IN ANIMAL MODELS

hESC-derived cardiomyocytes successfully engrafted in the peripheral scar tissue of ischemic rats and mice (33,34). Using activin A and BMP4, hESCs were differentiated into cardiomyocytes and then injected into ischemic infarcted rat hearts together with a pro-survival cocktail of factors that limited cardiomyocyte death after transplantation. This approach enabled consistent formation of myocardial grafts in the infracted rat heart. Also the engrafted human myocardium attenuated ventricular dilation and preserved regional and global contractile function after infarction, compared with controls receiving noncardiac hESC derivatives or a vehicle (34). Shim et al. (2007) (35) showed that hESCs sequentially treated with serum, activin, and retinoic acid during EB formation produced pancreatic endodermal cells that expressed islet markers PDX1 and HLXB9. After transplantation of these cells into streptozotocin-induced diabetic mice, the PDX1-positive cells differentiated into mature cells that released insulin and glucagon, resulting in amelioration of hyperglycemia and weight

loss. Yang et al. (2008) (36) recently reported that hESC-derived dopaminergic neurons survived transplantation to the neurotoxin 6-hydroxydopamine lesion rat striatum and in combination with the cells newly differentiated from their progenitors contributed to locomotive function recovery at five months. The animal behavioral improvement correlated with the dopamine neurons present in the graft.

Similarly, in parkinsonian rats that received WJSCs injected into the brain there were significant decreases in apomorphine-induced rotations from 4 to 12 weeks compared with controls. Following sacrifice of the rats, the number of tyrosine hydroxylase–positive cells in the midbrain correlated strongly with the behavioral observations (2). The authors hypothesized that the WJSCs probably released GDNF (a potent survival factor for dopaminergic neurons) as well as VEGF and ciliary neutrophic factor. In a different study WJSC-derived neurons [WJSCs treated with neuron-conditioned medium supplemented with hedgehog protein and FGF 8] were transplanted into parkinsonian rats, and five months later improvements were observed in behavior (29). Jomura et al. (2007) (37) transplanted WJSCs into the brains of rats with cerebral ischemia induced by cardiac arrest and showed significant neuronal loss due to the rescue phenomenon hypothesized by Weiss et al. (2006) (2).

WJSCs were found within and in close proximity to respiratory tumors when breast carcinoma cells were administered intravenously into SCID mice followed by intravenous administration of fluorescently labeled WJSCs. WJSCs had an affinity for homing into areas of tumor growth a feature also seen with neural stem cells (38). When β–human interferon produced from manipulated WJSCs was injected into SCID mice having such tumors, the tumor growth was significantly reduced (39). WJSCs also exhibit immunogenicity in vivo as shown in pigs. Repeated injections of porcine WJSCs into pigs elicited an immunogenic response (40). WJSCs also produced histological evidence of photoreceptor repair when administered into the retina of mice with retinal disease (41).

OTHER SOURCES OF UMBILICAL CORD STEM CELLS

Besides the isolation of stem cells directly from the Wharton's jelly by Weiss et al. (2006) (2) and Fong et al. (2007) (22), other workers have isolated mesenchymal multipotent stem cells from other areas within the human umbilical cord. MSCs were isolated from the perivascular tissues directly surrounding the umbilical blood vessels (HUCPVCs) (19,42), from the blood within the umbilical cord blood vessels [see Troyer and Weiss (2008) (3) for review of many such reports], from the subamnion (43), and from the subendothelium of umbilical veins (20). Since the Wharton's jelly extends from the subamnion through the intervascular and perivascular compartments, all these sources of MSCs may be actually WJSCs containing two distinct types of stem cells (type 1 and 11) as shown by Karahuseyinoglu et al. (2007) (18) and our studies (22). In fact the former authors divided their isolated stem cell populations of WJSCs into type 1 cells that were shorter fibroblast-like, expressing cytokeratin and other differentiation markers, and type 11 WJSCs, which were elongated fibroblasts that differentiated into neural cells more effectively. We showed changes of epithelioid to fibroblast-like morphology of WJSCs in conventional UCMSC and hESC culture conditions (22). Karahuseyinoglu et al. (2007) (18) reported that WJSCs had greater expansion capabilities and shorter population-doubling times compared with HUCPVs. They hypothesized that HUCPVs are more differentiated than WJSCs, thus suggesting why HUCPVs could not differentiate into neuronal cells.

Umbilical vein subendothelial MSCs (20) were shown to be similar to bone marrow MSCs and had multilineage potential (20,44–46).

HOW DO WJSCs COMPARE WITH FETAL AND ADULT STEM CELLS?

Recently Troyer and Weiss (2008) (3) reviewed the literature on WJSCs and concluded that they were a primitive stromal cell population with tremendous therapeutic potential. The same authors also reported that WJSCs shared several properties with fetal-derived MSCs. This was based on the fact that WJSCs had great expansion potential than adult MSCs, expressed HLA class 1 antigens, did not express HLA class II surface markers, were immunorepressive in mixed lymphocyte assays, and inhibited T-cell proliferation.

WJSCs also shared the basic characteristics of adult MSCs. Additionally, WJSCs expressed GD2 synthase, which was a marker specific for adult bone marrow MSCs (3). WJSCs were considered similar to adult MSCs in bone marrow because they were positive for the common adult MSC CD markers as well as express CD73, CD90, CD105, CD10, CD13, CD29, and CD44 (3). WJSCs were negative for CD34, CD45, CD14, CD33, CD56, and CD31 (2).

CONCLUSIONS

It is very clear from our studies and similar studies by other workers that bona fide stem cells do exist in the human Wharton's jelly. Some authors classified these stem cells as mesenchymal stromal cells and stated that they were a primitive stromal population as they grew as adherent cells with mesenchymal morphology, were self-renewing, expressed cell surface markers characteristic of MSCs, and were multipotent being able to be differentiated into neurons, bone, cartilage, cardiomyocytes, adipose, and endothelial cells (3). Although most workers classified other UCMSCs as multipotent MSCs in the absence of exposure of their cells to ESC markers, our studies clearly showed that WJSCs are widely multipotent being positive to not only MSC but also to many ESC markers. However, because they did not pass the full battery of ESC marker tests, they cannot thus be considered pluripotent like hESCs according to international standardized definitions of plasticity.

A striking feature of WJSCs is their prolific growth with short population-doubling times up to at least 50 to 55 passages (22). This is a tremendous advantage over hESCs when it comes to scaling up stem cell numbers for clinical application. Additionally, the intrinsic ability for WJSCs to exhibit this prolific growth on plastic instead of cell feeders makes them easier and less time consuming for scaling up numbers compared with hESCs. Growth on plastic also circumvents the risks of animal viral cross contamination from MEFs (22). It is interesting to note that porcine WJSCs grown on plastic also showed a heterogenous morphology of many spindle-shaped and round cells with high nuclear-cytoplasmic ratios (a feature of hESCs) in the presence of a very complex culture medium, containing ingredients over and above that used in conventional hESC medium. These hESC-like cells were also positive for alkaline phasphatase (47). These observations of these workers were consistent with those of our group on human WJSCs.

In contrast to bone marrow stem cells, WJSCs are embryonic, have great expansion capabilities, are well tolerated by the immune system, have short population-doubling times, can synthesize different cytokines, are not tumorigenic, and thus have tremendous therapeutic potential as an adjunct to hESCs for a variety of incurable diseases by transplantation therapy (Table 3).

Table 3 Similarities and Differences Between hESCs and WJSCs

HESCs	WJSCs
Embryonic in origin	Embryonic in origin
Positive for some CD markers	Positive for many CD markers
Positive for all ESC markers	Positive for some ESC markers
Pluripotent (*all body tissues*)	Widely multipotent (*bone, cartilage, adipose tissue, muscle, and neuronal cells*)
May induce teratoma formation	No teratoma formation
Long population-doubling time	Short population-doubling time
Takes time to scale up numbers	Can be scaled up in numbers rapidly
Unlimited self-renewal to many passages	Can be self-renewed to about 55 passages
Require cell feeders/matrices for growth	Can be grown on plastic without differentiation
hESC-derived tissues may be rejected unless personalized	WJSC-derived tissues may be rejected unless personalized
Therapeutic in preclinical animal models for the common human diseases	WJSC-derived tissues may not be rejected

Abbreviations: WJSCs, Wharton's jelly stem cells; ESC, embryonic stem cells; hESCs, human embryonic stem cells.

SUMMARY

Thus far, a variety of stem cells from preimplantation embryos, the fetus, and adult tissues and organs have been isolated in the human. They have been classified as HSCs, MSCs, and ESCs based on characterization tests and plasticity. ESCs are generally pluripotent, while MSCs are multipotent and HSCs unipotent. Stem cells of the reproductive system fall into very unique categories as some of the anatomical structures of this system have an embryonic origin, and as such they may be pluripotent or widely multipotent, serving as a complementary versatile source of stem cells to hESCs. hESC-derived tissues for transplantation therapy are charged with ethical sensitivities and are fraught with the problems of immunorejection, inadequate cell numbers, and teratoma formation from rogue undifferentiated hESCs in the differentiated tissue that have not completed the differentiation process. However recently, the issue of immunorejection may have been resolved with the development of personalized iPSC lines where pluripotent genes were used to reprogram adult tissues of patients. On the other hand, WJSCs of the umbilical cord, which are also of embryonic epiblast origin could be harvested in abundance given the number of umbilical cords discarded at birth and as such do not face ethical sensitivities. It was recently shown that WJSCs could be successfully isolated and grown from full-term umbilical cords. When WJSCs were grown on plastic, they did not differentiate unlike hESCs, and they exhibited two different morphologies based on the type of culture medium used. Their population-doubling times were shorter than hESCs, and they produced confluent monolayers in less than a week. When WJSCs were grown in hESC culture conditions on mouse feeders in the presence of hESC culture medium, they produced atypical colonies containing hESC-like cells and stained positive for most of the hESC stemness markers, showed normal chromosome makeups, produced EB-like structures, could be converted into neuronal derivatives, and did not induce tumors in SCID mice. They were also positive for many of the adult MSC CD markers, and although many of the hESC molecular genetic markers were detectable, they were expressed at low levels. WJSCs may not be pluripotent and as versatile as hESCs but are widely multipotent and have the advantages of being able to be scaled up in large numbers and do not produce teratomas.

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14 | Amniotic Fluid and Placenta Stem Cells

Anthony Atala

INTRODUCTION

Human amniotic fluid has been utilized in prenatal diagnosis for over 70 years. It has proven to be a safe, reliable, and simple screening tool for a wide variety of developmental and genetic diseases (1). The first reported amniocentesis took place in 1930 when attempts were being made at correlating cytologic examination of cell concentration, count, and phenotypes present in the amniotic fluid to the sex and health of the baby. Since then, development of karyotyping techniques and the discovery of reliable diagnostic markers such as α-fetoprotein, as well as the development ultrasound-guided amniocentesis, have greatly increased the safety and reliability of the procedure as a valid diagnostic tool (2–7).

However, there is now evidence that amniotic fluid may have more utility than only as a diagnostic tool and may be a source of a powerful therapy for a multitude of congenital and adult disorders. A subset of cells found in amniotic fluid has been isolated and found to be capable of maintaining prolonged undifferentiated proliferation as well as differentiate into multiple tissue types encompassing the three germ layers. Amniotic fluid–derived progenitor cells can be isolated from a small amount of the fluid obtained during amniocentesis, a procedure that is already performed in many pregnancies to screen for congenital abnormalities. Placenta-derived stem cells can be obtained from a small biopsy of the chorionic villi. Cell culture experiments with these two types of cells have provided evidence that they may have the potential to differentiate into various cell types, including adipogenic, osteogenic, myogenic, endothelial, neurogenic, hepatogenic, cardiac, and pancreatic lineages. In this respect, they meet a commonly accepted criterion for pluripotent stem cells, without implying that they can generate every adult tissue. This suggests that amniotic fluid and placenta contain a novel type of pluripotent cell, and because amniocentesis and chorionic villus sampling are already accepted techniques, the ethical concerns surrounding the isolation of other types of stem cells would be eliminated. Thus, this stem cell source may lead to a myriad of novel therapeutic applications.

DEVELOPMENTAL BIOLOGY OF THE AMNIOTIC FLUID AND PLACENTA

Gastrulation is a major milestone in early postimplantation development (8). At about embryonic day 6.5 (E6.5), gastrulation begins in the posterior region of the embryo. Pluripotent epiblast cells are allocated to the three primary germ layers of the embryo (ectoderm, mesoderm, and endoderm) and germ cells, which are the progenitors of all fetal tissue lineages as well as to the extraembryonic mesoderm of the yolk sac, amnion, and allantois (9–11,12). The latter forms the umbilical cord as well as the mesenchymal part of the labyrinthine layer in the mature chorioallantoic placenta (9,13,14). The final positions of the fetal membranes result from the process of embryonic turning, which occurs around day 8.5 of gestation and "pulls" the amnion and yolk sac around the embryo (15,16). At this time, the specification of tissue lineages is accomplished by the restriction of developmental potency and the activation of lineage-specific gene expression (16,17). This process is strongly influenced by cellular interactions and signaling (18,19).

The amniotic sac is a tough but thin transparent pair of membranes that holds the developing fetus until shortly before birth. The inner membrane, the amnion, contains the amniotic fluid and the fetus. The outer membrane, the chorion, envelops the amnion and is part of the placenta (15,20,21). The amnion is derived from ectoderm and mesoderm, and as it grows, it begins to fill with fluid (21). Originally this fluid is isotonic, containing proteins, carbohydrates, lipids, phospholipids, urea, and electrolytes. Later, urine excreted by the fetus increases its volume and changes its composition (22–25). The presence of the amniotic fluid ensures symmetrical structure development and growth. In addition, it cushions and protects

the embryo, helps maintain consistent pressure and temperature, and permits freedom of fetal movement, which is important for musculoskeletal development and blood flow (26). The fetus can breathe in the amniotic fluid, allowing normal growth and development of the lungs. The fluid is also swallowed by the fetus, allowing the gastrointestinal tract to develop, and as a result, components of the fluid pass via the fetal blood into the maternal blood.

Amniotic fluid also contains a mixture of different cell types. In 2002, it was reported that a subpopulation of cells cultured from amniotic fluid as well as from placenta have the potential to differentiate into various cell types (27), suggesting that they may contain potential stem cells. A number of different origins have been suggested for these cells (28). Cells of both embryonic and fetal origins and cells from all three germ layers have been reported to exist in amniotic fluid (29,30). However, studies have shown that the consistent presence of a Y chromosome in cell lines derived from cases in which the amniocentesis donor carried a male child implies that the actual amniotic fluid stem cells (AFSCs) originate in the developing fetus. The cells are thought to be sloughed from the fetal amnion and skin, as well as the alimentary, respiratory, and urogenital tracts.

CELLS DERIVED FROM AMNIOTIC FLUID AND PLACENTA

Amniotic fluid consists of a heterogeneous cell population that displays a wide range of morphologies. Cells of many shapes and sizes (6–50 μm and round to squamous in appearance) can be found in this fluid. In addition to different morphologies, these cells express widely varied behaviors. In the following sections, the isolation of specific types of cells from amniotic fluid and placenta are described.

Differentiatied Cells

Most of the cells that can be isolated from the amniotic fluid and placenta are terminally differentiated along an epithelial lineage, and as such, these cells have a very limited capacity for proliferation and differentiation. Interestingly, the population of differentiated cells in the amniotic fluid is very diverse and contains cells that express markers from all three embryonic germ layers (31–34). The source of these cells is not well defined, but it has been shown that they are mostly from the fetus. Current theories suggest that the cells are derived from the urine, digestive tract, pulmonary secretions, and epithelium of the fetus (35,36).

Mesenchymal Cells

Within the last decade, several groups published relatively simple protocols for isolating a nonspecific population of cells with mesenchymal-like characteristics from the amniotic fluid and placenta (20,37–39). These cells were able to proliferate in vitro and could be used to form a three-dimensional structure that was used in vivo to repair a tissue defect (38). In 't Anker et al. then proved that the amniotic fluid and placenta contain large numbers of fetal mesenchymal stem cells (MSCs) that have a phenotype and multilineage differentiation potential similar to postnatal bone marrow (BM)-derived MSCs (40,41). Flow cytometric analyses indicated that these cells were positive for SH2, SH3, SH4, CD29, and CD44; low positive for CD90 and CD105; and negative for CD10, CD11b, CD14, CD34, CD117, and EMA (42).

Importantly, the cells displayed major histocompatibility (MHC) class I molecules (HLA-ABC), but not MHC class II molecules (HLA-DR, DP, and DQ) (43). Li et al. studied the immunological role of these cells and found that when mononuclear cells from placenta were added to umbilical cord blood (UCB) lymphocytes that had been activated by exposure to potent stimuli, a significant reduction in lymphocyte proliferation occurred (43). This suggests that MSCs from the amniotic fluid and placenta may have important immunoregulatory properties, and as a result, they may have potential applications in allograft transplantation. In particular, the studies of Li. et al. suggest that these MSCs may reduce graft-versus-host disease (GVHD) when co-transplanted with UCB-derived hematopoietic stem cells (HSCs).

Other mesenchymal cell-like populations have been isolated from human placenta at term. While the cell surface markers of these cell types vary, the majority of them are able to differentiate readily into a number of cell types. Several studies suggest that amniotic fluid– and placenta-derived MSCs are a potential source of cells for cartilage tissue engineering. In 2005,

Zhang et al. used these cells to create a construct using these MSCs and a collagen scaffold. This was implanted into nude mice with osteochondral defects, and the construct was able to form cartilage-like tissue (44). Other studies have shown that these cells may be suitable sources for cardiomyocytes, vascular cells, neural cells, and glial cells (45,46).

Pluripotent Cells

In addition to mesenchymal-like cells, other types of progenitor cells exist in the amniotic fluid and placenta. During midgestation, the vascular tissues of the embryo (in particular the aorta-gonad-mesonephros region, the yolk sac, and the fetal liver contain a relatively large pool of pluripotent HSCs (47,48). While it may be possible to use HSCs themselves for future cell therapy, the discovery of the presence of pluripotent stem cells that resemble embryonic stem (ES) cells within the HSC population is even more exciting. These cells have been pointed out by our group and others.

Isolation and Characterization of Pluripotent Progenitor Cells from Amniotic Fluid and Chorionic Villi (Placenta)

Amniotic fluid progenitor cells are isolated by centrifugation of amniotic fluid obtained during amniocentesis. Chorionic villi placental cells are isolated from single villi under light microscopy. Amniotic fluid cells and placental cells are allowed to proliferate in vitro and are maintained in culture for four weeks. The culture medium consists of modified α-modified Earl's medium (18% Chang medium B, 2% Chang medium C with 15% embryonic stem cell–certified fetal bovine serum, antibiotics, and L-glutamine).

A pluripotential subpopulation of progenitor cells present in the amniotic fluid and placenta can be isolated through positive selection for cells expressing the membrane receptor c-kit (49,50). This receptor binds to the ligand stem cell factor. About 0.8% to 1.4% of cells present in amniotic fluid and placenta have been shown to be c-kit positive in analysis by fluorescence-activated cell sorting (FACS). Progenitor cells maintain a round shape for one week post isolation when cultured in nontreated culture dishes. In this state, they demonstrate low proliferative capability. After the first week the cells begin to adhere to the plate and change their morphology, becoming more elongated and proliferating more rapidly, reaching 80% confluence with a need for passage every 48 to 72 hours. No feeder layers are required either for maintenance or expansion. The progenitor cells derived show a high self-renewal capacity with >300 population doublings, far exceeding Hayflick's limit. The doubling time of the undifferentiated cells is noted to be 36 hours, with little variation with passages.

These cells have been shown to maintain a normal karyotype at late passages and have normal G1 and G2 cell cycle checkpoints. They demonstrate telomere length conservation while in the undifferentiated state as well as telomerase activity even in late passages (51). Analysis of surface markers shows that progenitor cells from amniotic fluid express human embryonic stage-specific marker SSEA4, and the stem cell marker Oct4, and did not express SSEA1, SSEA3, CD4, CD8, CD34, CD133, C-MET, ABCG2, NCAM, BMP4, TRA1-60, and TRA1-81, to name a few. This expression profile is of interest as it demonstrates expression by the amniotic fluid–derived progenitor cells of some key markers of the embryonic stem cell phenotype, but not the full complement of markers expressed by embryonic stem cells. This may indicate that the amniotic cells are not quite as primitive as embryonic cells, yet maintain greater potential than most adult stem cells. Another behavior showing similarities and differences between these amniotic fluid–derived cells and blastocyst-derived cells is that whereas the amniotic fluid progenitor cells do form embryoid bodies in vitro, which stain positive for markers of all three germ layers, these cells do not form teratomas in vivo when implanted in immunodeficient mice. Last, cells, when expanded from a single cell, maintain similar properties in growth and potential as the original mixed population of the progenitor cells.

Differentiation of Amniotic Fluid- and Placenta-Derived Progenitor Cells

The progenitor cells derived from amniotic fluid and placenta are pluripotent and have been shown to differentiate into osteogenic, adipogenic, myogenic, neurogenic, endothelial, hepatic,

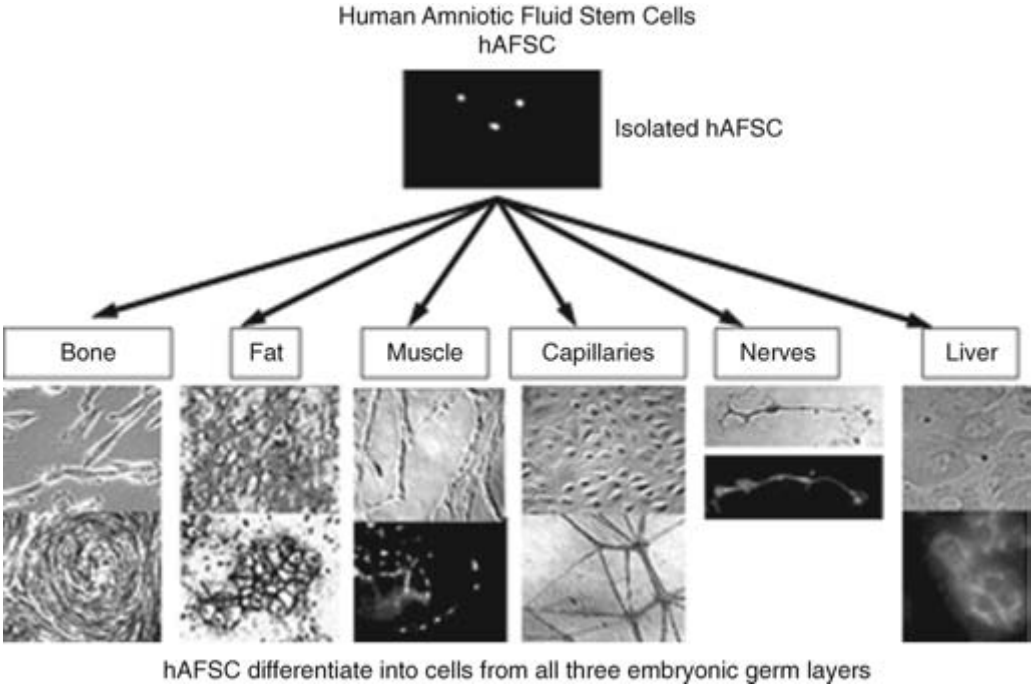

Figure 1 hAFSC are capable of differentiating into multiple cell types.

and renal phenotypes in vitro (Fig. 1). Each differentiation has been performed through proof of phenotypic and biochemical changes consistent with the differentiated tissue type of interest. We discuss each set of differentiations separately.

Adipocytes

To promote adipogenic differentiation, progenitor cells can be induced in dexamethasone, 3-isobutyl-1-methylxanthine, insulin, and indomethacin. Progenitor cells cultured with adipogenic supplements change their morphology from elongated to round within eight days. This coincides with the accumulation of intracellular droplets. After 16 days in culture, more than 95% of the cells have their cytoplasm filled with lipid-rich vacuoles. Adipogenic differentiation also demonstrates the expression of peroxisome proliferation-activated receptor $\gamma 2$ (PPAR$\gamma 2$), a transcription factor that regulates adipogenesis, and of lipoprotein lipase through reverse transcription-polymerase chain reaction (RT-PCR) analysis (28,52). Expression of these genes is noted in progenitor cells under adipogenic conditions but not in undifferentiated cells.

Osteocytes

Osteogenic differentiation was induced in progenitor cells with the use of dexamethasone, β-glycerophosphate, and ascorbic acid 2-phosphate (53). Progenitor cells maintained in this medium demonstrated phenotypic changes within four days, with a loss of their spindle-shaped phenotype and development of an osteoblast-like appearance with fingerlike excavations into the cytoplasm. At 16 days, the cells aggregated, showing typical lamellar bone-like structures. In terms of functionality, these differentiated cells demonstrate a major feature of osteoblasts, which is to precipitate calcium. Differentiated osteoblasts from the progenitor cells are able to produce alkaline phosphatase (AP) and to deposit calcium, consistent with bone differentiation. Undifferentiated progenitor cells lacked this ability. Progenitor cells in osteogenic medium express specific genes implicated in mammalian bone development [AP, core-binding factor A1 (CBFA1), and osteocalcin] in a pattern consistent with the physiological analog. Progenitor cells grown in osteogenic medium show activation of the AP gene at each time point. Expression of CBFA1, a transcription factor specifically expressed in osteoblasts and hypertrophic

chondrocytes and that regulates gene expression of structural proteins of the bone extracellular matrix, is highest in cells grown in osteogenic, inducing medium on day 8 and decreases slightly on days 16, 24, and 32. Osteocalcin is expressed only in progenitor cells under osteogenic conditions at eight days (54,55).

Endothelial Cells

Amniotic fluid progenitor cells can be induced to form endothelial cells by culture in endothelial basal medium on gelatin-coated dishes (56). Full differentiation is achieved by one month in culture; however, phenotypic changes are noticed within one week of initiation of the protocol. Human-specific endothelial cell surface marker (P1H12), factor VIII (FVIII), and KDR (kinase insert domain receptor) are specific for differentiated endothelial cells. Differentiated cells stain positively for FVIII, KDR, and P1H12. Progenitor cells do not stain for endothelial-specific markers.

Amniotic fluid progenitor–derived endothelial cells, once differentiated, are able to grow in culture and form capillary-like structures in vitro. These cells also express platelet endothelial cell adhesion molecule 1 (PECAM-1 or CD31) and vascular cell adhesion molecule (VCAM), which are not detected in the progenitor cells on RT-PCR analysis.

Hepatocytes

For hepatic differentiation, progenitor cells are seeded on Matrigel- or collagen-coated dishes at different stages and cultured in the presence of hepatocyte growth factor, insulin, oncostatin M, dexamethasone, fibroblast growth factor 4, and monothioglycerol for 45 days (57,58). After seven days of the differentiation process, cells exhibit morphological changes from an elongated to a cobblestone appearance. The cells show positive staining for albumin on day 45 post differentiation and also express the transcription factor HNF4α (hepatocyte nuclear factor 4α), the c-Met receptor, the multidrug resistance (MDR) membrane transporter, albumin, and α-fetoprotein. RT-PCR analysis further supports albumin production. The maximum rate of urea production for hepatic differentiation–induced cells is upregulated to 1.21×10^3 ng of urea per hour per cell from 50 ng of urea per hour per cell for the control progenitor cell populations (59).

Myocytes

Myogenic differentiation is induced in amniotic fluid–derived progenitor cells by culture in medium-containing horse serum and chick embryo extract on a thin gel coat of Matrigel (60). To initiate differentiation, the presence of 5-azacytidine in the medium for 24 hours is necessary. Phenotypically, the cells can be seen to organize themselves into bundles that fuse to form multinucleated cells. These cells express sarcomeric tropomyosin and desmin, both of which are not expressed in the original progenitor population.

The development profile of cells differentiating into myogenic lineages interestingly mirrors a characteristic pattern of gene expression reflecting that seen with embryonic muscle development (61,62). With this protocol, Myf6 is expressed on day 8 and suppressed on day 16. MyoD expression is detectable at 8 days and suppressed at 16 days in progenitor cells. Desmin expression is induced at 8 days and increases by 16 days in progenitor cells cultured in myogenic medium (63,64).

Neuronal Cells

For neurogenic induction, amniotic progenitor cells are induced in dimethyl sulfoxide (DMSO), butylated hydroxyanisole, and neuronal growth factor (65,66). Progenitor cells cultured under neurogenic conditions change their morphology within the first 24 hours. Two different cell populations are apparent: morphologically large flat cells and small bipolar cells. The bipolar cell cytoplasm retracts toward the nucleus, forming contracted multipolar structures. Over subsequent hours, the cells display primary and secondary branches and cone-like terminal expansions. Induced progenitor cells show a characteristic sequence of expression of neural-specific proteins. At an early stage the intermediate filament protein

nestin, which is specifically expressed in neuroepithelial stem cells, is highly expressed. Expression of III-tubulin and glial fibrillary acidic protein (GFAP), markers of neuron and glial differentiation, respectively, increases over time and seems to reach a plateau at about six days (67). Progenitor cells cultured under neurogenic conditions show the presence of the neurotransmitter glutamic acid in the collected medium. Glutamic acid is usually secreted in culture by fully differentiated neurons (68).

Renal Cells

End-stage kidney disease has reached epidemic proportions in the United States. Currently, dialysis and allogenic renal transplant remain the only treatments for this disease, but there are significant drawbacks to each. Dialysis can prolong survival via replacement of filtration functions, but other kidney functions are not replaced, thus leading to long-term consequences such as anemia and malnutrition (69). Currently, renal transplantation is the only definitive treatment that can restore the entire function of the kidney, including filtration, production of erythropoietin and 1, 25 dihydroxyvitamin D_3. However, transplantation presents with several limitations such as a critical donor shortage, complications due to chronic immunosuppressive therapy, and graft failure (70–72).

Over the last decade, stem cells and their possible role in the construction of bioartificial organs such as the kidney have been an area of intense research. Despite their potential in regenerative medicine applications, cells such as embryonic stem cells have ethical concerns associated with their use, and certain types of research with these cells has been banned. AFSCs, however, do not have these problems, and may represent an exciting new cell source for tissue engineering strategies.

In 2007, Perin et al. showed that AFSC could be induced to differentiate into renal cells when placed into an in vitro embryonic kidney environment (73). Human AFSCs (hAFSCs) were obtained from human male amniotic fluid and were labeled with either LacZ or green fluorescent protein (GFP) so that they could be tracked throughout the experiment. These labeled cells were microinjected into murine embryonic kidneys (12.5–18 days gestation) and maintained in a special coculture system in vitro for 10 days. Using this technique, it was shown that the labeled hAFSCs remained viable throughout the experimental period, and, importantly, they were able to contribute to the development of various primordial kidney structures including the renal vesicle, C- and S-shaped bodies. Studies using RT-PCR indicated that the implanted hAFSCs began to express early kidney markers such as zona occludens-1 (ZO-1), glial-derived neurotrophic factor, and claudin. Together, these data suggested that hAFSCs have the intrinsic ability to differentiate into a number of different cell types that make up the kidney. Therefore, hAFSCs could represent a potentially limitless, ethically neutral source of cells for tissue engineering and cell therapy applications aimed at regenerating failing renal tissue.

In Vivo Behavior of Amniotic Fluid Stem Cells

Our group has cultured AFSCs in neuronal differentiation medium for a time and then grafted them into the lateral cerebral ventricles of control mice and the ventricles of the *twitcher* mouse model, in which a progressive loss of oligodendrocytes leads to massive demyelination and neuronal loss (74). AFS cells integrated into the brains of both strains seamlessly, appeared morphologically indistinguishable from surrounding mouse cells, and survived efficiently for at least two months. Interestingly, more of the AFS cells integrated into the injured twitcher brains (70%) than into the normal brains (30%), hinting at the potential for CNS therapies. In this study the phenotypes of the implanted human cells were not assessed. However, the pattern of incorporation and morphologies of cells derived from the AFS cells appeared similar to those obtained previously in the same animal model after implantation of murine neural progenitor and stem-like cells 7. In that case the donor-derived cells were identified as astrocytes and oligodendrocytes.

From a tissue engineering perspective, osteogenically differentiated AFS cells were embedded in an alginate/collagen scaffold and implanted subcutaneously into immunodeficient mice. By 18 weeks after implantation, highly mineralized tissues and blocks of bone-like material were observed in the recipient mice using micro CT. These blocks displayed a density

somewhat greater than that of mouse femoral bone. This indicates that AFS cells could be used to engineer grafts for the repair of bone defects.

Amniotic Fluid and Placenta for Cell Therapy

Pluripotent stem cells are ideal for regenerative medicine applications as they have the capability to differentiate in stages into a huge number of different types of human cells. The discovery of a stem cell population in the amniotic fluid offers a very promising alternative source of stem cells for cellular therapy. The full range of adult somatic cells that AFS cells can produce remains to be determined, but their ability to differentiate into cells of all three embryonic germ layers and their high proliferation rate are two advantages over most adult stem cell sources. AFS cells represent a new class of stem cells with properties somewhere between embryonic and adult stem cell types. However, unlike ES cells, AFS cells do not form teratomas, and this low risk of tumorigenicity would be advantageous for eventual therapeutic applications. In addition, these cells are easily obtained without destruction of embryos, and thus their use may avoid some of the ethical concerns surrounding the use of ES cells. Finally, AFS cells could be used for both autologous and allogenic therapy through matching of histocompatible donor cells with recipients.

Amniotic fluid cells can be obtained from a small amount of fluid during amniocentesis at the second trimester, a procedure that is already often performed in many of the pregnancies in which the fetus has a congenital abnormality and to determine characteristics such as sex (75). Kaviani and coworkers reported that "just 2 milliliters of amniotic fluid" can provide up to 20,000 cells, 80% of which are viable (20). Because many pregnant women already undergo amniocentesis to screen for fetal abnormalities, cells can be simply isolated from this fluid and banked for future use.

In addition, while scientists have been able to isolate and differentiate on average only 30% of MSCs extracted from a child's umbilical cord shortly after birth, the success rate for amniotic fluid–derived stem cells is close to 100% (40,42). Furthermore, with amniotic fluid cells, it takes 20 to 24 hours to double the number of cells collected, which is faster than for umbilical cord stem cells (28–30 hours) and BM stem cells (>30 hours) (42). This phenomenon is an important feature for urgent medical conditions.

CONCLUSION

Pluripotent progenitor cells isolated from amniotic fluid and placenta present an exciting possible contribution to the field of stem cell biology and regenerative medicine. These cells are an excellent source for research and therapeutic applications. The ability to isolate progenitor cells during gestation may also be advantageous for babies born with congenital malformations. Furthermore, progenitor cells can be cryopreserved for future self-use. Compared with embryonic stem cells, progenitor cells isolated from amniotic fluid have many similarities: they can differentiate into all three germ layers, they express common markers, and they preserve their telomere length. However, progenitor cells isolated from amniotic fluid and placenta have considerable advantages. They easily differentiate into specific cell lineages and they avoid the current controversies associated with the use of human embryonic stem cells. The discovery of these cells has been recent, and a considerable amount of work remains to be done on the characterization and use of these cells. In future, cells derived from amniotic fluid and placenta may represent an attractive and abundant, noncontroversial source of cells for regenerative medicine.

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15 | Adult Stem Cells in the Human Endometrium

Caroline E. Gargett, Irene Cervelló, Sonya Hubbard, and Carlos Simón

INTRODUCTION

The human endometrium is a dynamic remodeling tissue undergoing more than 400 cycles of regeneration, differentiation, and shedding during a woman's reproductive years. The coordinated and sequential actions of estrogen and progesterone direct these major remodeling events to prepare the endometrium for blastocyst implantation. Endometrial regeneration also follows parturition, almost complete curettage and occurs in postmenopausal women taking estrogen replacement therapy. Adult stem/progenitor cells are likely responsible for endometrial regeneration. This chapter will review the evidence available to date for the existence of adult stem/progenitor cells in human endometrium. It will detail the functional approaches that have been used to identify candidate endometrial epithelial and mesenchymal stem/progenitor cells, in particular, cell cloning and side population (SP) studies. The importance of in vivo studies that demonstrate the ability to reconstruct endometrial tissue from isolated cell populations will also be emphasized. Identification of markers that distinguish endometrial epithelial and mesenchymal stem/progenitor cells from their more differentiated progeny is essential to progress this newly developing field of research. Approaches being undertaken to identify markers for endometrial mesenchymal stem/progenitor cells will be outlined, including the recent discovery that co-expression of two markers, CD146 and platelet-derived growth factor (PDGF) receptor-β, partially purifies human endometrial mesenchymal stem/progenitor cells. This $CD146^{+}PDGF\text{-}R\beta^{+}$ population has a surface phenotype similar to bone marrow and fat mesenchymal stem cells (MSC), and demonstrates multipotency by their capacity to undergo multilineage differentiation into fat, cartilage, bone, and smooth muscle. They have a perivascular location in the functionalis and basalis layers of the endometrium. Application of these fundamental studies to the current knowledge on the pathophysiology of a variety of common gynecological diseases associated with abnormal endometrial proliferation, including endometrial cancer, endometriosis, and adenomyosis will also be discussed. Mention will be made of the possible use of endometrial stem/progenitor cells in autologous tissue engineering applications relevant to urogynecology. Finally, the future directions of human endometrial stem/progenitor cell research will be suggested.

REGENERATIVE CAPACITY OF HUMAN ENDOMETRIUM

In humans, the failure of an embryo to implant into the receptive endometrium at the appropriate time results in the sloughing off of the functional layer of the endometrial lining, which is regenerated in the next menstrual cycle (Fig. 1). Thus, the human endometrium is a dynamic remodeling tissue undergoing more than 400 cycles of regeneration, differentiation, and shedding during a woman's reproductive years (2–4). The first stage of endometrial repair involves migration of epithelial cells from protruding stumps of basal glands over the denuded surface within 48 hours of shedding (2,3). Endometrial repair does not appear to require estrogen as this process occurs while circulating estrogen levels are very low and when epithelial cells lack estrogen receptor-α (ERα) expression (3). As estrogen levels rise during the proliferative stage, ERα and progesterone receptors (PR) are induced in the epithelium and stroma. The functionalis mucosa grows 4 to 10 mm within 4 to 10 days (4), with extensive proliferation of glandular epithelial cells and, to a lesser extent, stromal cells (Fig. 1) (5–7). Following ovulation, proliferation gradually ceases and the estrogen-primed functionalis commences differentiation under the influence of progesterone, which suppresses functionalis, but not basalis ERα and PR (8,9). The differentiating endometrial glands secrete large quantities of glycogen and histotrophic secretory products in preparation for an implanting blastocyst (8,10,11). PR persist on stromal cells in the functionalis, which proliferate and

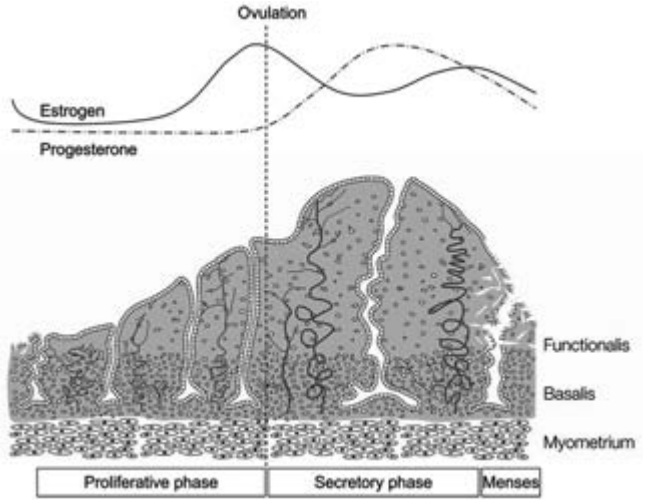

Figure 1 Schematic of the human menstrual cycle. The functionalis layer in human endometrium undergoes cycles of growth, differentiation, and shedding, processes regulated by cyclical changes in circulating sex steroid levels. The basalis layer shows little change and is relatively insensitive to sex steroid action. *Source*: From Ref. 1.

differentiate into predecidual cells surrounding/around spiral arterioles and beneath the luminal epithelium. Endometrial differentiation is also accompanied by dramatic changes in gene expression profiles (12). When implantation fails to occur, stromal decidualization becomes a terminal differentiation. The demise of the ovarian corpus luteum and subsequent fall in circulating estrogen and progesterone levels trigger menstruation, and the functionalis is shed (Fig. 1) (9,13). The remaining basalis layer of the endometrium, which is relatively insensitive to sex steroid hormone actions and undergoes little proliferation or differentiation (8), acts as a germinal compartment providing a source of endometrial cells for regenerating the new functionalis in the subsequent cycle (5,14).

When menstrual cycles cease at menopause, the endometrium becomes very thin and atrophic, containing a few glands in a stroma resembling the basalis (15,16). Similarly, women taking oral contraceptive pills (OCP) do not exhibit cyclic changes in circulating sex steroid hormones and their endometrium does not undergo cyclical growth, differentiation, and regression. Histologically, OCP endometrium appears inactive, with similar morphology to postmenopausal endometrium. However, when postmenopausal women take estrogen replacement therapy or women cease OCP medication, their endometrium regenerates. Endometrial regeneration also follows parturition, extensive resection, and unsuccessful endometrial ablation therapies for menorrhagia (17).

ENDOMETRIAL STEM/PROGENITOR CELL HYPOTHESIS

The monthly regeneration of the human endometrium and its reconstruction following parturition are at least equivalent to the level of cellular turnover that occurs in other highly regenerative organs such as blood-forming tissue of the bone marrow, epidermis, and intestinal epithelium (17). In these regenerative tissues, adult or somatic stem cells, responsible for provision of replacement cells to maintain tissue homeostasis, have been identified. The concept that basalis endometrium harbors stem/progenitor cells responsible for the remarkable regenerative capacity of endometrium was proposed many years ago (14,18,19). Indirect evidence for the existence of adult stem/progenitor cells in the endometrium has accumulated over the intervening years (reviewed in Ref. 17). Attempts to isolate, characterize, and locate endometrial stem/progenitor cells have recently been undertaken as experimental approaches to identify adult stem cells in other tissues (17), resulting in the identification of rare populations of epithelial stem/progenitor cells and mesenchymal stem-like cells in human endometrium.

Adult Stem Cells and Their Properties

Somatic stem cells are undifferentiated cells present in most adult tissues. Their rarity, the lack of distinguishing morphological features, and current lack of known specific markers make them difficult to identify in many tissues. Adult stem cells are defined by their functions: high proliferative potential, self-renewal, and differentiation into one or more lineages (20). The retention of a DNA synthesis label [bromodeoxyuridine (BrdU)] for prolonged periods of time is another property of adult stem cells, since, paradoxically, they proliferate less frequently than their daughter cells (21). Adult stem cells maintain tissue homeostasis by providing replacement cells in regenerating tissues, in routine cellular turnover, and for repair after acute injury (22,23). The balance between adult stem cell self-renewal and differentiation is strictly regulated by the stem cell niche, comprising the adult stem cells, surrounding niche cell(s) and extracellular matrix, to ensure an appropriate balance between stem cell replacement and provision of sufficient differentiated mature cells to maintain tissue homeostasis for organ function (22).

Endometrial Epithelial Stem/Progenitor Cells

Cell Cloning Studies

The first published evidence for the existence of endometrial epithelial stem/progenitor cells in human endometrium came from cell cloning studies, where single-cell suspensions were seeded at cloning density in culture (24). Rare clonogenic epithelial cells were identified in normal cycling and inactive perimenopausal endometrium and in endometrium of women on oral contraceptives (24,25), suggesting that clonogenic epithelial cells may be responsible for regenerating glands in cycling and atrophic endometrium (26). These studies found that 0.22% of human endometrial epithelial cells had colony-forming unit (CFU) activity. Two types of CFU formed: large (0.09%) and small (0.14%), leading to the hypothesis that large CFU are initiated by stem/progenitor cells possibly located at the base of the glands in the basalis (Fig. 2). Small CFU are possibly initiated by more differentiated transit amplifying cells, likely located in the functionalis layer and responsible for the extensive proliferation observed in the first half of the menstrual cycle (17,24,27). Differential expression of epithelial markers was noted between large

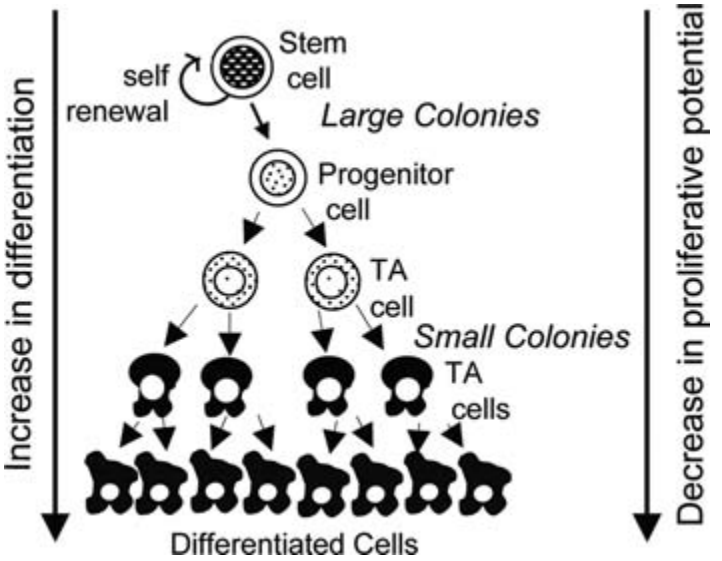

Figure 2 The relationship of endometrial colony-forming cells to the hierarchical model for stem cell differentiation. Stem cells have the capacity to self-renew and replace themselves as well as differentiate into committed progenitors through asymmetric cell divisions. Progenitors proliferate and give rise to more differentiated rapidly proliferating transit amplifying cells, which finally differentiate to produce a large number of terminally differentiated functional cells with no capacity for proliferation. We postulate that the large colonies are initiated by putative stem/progenitor cells and the small colonies by putative transit amplifying cells. *Source*: From Ref. 24.

and small CFU. Small CFU expressed epithelial differentiation markers, cytokeratin, epithelial cell adhesion molecule (EpCAM), and α6-integrin, but only the latter was expressed in cells of large CFU, which comprised small cells with a high nuclear:cytoplasmic ratio, suggesting an undifferentiated phenotype (24). The percentage of endometrial epithelial CFU did not vary with menstrual cycle stage, indicating their continued presence in human endometrium (25). The growth factor requirements of human endometrial epithelial CFU have been characterized in serum-free culture conditions. Fibroblast feeder layers were necessary for serum-free growth, indicating the importance of surrounding stromal cells, which are likely the niche cells that regulate clonogenic epithelial cell fate decisions. Epidermal growth factor (EGF), transforming growth factor-α (TGFα), and PDGF-BB were requisite growth factors for human endometrial epithelial CFU activity, while insulin-like growth factor-1 (IGF-1), leukaemic growth factor (LIF), stem cell factor, and hepatocyte growth factor were weakly supportive and basic fibroblast growth factor (bFGF) was without effect (24). This data suggests that EGF receptors are present on endometrial epithelial CFU. However, it is likely that PDGF-BB exerted its mitogenic effects on epithelial CFU indirectly via the PDGF receptor-β on the fibroblast feeder cells (27).

Side Population Cells

Another approach to the identification and isolation of adult stem cells in adult tissues or organs in the absence of known specific markers for these cells is to use the Hoechst dye exclusion method. A specific SP displaying low red and low blue fluorescence can be identified by dual-wavelength flow cytometry after incubating the target cells with the DNA-binding dye Hoechst 33342 (28). This phenomenon is due to the expression of a cell membrane transporter related to immature cells, breast cancer resistance protein 1 (BCRP1) (29). The SP phenotype is thought to be a universal marker of adult stem cells and has been used to isolate adult stem cells from many adult tissues (30).

Recently, an epithelial SP population was identified in human endometrium (31,32). The mean percentage of SP cells in the epithelial fraction was 0.21% during the menstrual phase, 0.15% during the proliferative phase, and 0.02% in the secretory phase (31). After five months in 3D culture conditions, the endometrial epithelial SP cells formed gland-like CD9-expressing structures, indicating their ability to differentiate (31). A study by Cervello et al. corroborates the existence of epithelial SP cells in the human endometrial epithelium (Fig. 3) (32). Furthermore, the isolated SP cells expressed typical undifferentiation markers such as c-*KIT* and *OCT*-4 at the mRNA level, and they had telomerase activity intermediate between human embryonic stem cells and differentiated cells. Phenotypic analysis of these SP cells revealed that more than 50% were $CD90^{+}$ [mesenchymal and endometrial stromal cell marker (42)] and negative for CD34 (endothelial and hemopoietic stem cell marker) and CD45 (leukocyte marker) expression (32), suggesting that they are of mesenchymal origin. Clonogenic and in vivo endometrial tissue reconstitution studies are necessary to further demonstrate the differentiation potential of the SP cells isolated from the epithelial fraction of human endometrium (1).

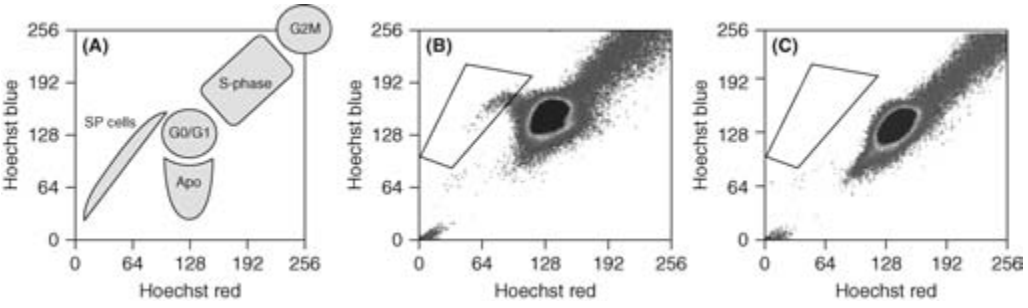

Figure 3 Identification of SP cells in the human endometrium. (**A**) Typical diagram showing the distribution of the Hoechst-retaining cells in a FACS histogram. (**B**) SP cells obtained from human endometrium. (**C**) Human endometrial cells treated with verapamil where SP region disappears.

Label-Retaining Cells

Label-retaining cells (LRC) have been identified as candidate adult stem cells in vivo in mouse endometrium (33–35). The LRC approach identifies adult stem cells by their quiescent, slowly cycling nature, since they only undergo cell division during tissue turnover to initiate replacement of lost cells. It is based on pulse labeling the majority of tissue cells with a DNA synthesis label (BrdU) during a time when adult stem cells are proliferating and a subsequent chase of the label over long periods of time. Slow-cycling stem cells retain the label, while rapidly dividing transit amplifying cells dilute the label to undetectable levels. Immunohistochemistry localizes $BrdU^+$ LRC, revealing their location and the stem cell niche. Only one study to date has demonstrated epithelial LRC in the mouse endometrium (33). During a chase period of 56 days, the BrdU label diluted rapidly because of extensive proliferation of luminal epithelium as nascent glands developed during neonatal and prepubertal endometrial growth and during subsequent estrus cycles. Epithelial LRC, comprising 3% of mouse endometrial epithelial cells, were observed as separate cells in the luminal but not glandular epithelium, suggesting that luminal epithelial stem/progenitor cells are responsible for the growth of glands during development and in the cycling adult mouse (33). They may also have an important role in regenerating luminal epithelium, which undergoes substantial proliferation and apoptosis during the estrus cycle (36).

Endometrial Stromal/Mesenchymal Stem-Like Cells

Cell Cloning Studies

Several published studies support the existence of stromal/mesenchymal stem-like cells in human endometrium. Initial studies identified a small population (1.25%) of freshly isolated human endometrial stromal cells possessing colony-forming ability (24). These CFU are not only retained in culture but their proportion increases to 15% of stromal cells after prior expansion in culture at normal seeding densities (37). Similar to epithelial CFU, two types of stromal CFU formed from freshly isolated cells, with only 0.02% of stromal cells initiating large CFU, supporting a stromal cellular hierarchy hypothesized to exist in human endometrium (17). Both large and small stromal colonies expressed fibroblast markers, with some cells expressing α smooth muscle actin (αSMA), indicative of myofibroblast differentiation. Four growth factors supported stromal CFU in serum-free cultures: bFGF, EGF, TGFα, and PDGF-BB (24,25), indicating that stromal CFU are different from epithelial CFU and that there are separate epithelial and stromal adult stem cells in human endometrium.

Multilineage Differentiation

A key adult stem cell property is the ability to undergo multilineage differentiation. MSC have been identified in bone marrow and fat, and these cells differentiate into at least three mesenchymal lineages in vitro under appropriate induction conditions (38). Various human endometrial stromal cell populations can be induced to differentiate into one or more of these mesenchymal lineages, suggesting that MSC-like cells may reside in human endometrium. Various studies have demonstrated that some stromal cells in a heterogeneous population of cultured human endometrial stromal cells differentiate into fat or chondrocyte lineages (37,39). Since mixed populations of stromal cells were examined, these studies were unable to determine whether individual stromal cells are multipotent. Similarly, a recent study examining the differentiation potential of a $CD146^+PDGF\text{-}R\beta^+$ fraction of endometrial stromal cells enriched eightfold for human endometrial stromal CFU showed that these cells differentiated into four mesenchymal lineages: adipogenic, chondrogenic, osteoblastic, and myogenic (40). Although the $CD146^+PDGF\text{-}R\beta^+$ cells are a partially purified population, the multilineage capacity of individual cells was not examined. Recently the differentiation capacity of single human endometrial stromal cells was demonstrated proving that human endometrium contains truly multipotent MSC (1).

Side Population Cells

Rare SP cells were also identified in the human endometrial stromal compartment. The greatest number (3.9%) was observed during the menstrual phase, probably because of the

mobilization of this population at this specific time (31). Much lower percentages of stromal SP cells were identified in the proliferative (0.06%) and secretory (0.1%) phases. The expression of stromal markers CD13 and vimentin was confirmed in the isolated stromal SP cells, which did not express E-cadherin or CD9, markers of epithelial cells. The human endometrial stromal SP cells proliferated slowly in long-term cultures and differentiated into stromal-like clusters after six months in 3D matrigel culture. Cervello et al. confirmed the presence of SP cells in the human endometrial stromal compartment ($CD13^+$ cells). They found 0.01% to 1.36% SP cells on more than 50 samples. These stromal SP cells expressed the typical undifferentiation markers, c-KIT and OCT-4, suggesting a degree of undifferentiation in this subset of cells. Further phenotypic analysis for CD90 confirmed that endometrial stromal SP cells are of mesenchymal and endometrial origin (42). This data supports the hypothesis for the existence of endometrial stromal stem/progenitor cells in the human endometrium, although further studies are required to determine if stromal SP cells form CFU and express defining MSC properties such as multilineage differentiation.

SP cells have also been identified in freshly isolated human myometrial cells (43). Myometrial SP cells were $CD34^+CD45^-$, indicating that they were not hemopoietic stem cells. They were relatively undifferentiated as they expressed lower levels of ERα, PR, and smooth muscle cell markers, calponin and smoothelin, compared with main population (MP) myometrial cells, and spontaneously differentiated into mature myometrial cells expressing αSMA and calponin under hypoxic conditions (43). Some also underwent multilineage differentiation into osteogenic and adipogenic lineages when cultured in appropriate differentiation induction media. Myometrial SP cells expressed some bone marrow MSC surface markers including STRO-1, CD90, CD73, and CD105, but not CD44. Human myometrial SP cells transplanted into the uterine horns of non obese diabetic/severe combined immunodeficient/γc-null (NOD/SCID/γc-null (NOG) mice (44) supplemented with estrogen incorporated into myometrium and co-expressed vimentin and αSMA. These cells also induced the expression of a pregnancy marker, oxytocin receptor mRNA, in pregnant but not nonpregnant uterus of NOG mice previously transplanted with SP cells, indicating the functional capacity of the myometrial SP cells (43). This study supports the existence of myometrial MSC-like cells with ability to produce mature myometrial cells in vitro and contribute to myometrial tissue in vivo. The relationship of myometrial SP cells with MSC-like properties to endometrial SP cells or the $CD146^+$PDGF-Rβ^+ mesenchymal stem/progenitor cells identified in human endometrium is not currently known. Perhaps myometrial SP cells are derived from müllerian duct mesenchyme during fetal uterine development since the inner myometrial smooth muscle layer develops from müllerian duct mesenchyme, the endometrial stroma primordium.

Label-Retaining Cells

Candidate stromal stem/progenitor cells have been identified in mouse endometrium as stromal LRC (33–35). Between 6% and 9% of the stromal cells were identified as LRC after at least 12-weeks chase of the BrdU label. A large proportion of these were located near blood vessels close to the endometrial-myometrial junction (33,34), correlating with their postulated basalis location in human endometrium. Stromal LRC were further characterized for expression of various markers. They were not leukocytes or of bone marrow origin as they did not express CD45 (33). Some expressed ERα (35), and some expressed typical markers of undifferentiation, c-Kit and Oct-4 (36), although in another study, c-Kit was not expressed by endometrial stromal LRC (35). The continued decrease in the percentage of LRC as the chase period lengthened indicates that the LRC also undergo symmetric cell divisions in adulthood or do not necessarily retain their template DNA strands, as has been demonstrated for LRC in the intestine (45).

Menstrual Blood Stem/Progenitor Cells

There is increasing evidence that endometrial stem/progenitor cells may be shed in menstrual blood (46), although the number of samples reported to date is extremely small (47,48) and further characterization of the stem cell status of isolated cells requires verification. Menstrual blood not only contains fragments of shed endometrial functionalis,

where adult stem cells are not expected to reside, but also peripheral blood, which may contain a small number of hemopoietic stem cells, MSC, or endothelial progenitor cells. Furthermore, stem cells are rare cells in any tissue, and it is difficult to understand a physiological system dispensing with these important cells so readily each month. Nevertheless, the shedding of endometrial stem/progenitor cells into menstrual blood requires more investigation.

Endometrial Stem/Progenitor Cell Reconstitution of Endometrial Tissue In Vivo

The functional proof of the regenerative capacity of the putative endometrial adult stem cell population is the reconstitution of the original tissue in vivo. Functional endometrium has been regenerated from singly dispersed unfractionated human endometrial cell suspensions xenotransplanted beneath the kidney capsule of ovariectomized and estrogen-supplemented NOG mice lacking T, B, and natural killer cells (49). Well-organized endometrial tissue comprising glandular structures expressing typical epithelial markers such as cytokeratin and CD9, stroma positive for CD10 and CD13, and myometrial layers was reproduced (49). In this animal model, both compartments underwent typical hormone-dependent changes such as production of tortuous glands and stromal decidualization, characteristic of secretory phase, on simulation of the menstrual cycle with cyclic administration of estrogen and progesterone (49). Grafts collected after hormonal withdrawal contained large blood-filled cysts similar to red spot lesions of active endometriosis. Immunohistochemical staining of the cystic lesion revealed that the glandular structures were partly destroyed and that hemorrhage had occurred in the degenerated stroma (49). This animal model provides an excellent in vivo assay system to test whether candidate human or mouse endometrial stem/progenitor cell populations, such as the SP cells or $CD146^{+}PDGF\text{-}R\beta^{+}$ stromal cells, can reconstitute endometrial tissue (50).

Markers of Endometrial Stem/Progenitor Cells

Currently, there are no markers for endometrial epithelial stem/progenitor cells and they cannot be distinguished from their mature progeny, the pseudostratified epithelium comprising the glands and luminal epithelium.

However, MSC-like cells were recently isolated from human endometrium using co-expression of two perivascular cell markers, CD146 and PDGF receptor-β (PDGF-Rβ) (40). The fluorescent activated cell sorted (FACS)-sorted $CD146^{+}PDGF\text{-}R\beta^{+}$ subpopulation of endometrial stromal cells was enriched eightfold for CFU compared with unsorted stromal cells. That PDGF-BB supports stromal CFU activity further supports the use of PDGF-Rβ as a marker for the prospective isolation of MSC-like cells from human endometrium. These $CD146^{+}PDGF\text{-}R\beta^{+}$ cells expressed typical MSC surface markers, CD29, CD44, CD73, CD90, and CD105, and were negative for hemopoietic and endothelial markers (CD31, CD34, and CD45) (40). STRO-1, the classic marker used to prospectively isolate bone marrow MSC, was not expressed by these cells nor by clonogenic stromal CFU (42). This study also demonstrated multilineage differentiation of $CD146^{+}PDGF\text{-}R\beta^{+}$ cells into adipogenic, myogenic, chondrogenic, and osteoblastic lineages when cultured in appropriate induction media (Fig. 4). This data suggests that the $CD146^{+}PDGF\text{-}R\beta^{+}$ subpopulation of endometrial stromal cells contains MSC-like cells similar to MSC of bone marrow, fat, and dental pulp (51–53). Furthermore, confocal microscopy demonstrated that CD146 and PDGF-Rβ co-expressing cells were located perivascularly in the functionalis and basalis (Fig. 5) (40). Whether MSC-like cells in the basalis and/or functionalis are involved in regenerating endometrium awaits the identification of more specific markers. This finding also suggests that it is possible that endometrial MSC-like cells are shed during menstruation. The demonstration of endometrial tissue reconstitution and differentiation in vivo would further support the existence of MSC in human endometrium.

A number of studies have examined stem cell marker expression in human and mouse endometrium by immunotechniques. These studies, while valuable, require further analyses to validate whether the cells expressing these markers function as endometrial stem/progenitor cells (50). Unfortunately, there are no specific markers of adult stem cells, although some pluripotency genes indicate the likely presence of adult stem cells. OCT-4, a transcription

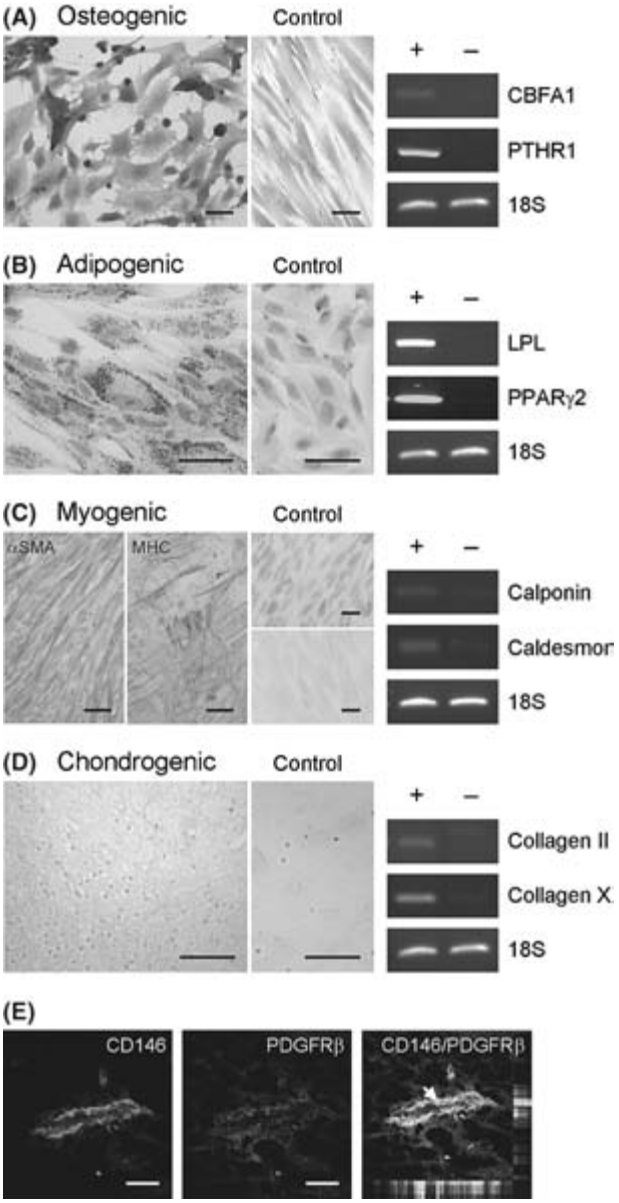

Figure 4 Multilineage differentiation of CD146$^+$PDGF-Rβ^+ human endometrial stromal cells and their perivascular location. FACS-sorted CD146$^+$PDGF-Rβ^+cells were cultured as monolayers or micromass pellets (chondrogenic) for four weeks in differentiation induction or control media. (**A**) Osteogenic-differentiated cells express alkaline phosphatase (*left panel*) and osteoblastic lineage–specific genes. (**B**) Adipogenic-differentiated cells are visualized as oil red O–stained fat droplets (*left panel*) and express adipocyte-specific genes. (**C**) Myogenic-differentiated cells express smooth muscle proteins, α αSMA and smooth muscle-specific myosin heavy chain (MHC), and calponin and caldesmon mRNA. (**D**) Chondrogenic-differentiated cells in a micromass pellet section showing Alcian blue–stained cartilage matrix (*left panel*) and mRNA for chondrocyte lineage specific genes. (**E**) CD146 and PDGF-Rβ co-localize on perivascular cells of blood vessels (*white arrows*) in the functionalis (shown) and basalis layers. Co-localization of the two surface markers is shown in the *x/z* and *y/z* planes of the merged images. *Source*: From Ref. 40.

factor and marker of pluripotent human embryonic stem cells (55) and, more recently, of adult stem cells (56), was expressed in some human endometrial samples (57). More OCT-4$^+$ cells were observed during the proliferative stage. However, this study did not quantify or

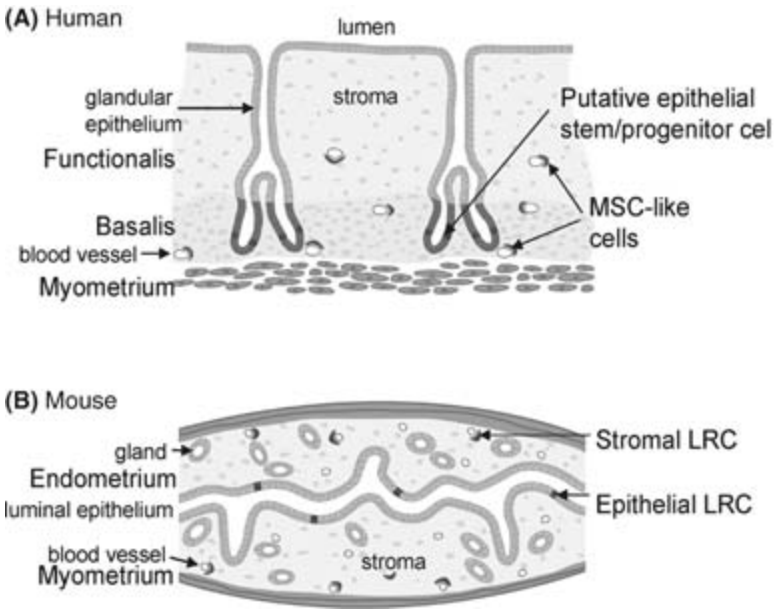

Figure 5 Schematic showing the possible location of candidate endometrial stem/progenitor cells in human and mouse endometrium. (**A**) In human endometrium, it is hypothesized that epithelial stem/progenitors are located in the base of the glands in the basalis. Recent data indicates that MSC-like cells are located near blood vessels possibly in both the basalis and/or the functionalis. (**B**) In mouse endometrium, candidate epithelial and stromal stem/progenitor cells (label-retaining cells, LRC), which rapidly proliferate during estrogen-stimulated endometrial growth, are located in the luminal epithelium and mainly near blood vessels at the endometrial-myometrial junction, respectively. *Source*: From Ref. 54.

determine the identity or location of the OCT-4^+ cells. Pou class 5 transcription factor 1 (pou5f1) was co-localized in 0.19% of stromal LRC in the lower region of the murine endometrial stroma, suggesting the highly undifferentiated status of this specific subset of LRC (34). Nested PCR confirmed the presence of *Pou5f1* mRNA in mouse uterus during the prepubertal period (day 21) and in adulthood (day 50) (34).

Musashi-1, an RNA-binding protein in neural stem cells and an epithelial progenitor cell marker that regulates stem cell self-renewal signaling pathways (58), was recently localized to single epithelial and stromal cells as well as small clusters of stromal cells in human endometrium (59). Musashi-1-expressing cells were greater in number in the basalis compared with the functionalis in the proliferative stage of the menstrual cycle, suggesting their possible stem/progenitor cell function. Notch-1 and telomerase, key downstream targets of Musashi-1, co-localized with Musashi-1^+ endometrial cells. Telomerase activity has been identified in adult stem cells in other tissues (60). Interestingly, the stromal Musashi-1^+ cells were not found in a perivascular location (42), although some were found in a periglandular region, similar to some stromal LRC in mouse endometrium (33). It is now important to determine whether Musashi-1 is expressed in endometrial stromal CFU and in $CD146^+$PDGF-Rβ^+ cells, although the latter would appear unlikely given their different localizations.

A recent flow cytometric analysis identified cells with a hematopoietic stem cell phenotype ($CD34^+CD45^+$) in human endometrial cell suspensions that co-expressed CD7 and CD56 and appear to be lymphoid progenitors (61). Whether these cells function as hemopoietic stem cells and generate endometrial leukocytes in the endometrium or contribute to the SP is unknown.

A stem cell marker of hemopoietic stem cells is the proto-oncogene c-KIT (CD117), which encodes a 145-kDa transmembrane tyrosine kinase receptor specific for its ligand stem cell factor (62). While c-KIT was co-localized to 0.32% of stromal LRC in the lower region of murine endometrium (34), it was not detected in endometrial stromal LRC in another study (35). Neither human endometrial epithelial nor stromal CFU responded to stem cell factor in CFU assays, suggesting that c-KIT may not be important in the function of human endometrial stem/progenitor cell function (24,25).

Indirect Evidence for Endometrial Stem/Progenitor Cells

Monoclonality of Endometrial Glands

Evidence indicates that endometrial glands are monoclonal in origin, suggesting that they arise from a single progenitor or stem cell. In almost half of histologically normal proliferative endometrial samples, rare glands have been observed that fail to express phosphatase and tensin homolog (PTEN) protein (PTEN-null glands) because of a mutation of and/or deletion in the *PTEN* gene (63). These PTEN-mutant glandular clones persist in the basalis region between menstrual cycles to regenerate their respective glands in the functional layer in subsequent cycles. PTEN-null glands are increased in the endometrium of women in conditions arising from unopposed estrogen, particularly endometrial hyperplasia, a monoclonal epithelial proliferative disorder (64,65). In a separate study, monoclonality was detected in carefully dissected individual endometrial glands using a polyerase chain reaction (PCR)-based assay for nonrandom X chromosome inactivation of the androgen receptor gene (66). Furthermore, adjacent glands up to 1 mm apart shared clonality, indicating that well-circumscribed regions of endometrium were derived from the same precursor cell, suggesting that several glands share the same stem cell. This raises questions on the precise locality of candidate human epithelial stem/progenitor cells.

Gland Methylation Patterns

Another retrospective approach indicating adult stem/progenitor cell activity in endometrium analyzed methylation patterns in endometrial glands (67). Epigenetic changes to DNA sequences arising during cell division encode a cellular history in individual glands, which reflect the methylation patterns arising in resident stem/progenitor cells, since these are inherited in subsequent cell divisions and retained. In contrast, those arising in more mature progeny are lost during shedding. Recently, methylation patterns observed in individual glands from cycling and atrophic human endometrium were subjected to mathematical modeling, which supported the concept that an individual gland contains a stem cell niche with an unknown number of long-lived stem cells rather than a single stem cell (67). Further evidence of gland diversity in aging endometrial glands indicated that a reservoir of stem cells remained in atrophic endometrium, supporting the data from clonogenicity studies of human endometrium (25).

Source of Endometrial Stem/Progenitor Cells

Remnant Fetal Stem Cells

The embryonic female reproductive tract has its origins in the intermediate mesoderm, which begins to form soon after gastrulation. As this embryonic tissue proliferates, it is thought that some cells undergo mesenchymal to epithelial transition to give rise to the coelomic epithelium that later invaginates to form the paramesonephric or müllerian ducts (68). These ducts comprise surface epithelium and underlying urogenital ridge mesenchyme. During fetal life, the glands commence developing as the undifferentiated uterine surface epithelium invaginates into the underlying mesenchyme, and the inner myometrium commences to form as smooth muscle cells differentiate from the mesenchyme (10).

A small number of fetal epithelial and MSC are thought to remain in the adult endometrium and contribute to tissue replacement during its cyclic regeneration (69). Whether there is an ultimate uterine stem cell that has the capacity to replace all endometrial and myometrial cells, including epithelial, stromal, vascular, and smooth muscles, or whether there are separate epithelial and MSC is not currently known. The different phenotypes, growth factor dependence, and frequency of clonogenic endometrial epithelial and stromal cells suggest that there are at least two endometrial progenitor cells. However, this does not exclude the possibility of an unidentified, more primitive precursor in human endometrium.

Circulating Stem Cells from the Bone Marrow

There is increasing evidence that bone marrow–derived cells may also be a potential source of cells for endometrial regeneration (70–72). Significant chimerism ranging from 0.2% to 52% was detected in the endometrial glands and stroma of four women who received single-antigen

histocompatability locus antigen (HLA)–mismatched bone marrow transplants, suggesting that bone marrow stem cells contributed to endometrial regeneration in a setting of cellular turnover and inflammatory stimuli (69). It is not known if the source of the donor bone marrow cells contributing to chimeric endometrial tissue is hemopoietic or MSC. Further evidence for bone marrow stem cell contribution to endometrial repair comes from gender-mismatch bone marrow transplant studies in mice, where less than 0.01% of cytokeratin-positive endometrial epithelial cells and less than 0.1% of stromal cells contained a Y chromosome (72). Bone marrow cell contribution to endometrial repair is very modest, and engraftment of the endometrium seems more likely during repair after injury. Bone marrow cell contribution to endometrial regeneration may also have a role in the extensive endometrial epithelial growth and regeneration occurring during pregnancy and after parturition. Recently, circulating $CD45^+$ bone marrow cells were shown to contribute 82% of mouse uterine epithelium during pregnancy in a novel double reporter *CD45/Cre-Z/EG* transgenic mouse used to track the fate of $CD45^+$ green fluorescent protein (GFP) cells in female mice (71). These preliminary results need to be interpreted with caution as data was obtained only from a single pregnant reporter mouse. However, small but increasing numbers of GFP^+ endometrial epithelial cells were also observed in the luminal epithelium as the mice aged, ranging from 0% in 1- and 6-week-old to 0.5% in 12-week-old and 6% in 20-week-old mice (71). Although there were insufficient animals per group for statistical analysis, these data suggest increasing contribution of bone marrow–derived cells to the endometrial epithelium over time. Clonal expansion of transplant donor cells in the endometrium was apparent as GFP^+ cells were often found in clusters. Endometrial epithelial and stromal LRC did not express CD45 (33), but expression of this hemopoietic marker may be lost if bone marrow cells incorporate and transdifferentiate into endometrial epithelium. The role of estrogen and progesterone in recruiting bone marrow cells has not been examined, although progesterone may have a role during pregnancy.

ENDOMETRIAL STEM/PROGENITOR CELLS: CLINICAL PERSPECTIVE

Since adult stem cells regulate tissue homeostasis, it is expected that abnormal functioning of endometrial stem/progenitor cells and/or their surrounding niche cells may also be involved in the initiation and progression of gynecological diseases associated with abnormal endometrial proliferation, such as endometriosis, adenomyosis, endometrial hyperplasia, and endometrial cancer (17). Furthermore, epithelial and stromal CFU are present in non-cycling and perimenopausal endometrium (25) and may be responsible for regenerating endometrium in women given estrogen replacement therapy (17).

Cancer Stem Cells in Endometrial Cancer

The cellular composition of any cancer is quite heterogenous. Individual tumor cells vary in their ability to initiate tumors, their expression of markers and level of differentiation, their ability to generate tumor cells, and their life span. Like their normal tissue counterparts, there is a cellular hierarchy in tumors with the rare stem cell or cancer stem cell (CSC) at the apex (73). CSC have been extensively characterized in leukemias and are currently being characterized in many solid human tumors including breast, glioblastoma, colon, pancreas, prostate, and ovary (74). Controversy exists on the origin of the CSC: is it transformation of a normal adult stem cell, or do more mature cells gain mutations that enable them to acquire self-renewal properties of adult stem cells? A CSC is defined as a self-renewing cell within a tumor that has the capacity to regenerate the phenotypic diversity of the original tumor (74). Thus, CSC are able to initiate, maintain, and propagate tumors in vivo producing heterogeneous tumor cell progeny, initiate clones in vitro, undergo self-renewing cell divisions, and have high proliferation potential. In cancers where CSC have been identified, they comprise less than 1% of the total cell population.

Endometrial cancer is characterized by abnormal endometrial epithelial cell proliferation. It affects around 6430 women each year in the United Kingdom, resulting in approximately 1630 deaths (75). Endometrial cancer is the most common gynecological malignancy in the western world. There are two types of endometrial adenocarcinoma. Type I generally effects pre- and perimenopausal women, is estrogen dependent, and is associated with mutations in P*TEN*, K-*RAS*, and β-catenin genes or MSI (microsatellite instability) (76). Type II normally

affects postmenopausal women, is estrogen independent, and is associated with mutations in p53 and HER-2/neu (76).

Evidence for endometrial CSC has recently been obtained from studies in human endometrial cancer cell lines and isolated primary endometrial cancer cells (77,78). The endometrial cancer cell line, AN3CA, possessed a small population of SP cells (0.02%) (77). Further investigation revealed that AN3CA SP cells were relatively quiescent as they were mainly in the G_1 stage of the cell cycle and had low proliferative activity during the initial stages of cell culture. The AN3CA SP cells displayed adult stem cell functional properties of self-renewal and differentiation in vitro as they maintained similar proportions of SP cells after many passages in culture. The AN3CA SP cells were also relatively resistant to the chemotherapeutic agent paclitaxol compared with the MP cells (77). It remains to be seen if these properties are present in tumor cells isolated from primary human endometrial carcinomas. Tumor-initiating cells were also demonstrated in the SP cells but not MP cells after both cell fractions were injected subcutaneously into male NOD/SCID mice with estrogen implants (77). All of the SP injections resulted in tumors, whereas the MP injections failed to produce tumors (77).

CSC as tumor-initiating cells have also been demonstrated in unsorted cells isolated from primary human endometrial carcinomas, which have been transplanted into NOD/SCID mice either subcutaneously (77) or under the kidney capsule (78) and produced tumors in both locations. When transplanted in limiting dilution (10^6–10^3 cells), not all dilutions resulted in tumors, suggesting that not all cells within the tumor have the same capacity to initiate tumor growth (78). Similarly, not every cell within the tumor was able to produce a clone in vitro in cell cloning studies (78), evidence supporting the concept of the cellular heterogeneity of primary endometrial carcinomas. An alternative explanation is that some tumor cells lack the ability to adapt to the new environment in vitro and in vivo, and hence ability to grow and proliferate.

In vivo self-renewal of endometrial CSC was demonstrated by the formation of new secondary tumors after regrafting cells isolated from the original tumor transplants (77). Fewer tumor cells were required to initiate tumors on successive serial transplantation of secondary and tertiary tumors, indicating that increasing numbers of CSC with self-renewal capacity were produced. Self-renewal of endometrial CSC has also been demonstrated in vitro by serial cloning of initial individual CFU recultured at clonal seeding densities (<10 cells/cm^2) (78). Secondary, tertiary, and quaternary clones were produced. The fact that only some cells re-cloned indicates that only a few cells within a clone have self-renewal capacity.

The transplanted endometrial cancer cells produced tumors with similar histology and phenotype as the parent tumor (Fig. 6) (77,78), indicating the ability of the tumor-initiating cells to differentiate into the heterogenous mix of cells comprising the original tumor. Differentiation was demonstrated by investigating differentiation markers by immunohistochemistry. The distribution of EpCAM$^+$ epithelial cells was similar in parent tumor and transplants, and the pattern of cells expressing ERα and PR was similar to that observed in the parent tumor (78). While rare cells isolated from primary human endometrial cancers have CSC properties of tumorigenicity, self-renewal, differentiation, and clonogenicity, markers of these endometrial CSC have not yet been discovered. Identification of such markers will allow the prospective isolation of endometrial CSC and investigation into their role in the development and progression of endometrial carcinoma.

Endometriosis

Endometriosis is characterized by the growth of ectopic endometrial tissue on pelvic organs and the peritoneum (79). It is thought that retrograde menstruation, which occurs in most menstruating women, deposits menstrual debris into the peritoneal cavity. However, it is not known why only 6% to 10% of women develop endometriosis and its associated symptoms of inflammation, pain, and infertility. It has been postulated that in women who develop endometriosis, endometrial stem/progenitor cells are inappropriately shed during menstruation and reach the peritoneal cavity where they adhere and establish endometriotic implants (17,26,80). This assertion is supported by the demonstration of clonogenic cells in a long-term culture derived from a sample of endometriotic tissue (81) and monoclonality of some

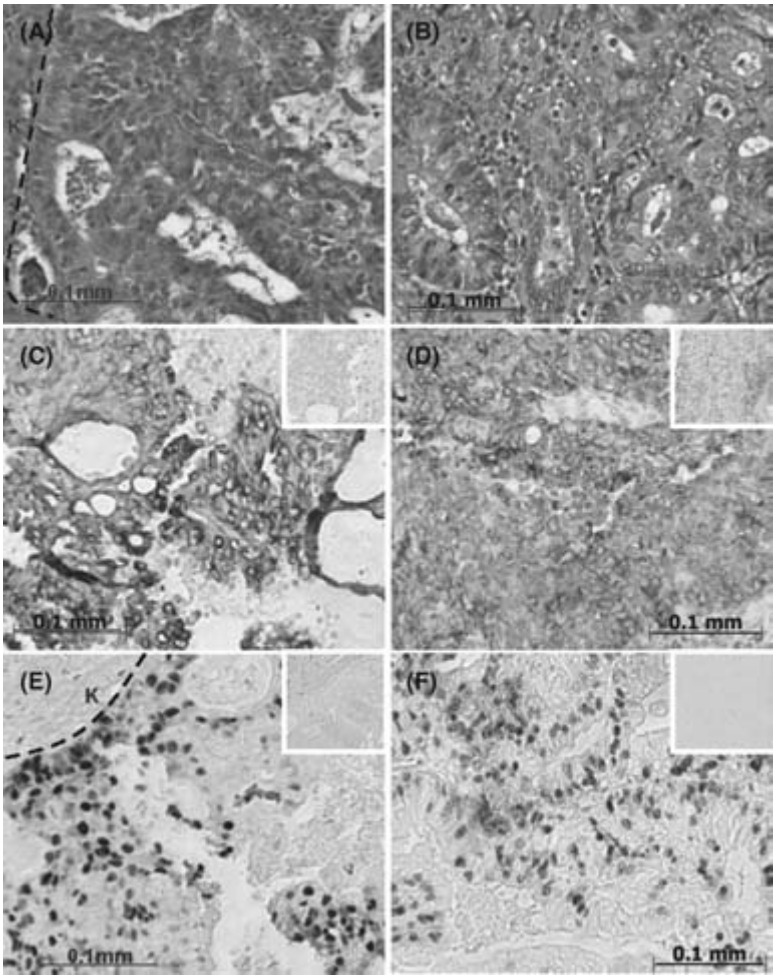

Figure 6 Endometrial tumors from transplanted endometrial carcinoma cells (H&E stain). (**A**) of a tumor produced from transplanting human endometrial carcinoma cells (5 x 10^5) under the kidney capsule of NOD/SCID mice after 12 weeks and (**B**) the parent tumor. Similar tumor morphology and expression pattern of markers were observed in tumors derived from transplanted endometrial cancer cells (**C**, **E**) and parent tumors (**D**, **F**), indicating the presence of tumor-initiating cells with the capacity to produce differentiated tumor cell progeny. (**C**, **D**) Cytokeratin. (**E**, **F**) Progesterone receptor. Insets: negative controls.

endometriotic lesions (82). Bone marrow stem cells may contribute to the progression of endometriosis lesion development as demonstrated recently in a mouse model (72). Some forms of endometriosis may arise from remnant fetal müllerian cells, which may behave like stem cells to establish ectopic growth of endometrial tissue. Clearly, the role of endometrial stem/progenitor cells or bone marrow stem cells in the development of endometriosis will require an extensive research effort.

Adenomyosis

Adenomyosis, a condition affecting 1% of women, results from extensive myometrial invasion by the basal endometrium. It is associated with smooth muscle hyperplasia and is also considered to arise from fetal müllerian cells (83).

It is possible that endometrial stem/progenitor cells or their niche cells demonstrate abnormal behavior in adenomyosis, or these putative stem cells have an abnormally orientated niche such that their differentiating progeny are directed toward the myometrium rather than

functionalis, producing pockets of endometrial tissue deep within the myometrium. Alterations in the putative endometrial stem cell niche, particularly in the niche cells regulating stem cell fate decisions may result in excessive smooth muscle differentiation of putative endometrial stem/progenitor cells producing the observed myometrial hyperplasia. Much research is required to establish a role for endometrial progenitors or myometrial SP cells in the pathogenesis of adenomyosis.

Tissue Engineering Applications

There is great interest in the use of both embryonic and adult stem cells in tissue engineering applications for restoring function to aging or diseased tissues and organs. Medical advances have ensured increasing longevity, and the aging population has many tissues in need of repair (84). The failure of artificial implants to last longer than 10 to 15 years and the problems associated with nondegradable synthetic materials make cell-based therapies for tissue replacement attractive (85). There is now a focus on using a combination of temporary biological scaffold materials to provide initial support and stem cells to promote appropriate tissue genesis and regeneration of functional tissue (85). This is particularly important for the provision of supportive tissues and could be adapted for tissue engineering support of the female reproductive tract. Pelvic floor prolapse is a major problem resulting in 10% of women requiring surgery, with approximately 30% of these requiring repeat surgery (86). The use of artificial and biological scaffolds for pelvic floor prolapse surgery has improved outcomes to a limited degree. Thus, the use of tissue constructs comprising scaffolds and autologous endometrial mesenchymal stem/progenitor cells may provide a possible solution for treatment of pelvic floor prolapse in future (26).

CONCLUSIONS AND FUTURE DIRECTIONS FOR HUMAN ENDOMETRIAL STEM/PROGENITOR CELL RESEARCH

There is now sufficient evidence to conclude that rare populations of adult epithelial and mesenchymal stem/progenitor cells exist in normal human and mouse endometrium and rare CSC are present in endometrial adenocarcinoma. While some evidence has been obtained from in vivo studies in mice, identifying candidate stem/progenitor cells as LRC, all evidence collected to date for human endometrium has been from in vitro studies. It is most important that the capacity of these rare cells to reconstitute endometrial tissue in vivo using xenotransplantation approaches is undertaken. Such a model has already been published and could be adapted to examine putative endometrial stem/progenitor populations (49). Whether there is a single more primitive endometrial stem cell that produces all cell types in the uterus is yet unknown. Whether the bone marrow is a source of endometrial stem/progenitor cells as a major or minor contributor needs to be determined under physiological conditions. There is also a pressing need to identify definitive markers for endometrial epithelial stem/progenitor cells and find markers that further purify the $CD146^{+}PDGF\text{-}R\beta^{+}$ endometrial MSC-like cells. Further characterization of the endometrial stem cell niches and the signaling pathways involved in the regulation of the resident stem/progenitor cells is also required. Investigation into the possible roles of developmental pathways involving bone-morphogenetic protein, Hedgehog, Notch, and Wnt signaling in endometrial stem/progenitor cell self-renewal and cell fate differentiation decisions, would be a valuable starting point as these molecules or pathways have already been detected in endometrium or have important roles during endometrial development or decidualization when stromal cells undergo terminal differentiation. More extensive studies examining how estrogen, progesterone, and the growth factors EGF, TGFα, PDGF, and bFGF interact with endometrial stem/progenitor cells and their niche cells would be useful. How estrogen and progesterone interact with endometrial stem/progenitor cells or their neighboring niche cells needs to be explored.

Such additional knowledge will assist the investigation into the role of endometrial stem/progenitor cells in gynecological disorders associated with abnormal endometrial proliferation and will not only increase our understanding of the pathophysiology of endometriosis, adenomyosis, endometrial hyperplasia, and endometrial cancer but it also has the potential to change the way these hormone-dependent diseases will be treated in future.

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16 Stem Cell Populations in Adult Bone Marrow: Phenotypes and Biological Relevance for Production of Somatic Stem Cells

Agustín G. Zapata

INTRODUCTION

Bone marrow (BM) has been acknowledged many years as the main hematopoietic locus of adult mammals, including in humans. Apart from mature and developing blood cells belonging to all hematopoietic cell lineages, a poorly defined heterogeneous cell population of stromal cells and hematopoietic stem cells (HSC) are the main cell components of BM.

HEMATOPOIETIC STEM CELLS

There is total agreement that HSC represent the primitive hematopoietic cell progenitors capable of producing all blood cell lineages (1). However, in the past years, different results, largely obtained in laboratory rodents, have suggested that total or purified BM cell fractions were also able to generate other nonblood cell lineages under certain conditions, including skeletal muscle cells (2), hepatocytes (3), neural cells (4), cardiomyocytes (5), vascular endothelium (6), and epithelium of the skin, lung and gut (6).

These results were firstly explained on the basis that HSC could transdifferentiate into cell progenitors to different nonhematopoietic tissues according to two possible mechanisms: a direct commitment of HSC to other cell lineages under the influence of "new microenvironments" or a dedifferentiation process of HSC that could result in their conversion into multipotent or even pluripotent stem cells. However, other studies have refused to assume the capacity of HSC to clonally produce other cell types, apart from those belonging to the lymphohematopoietic system (7). On the other hand, tissue-derived stem cells can undergo fusion, with HSC favoring the differentiation of the first ones (8,9). However, there is a general agreement that cell plasticity is a very rare process: HSC typically show low levels of engraftment and transdifferentiation within the environment of altered tissues. It is difficult, therefore, to assume that they contribute significantly to tissue regeneration (10).

In addition, injected BM cells could promote tissue repair by secretion of various growth factors: chemokines and cytokines that could stimulate proliferation and differentiation of tissue endogenous stem-like cell progenitors inhibit apoptosis, decreasing local inflammatory and immune reactions and increasing neoangiogenesis (11). On the other hand, it is possible that BM houses other stem cell populations, apart from HSC, that could be truly responsible for of the effects of BM cells on tissue repair. Remarkably, the idea that BM could contain heterogeneous populations of stem cells has received little attention (12).

It is important therefore to reanalyze the composition of different cell populations of adult BM and to review their biological characteristics including plasticity and differentiation potentiality.

NONHEMATOPOIETIC CELLS OF ADULT BONE MARROW

In the past years different nonhematopoietic cell types have been isolated from adult BM (13). They share common features, which suggest that they could correspond to stem cells, including the capacity to differentiate into various cell lineages belonging to the three embryonic germ layers, and do not express typical hematopoietic cell markers but exhibit variable expression of molecules specific to embryonic stem cells. Moreover, most of these BM cells are generated after extensive in vitro culture, making it possible that they actually do not exist in vivo but are culture-induced products. Accordingly, it is difficult to establish their

relationships with the nonhematopoietic stromal cells that occur in situ in the BM as well as between the different cell types of BM stem cells described in the literature.

On other hand, it is important to remark that studies on some of these cells [multipotent adult progenitor cells (MAPC), marrow-isolated adult multilineage inducible (MIAMI) cells, very small embryonic-like (VSEL) cells] have been largely performed by only one research group, whereas other researchers have had difficulties to culture them. In this respect, it seems necessary to confirm results on their biology and capacities by independent laboratories.

Multipotent Adult Progenitor Cells

They were the first to be isolated from adult BM cultures of humans, mice, and rats and, more recently, from mouse muscle and brain and from swine BM (14) as fibroblastic, adherent mononuclear $CD45^{-}$ cells, although it remains to be determined whether they really exist in vivo (13). MAPC appear to be a clonal population of stem cells that can be ex vivo expanded without signs of senescence, maintaining unchanged telomere length and retaining its potential of differentiation. However, the growth of these cells seems to be particularly dependent on selected serum batches and oxygen levels. Murine MAPC are c-kit $(CD117)^{+}$, stage-specific embryonic antigens $(SSEA)\text{-}1^{+}$, Flk^{lo}, and $Thy\text{-}1^{lo}$ cells. They do not express, however, major histocompatibility complex (MHC) class I and II molecules, CD34, CD44, CD45, CD90, or Sca-1. Human MAPC express Oct-3a but not CD44 or MHC class I antigens.

Data on the MAPC pluripotency to differentiate into distinct cell lineages are controversial (13,15). MAPC can differentiate into mesenchymal cell lineages (osteoblasts, chondroblasts, adipocytes, skeletal muscle cells), but their capacity to produce endothelial cells, smooth muscle cells, neuroectodermal cells, and hepatocytes (13) in vitro has also been reported. In addition, MAPC injected into a blastocyst contribute to almost all somatic tissues of born mice, although their degree of contribution is low, and they are unable to form embryonic bodies or teratomas. On the other hand, when MAPC are grafted in sublethally irradiated mice, they participate at low levels in the generation of gut, lung, and liver epithelia and the hematopoietic system (16). A total recovery of the hematopoietic system requires large numbers of MAPC (17), whereas MAPC supplied with VEGF differentiate to endothelial-like cells (18). Furthermore, it has been reported that an important proportion of MAPC has abnormal karyotypes, and their presumptive high plasticity could therefore be a consequence of epigenetic and/or genetic alterations (16).

On the other hand, the relationships between MAPC and other non-HSC reported in adult BM are unclear. It has been proposed that MAPC could be more primitive than mesenchymal stem cells (MSC), but their relationships with the described MSC subpopulations (see later in the chapter) are unknown (15).

Bone Marrow–Derived Multipotent Stem Cells

Human BM-derived multipotent stem cells (BMSC) were recently described by Yoon and colleagues (19) as a BM-derived cell population capable of expanding to a population of more than 140 doubling and to differentiate into cells of all three germ layers. Remarkably, these cells express different markers from those used for defining HSC, MSC, or MAPC. They do not express CD29, CD44, CD73, or the transcription factor Oct-3a, which is a key factor for maintaining MAPC functions. In addition, they express weakly typical MSC markers such as CD90, CD105, and CD117.

After being grafted intramiocardially in a model of acute myocardial infarction, BMSC appear to differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells, improving cardiac function (19).

Multipotent Adult Stem Cells

A very rare cell population named multipotent adult stem cells (MASC) has been recently isolated from the mononuclear cell fraction of human liver, heart, and BM (20). They express CD13, CD49a, CD49b, CD90, CD73, CD44, CD29, CD105, and MHC class I antigens, but not HLA-DR molecules, CD14, CD34, CD45, CD38, CD133, or c-kit. Furthermore, they express

typical transcription factors of embryonic stem cells, such as Oct-3/4, Nanog, and Rex-1, and exhibit high telomerase activity and a wide range of differentiation potential. However, their relationships with the other non-HSC of BM are totally unknown.

SSEA-1$^+$ Cells

Anjos-Afonso and Bonnet (21) have also reported very recently the presence of SSEA-1$^+$ cells in BM cultures and even directly in BM, although, in this last case, they cannot be expanded in long-term cultures. Whereas SSEA-1$^-$ cells seem to be MSC, although SSEA-1 antigen has been used to isolate MSC from BM (see later in the chapter), SSEA-1$^+$ cells appear to be very similar to MAPC. Thus, SSEA-1$^+$ cells can only be maintained in vitro under conditions similar to those used to culture mouse MAPC, losing both Oct-3a/4 and Nanog expression and their differentiation capacities in any other culture conditions. In vitro SSEA-1$^+$ cells differentiate not only to mesenchymal cells but also to other ones that exhibit phenotype and functional properties of astrocytes, endothelial cells, and hepatocytes. After in vivo grafting, they also contribute to mesodermal lineages, including low levels of both hematopoietic cells and endothelial cells.

Marrow-Isolated Adult Multilineage Inducible Cells

MIAMI cells are also obtained after culture in similar conditions to those used for producing MAPC (22). They have been isolated from BM of both young and old men, whereas other presumptive BM non-HSC occur largely in young mice (12,15) (see later in the chapter).

Like MAPC, MIAMI cells may be extensively cultured without ageing or loosing their differentiation potentialities and exhibit a similar phenotype expressing telomerase, Oct-3a, and Rex-1 but not CD34, CD36, CD45, CD117, and HLA-DR. They are positive for the following markers: CD29, CD63, CD81, CD122, CD164, c-Met, BMPR1b, and NTRK3.

MIAMI cells differentiate in vitro to osteoblasts, chondroblasts, adipocytes, immature neural cells, and presumptive pancreatic-like cells, but the functional properties of these in vitro–produced cells have not been tested. Besides, although the expression of several markers typical of cells from the three germ layers suggest that MIAMI cells could be pluripotent, their in vivo capacities have not been determined.

Mesenchymal Stem Cells or Multipotent Mesenchymal Stromal Cells

In the past years, MSC have become one of the most promising BM stem cells for cell therapy. Their origins, mechanisms of functioning, and true differentiation potentialities are, however, controversial.

MSC were initially named colony-forming unit–fibroblasts (CFU–F) (23), then marrow stromal cells, and, more recently, either MSC or multipotent mesenchymal stromal cells (24). Apart from the BM, MSC have been isolated from peripheral blood (25), adipose tissue (26), skin (27), trabecular bone (28), fetal blood, liver, BM (29), lung (30), amniotic fluid (31), umbilical cord, synovium, and dental pulp (24). Accordingly, it has been proposed that MSC reside within the connective tissue of most organs (32). All these MSC populations are not totally equivalent especially in terms of the in vivo differentiation potential (33).

BM MSC derive from cell suspensions containing both HSC and BM stroma after culture at low density. In those conditions, adherent cells are easily separated from the nonadherent HSC and under appropriate culture conditions form distinct clonogenic CFU–F that show a high, although limited, self-replication capacity. In addition, MSC appear to be relatively stable as primary cultures, although spontaneous transformation has been reported in long-term cultures (34).

The proportion of obtained colonies is, however, highly dependent on the culture conditions, and there is a great variability in the requirements from one species to another. That presumably reflects the high heterogeneity described in these cultures and their derived colonies. However, because a standard, unique isolation method for MSC does not exist, it is difficult to directly compare the available results.

Indeed, morphological, phenotypic, and functional heterogeneity has been reported to occur in MSC cultures. Two morphologically different MSC types have been reported (35):

Small, spindle-shaped, rapidly self-renewing multipotent cells
More mature, slowly replicating, larger cells

Human MSC cultured under serum deprivation conditions produce a unique subpopulation similar in size to the above-described smaller, spindle-like cells, although they proliferate at a slower rate and express mRNA for embryonic markers (15).

MSC cloned as single cell–derived colonies show variable degree of plasticity. All tested colonies undergo osteogenic differentiation, and most of them also produce adipocytes, but only two out of six are able to have chondrogenic differentiation (36).

These results suggest that all MSC colonies do not contain multipotent stem cells and confirm the relevance of employed protocols in both transplantation and in vitro assays to critically determine the range of differentiation of MSC.

Most of the subsets of MSC express CD29 and CD105 or both cell markers, but it is important to remember that there are no real specific markers for MSC. Enriched MSC have been isolated from peripheral and cord blood by using CD133 as a marker and from BM through selection of SSEA-1 or SSEA-4, or CD271 (NGF receptor). The mAb Stro-1 has been used in some studies to enrich for MSC, but all MSC do not express Stro-1 antigen, and this molecule is weakly expressed in hematopoietic cells (37). On the other hand, the phenotypic analysis of BM MSC has been largely carried out on MSC expanded ex vivo, but not directly in CFU–F cells. Thus, the phenotype of MSC progenitors that initiate the culture is unclear.

MSC (24) express CD29, CD44, CD49a–f, CD51, CD73, CD90, CD105, CD106, CD166, CD133, CD271 (loNGF receptor), Stro-1, SSEA-1, SSEA-4, and 3G5 and are negative for typical hematopoietic markers such as CD11b, CD14, CD34, and CD45. Cells expressing these markers are capable of differentiation into connective tissue cell lineages, one of the functional characteristics of MSC.

MSC, whose effects on immune reactions and tissue repair will be later discussed, express specific receptors for numerous cytokines (38), including IL1, IL3, IL4, IL6, IL7, IL15, IFNγ, and TNFα. Also, human MSC express receptors for a wide range of chemokines, including CCR1, CCR7, CCR9, CXCR4, CXCR5, CXCR6, although some of them, that is, CCR9 and CXCR6 are only expressed by some MSC subsets (39).

As mentioned earlier for other BM stem cells, the in vivo and in vitro differentiation capacities of the MSC are a matter of discussion. In this case, however, numerous assays have been performed by using MSC not only in rodents but also in big animals, such as horses, cows, pigs, dogs, sheep, and even in humans (40). Classically, MSC were described as adherent cells from adult BM that can be cultured and expanded and produce bone, adipocytes, and in general, mesenchyme-derived tissues (23), but, more recently, there have been reports about their capacity to differentiate in vitro into skeletal myoblasts, cardiomyoblasts, neuroectoderm, hepatocytes, etc. (41).

Molecules involved in these processes are largely unknown, although a few data can be found in literature. TGFβ and Wnt families of molecules appear to be key for the development and maintenance of MSC (42). Depending on the molecule, different members of TGFβ family promote or inhibit MSC proliferation, apoptosis, or differentiation (38). Wnt signaling is essential for the differentiation of MSC into osteoblasts. The secreted soluble factor Dickkopf-1 involved in the Wnt signaling inhibits the differentiation of MSC to osteoblasts and, at the same time, predisposes undifferentiated MSC to proliferate (43) in vitro. The transcription factor Runx1 appears to drive MSC to differentiate in osteoblasts, whereas PPARγ induces differentiation into adipocytes (44,45), both being processes that are mutually exclusive and apparently regulated by the transcriptional modulator TAZ (46).

In vivo systematic administration of MSC results in the production of various epithelial cell types, although other groups have claimed that MSC do not contribute significantly to epithelial regeneration (47). In fact, evidence on MSC differentiation in cells of other lineages came in most cases just from the expression of specific tissue transcripts and/or proteins, but their functional capabilities have been rarely assayed.

MSC are, however, one of the few adult stem cells that are currently in use in clinics. On the basis of their osteogenic potential, MSC have been used for recovery of various skeletum problems (48). Also, MSC could be useful for cartilage repair in osteoarthritis patients (49). Furthermore, production of new cardiomyocytes and neoangiogenesis after MSC infusion has been reported in the human heart (50), and MSC implantation is capable of repairing digestive fistula in 75% of cases (51).

Although underlying mechanisms on the capacity of MSC to trigger tissue repair remain unknown, these cells could be more efficient for tissue regeneration than other BM stem cells, including HSC. One of the most important issues in Regenerative Medicine is the efficacy of stem cells to graft and survive in the damaged tissues. It is generally assumed that the greater the ability of the exogenous cells to survive, the greater the therapeutic response. In damaged tissues a hypoxic and inflammatory environment does not favor the survival of provided cells, although MSC have immunosuppressor capacities (see later in the chapter). In infracted hearts, Mangi and colleagues (52) improved the MSC survival and differentiation by transfecting them with the gene encoding Akt. In a similar model, both coadministration of IGF-1 (53) and modification of MSC with a hypoxia-regulated heme oxygenase I vector (54) enhanced MSC survival and their ability to target myocardium reparation. In any case, it is unknown whether MSC, as other assayed stem cells, act directly by in situ transdifferentiation or fusion with resident myocardioblasts or indirectly through a trophic action by secretion of promyogenic factors, such as VEGF and FGF (55).

In this regard, it is important to know whether MSC could migrate from the BM (or other sources) to the damaged tissues. MSC have been isolated from the peripheral blood and are believed to migrate to sites of injury (56). In fact, MSC use both P-selectin and VCAM-1 to interact with endothelial cells and thus extravasate into organs, such as the liver and spleen (57). Other molecules involved in MSC migration are CC chemokines (58), β-integrins (59), and CD44 (60).

The relationships between MSC and the in situ components of BM, such as HSC and stromal cells, are unclear. HSC and MSC have been considered different, unrelated cell lineages because they cannot be detected in both recipient BM MSC in heterotopic BM transplantation (61) and donor BM MSC after systemic BM transplantation (62). Other results suggest, however, the existence of certain direct or indirect connections. It has been reported that MSC-derived marrow stromal cells provide growth factors that are essential for hematopoiesis (37). Singer and colleagues (63) reported a BM-adherent cell population that contain cells with both hematopoietic and stroma-like features, and several BM $CD34^-$ cells could differentiate into $CD34^+$ progenitors, initiate multilineage hematopoiesis, and reconstitute the lymphohematopoietic system. In this respect, Huss (64) proposed that $CD34^-$ fibroblast-like cells exhibit hematopoietic pluripotency, although other results question the existence of this common stem cell because HSC contamination of the MSC fraction cannot be ruled out (65).

On the other hand, MSC could correspond in situ with the adventitial reticular cells that cover the outside of sinusoidal blood vessels in the BM, connecting endothelial cells with the meshwork of reticular cells that constitute the BM nonhematopoietic stroma, but evidence is indirect and nonconclusive; other authors named pericytes to these adventitial reticular cells and assumed that they have a common origin with smooth muscle and endothelial cells deriving from common vascular cells (66). More recently, these cells have been described as CXCL12 secretory reticular cells capable of attracting HSC (67). All these results obviously need further confirmation and question the homogeneity of BM MSC population. Thus, it could be more important to determine whether all MSC are stem cells or some of them represent in vitro merely mechanically supporting BM stromal cells.

One of the most remarkable properties of MSC, which could explain their above-mentioned anti-inflammatory effects in damaged tissues, is their immunosuppressive capacities. In vivo administration of purified haploidentical human MSC after allogeneic stem cell transplantation reverses the GVHD. Other studies in mice have failed, however, to show any effect of MSC supply on GVHD (68). Syngeneic MSC appear to induce tolerance against host and donor minor antigens, but grafting of allogeneic MSC and allogeneic BM cells not only failed to prevent graft rejection but also increased it (69). Moreover administration of syngeneic MSC reduces the severity of EAE, an

experimental model for human multiple sclerosis, with reduced demyelination and decreased infiltrates of T lymphocytes and macrophages (70).

The immunosuppressive effects of MSC affect every step of the immune response from the antigen presentation to the activation of T and B lymphocytes. In addition, certain MSC-NK cell cross talk has been reported. Allogeneic MSC appear to inhibit the generation of mature DCs as well as to induce a significant reduction of the immune capacities of mature DCs (71). On the contrary, IFNγ stimulates both mouse (38) and human MSC (72) to act as antigen-presenting cells, thereby becoming capable of processing soluble exogenous proteins to present antigenic peptides on MHC class II molecules and to activate in vivo and in vitro antigen-specific primary T cells (38). On the other hand, ex vivo–cultured MSC suppress both T- and B-cell proliferation induced by different specific and nonspecific stimuli (73) as well as the IL2- and IL15-induced proliferation of resting NK cells, although only partly that of preactivated NK cells (74). On the contrary, MSC are particularly susceptible to NK-mediated lysis, a finding that could be related with the fact that human MSC broadly express NK receptor ligands (74). Furthermore, MSC express toll-like receptor (TLR)-2 and other possible TLRs, and TLR ligands significantly affect in vitro proliferation, migration, and differentiation of MSC (75).

Soluble factors (73) [i.e., hepatocyte growth factor, prostaglandin E2, TGFβ, indoleamine 2,3-dioxygenase (IDO), nitric oxide, IL 10] secreted by MSC or by the immune cells in response to MSC appear to be involved in these effects of MSC on the immune system. It is possible that the nature of these factors varies depending on the nature of the received stimuli by MSC. Thus, TNFα enhances the production of immunosuppressive prostaglandins by MSC, whereas IFNγ induces MSC to produce prostaglandins and IDO (73).

Very Small Embryonic-Like Stem Cells

As mentioned earlier, most of non-HSC described in the BM correspond to ex vivo–cultured expanded cells; however, the so-called VSEL stem cells were isolated from BM by FACS employing a novel size-based approach controlled by size bead markers (12,15). Since most sorting protocols exclude events smaller than 6 μm, VSEL cells (about 3.6 μm diameter) were usually excluded from the BM-sorted cell population.

They constitute a rare, homogeneous Sca-1^+ Lin^- $CD45^-$ MHC class I^- HLA-DR^-, $CD90^-$ $CD105^-$ $CD29^-$ cell population that represents about 0.01% of murine BM mononuclear cell fraction, and it has also been isolated from human cord blood. In addition, VSEL cells express pluripotent markers present in embryonic stem cells, such as Oct-4, CXCR4, Nanog, Rex-1, SSEA-1, Stella, Fragilis, Nobox, and Rif telomerase, and ultrastructurally are similar to the embryonic stem cells showing scarce cytoplasm and abundant euchromatin. They are also mobile cells that respond to CXCL12, adhere to fibronectin and fibrinogen, and interact with BM-derived stromal fibroblasts.

Five percent to ten percent purified VSEL cells cultured on a C2C12 murine sarcoma cell feeder layer form spheres resembling embryonic bodies, which in cultures favoring differentiation give rise to cells belonging to the three germ cell layers. These spheres could also be obtained from fetal liver, spleen, and thymus from young but not old mice. In fact, the numbers of VSEL cells of BM are higher in long-lived mice than in short-lived strains. In vitro, in the presence of cytokines VSEL cells, they generate cells with phenotype of cardiomyocytes, neural cells, and pancreatic cells.

It has been proposed (12) that during late phases of embryogenesis some migrating/circulating stem cells similar to epiblast-derived cells could colonize the BM, turning into the VSEL cells found in the adult BM. $CXCR4^+$ VSEL cells could be attracted by stromal fibroblasts that secrete CXCL12. On this basis, it has been speculated (12,15) that the capacity of BM-derived fibroblast cells, largely MSC and MAPC, to differentiate into other cell lineages could really be due to contamination by VSEL cells. However, it remains to be determined if these cells in physiological conditions contribute to the renewal of other adult BM stem cells, including HSC.

Endothelial Progenitor Cells

Finally, endothelial progenitor cells (EPCs) (76) have been reported in human (phenotype: $CD133^+$ $CD34^+$ $CD117^+$ VE-$cadherin^+$ $VEGFR2^+$ $CD146^+$ vWF^+ CD 31^+ cells) and mouse BM

(phenotype: Sca-1^+ $CD117^+$ Lin^- $VEGFR2^+$ VE-$cadherin^+$ $Tie2^+$ $CD146^+$ vWF^+ $CD31^+$ cells) as stem cells capable of being mobilized to peripheral blood as a source of cell progenitors involved in the neovascularization of injured tissues (77). However, the degree of contribution of these cells to the repair of damaged tissues requires further demonstration.

CONCLUSIONS

In physiological conditions, the contribution of the above-described different stem cell populations of BM to repair appears not to be very important, and, in any case, it remains to be conclusively determined which cell progenitor population housed in the BM has this repair capacity as well as what the mechanism (i.e., transdifferentiation, cell fusion, production of trophic factors) employed by these cells to perform their effects is.

A better knowledge of the underlying processes that occur in damaged tissues during reparation as well as clonal or semiclonal assays by using highly purified BM stem cells, the phenotype of which must be clearly determined at the time of implantation, are necessary for resolving the many unanswered questions about the contribution of adult stem cells to tissue repair and their possible successful use in human cell therapy.

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17 Models of Trophoblast Development and Embryo Implantation Using Human Embryonic Stem Cells

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INTRODUCTION

Whereas gamete production and function is reflected in successful fertilization, the defining moment when the developmental potential of gamete union and the early embryo is tested is at implantation. Notwithstanding the necessity of a beneficial milieu of the oviduct and uterus, preimplantation embryo development is to a large extent autonomous, relying heavily on stored factors residing in both gametes and subsequent zygote, as witnessed by the efficient development of the early embryo in comparatively simple culture conditions in vitro (1). In contrast, at implantation a complex dialogue of signaling factors enables the mother to interrogate the credentials of the embryo and to terminate gestation if a sufficient standard (genetic, epigenetic, biochemical, etc.) is not forthcoming. Indeed it is estimated that in fertile women 60% to 80% of all embryos fail to implant (2,3). This highly regulated selection process is absolutely crucial to allow for a healthy pregnancy with optimal fetal development and prevention of the immunological rejection of the fetus by the mother. Investment in fetal development, which may ultimately fail, has dire consequences for the reproductive success of the female mammal. Thus implantation processes have been subject to very strong selective pressure during evolution, resulting in a finely balanced system. When this balance at implantation goes awry in women, then it can cause implantation failure and placental dysfunction (4). Aberrant and inappropriate implantation may lead to miscarriage or a deficient placenta, resulting in maternal disease such as preeclampsia (5) and a baby with low birth weight, thereby potentially increasing the risk of disease in the offspring throughout life (6). In contrast, recurrent failure of the embryo to implant is an important cause of infertility, often with psychological repercussions. This condition affects about 1% of all women where three or four consecutive pregnancies before 20 weeks of gestation are lost (7), about a threefold higher incidence than that expected by chance alone.

Most of our current understanding of human trophoblast development and implantation is either inferred from clinical findings of later stages of pregnancy when fetal and placental development can be monitored or gained indirectly from histological observation of tissue samples. Experimentally, we can extrapolate findings from animal models and in vitro cellular studies. The first signs of embryonic differentiation are apparent during the morula stage, when cell division creates two distinct cell phenotypes. Outer cells become polarized and then give rise to trophectoderm, whereas cells in the interior stay unpolarized and give rise to the inner cell mass. However, these initial events may not irreversibly form extraembryonic and embryonic lineages, since in the mouse the polarized outer cells may contribute to the inner cell mass (8), while inner cells of the morula can retain their capacity to differentiate into trophectoderm. A very similar process seems to occur in the human embryo. Thus, whether a cell becomes polarized or not provides the molecular signal to specify this initial embryo differentiation, implicating both positional and transcriptional cues as essential. An alternative hypothesis is that trophectoderm and embryonic lineages may result from prepatterning in the early cleavage-stage embryo or even in the oocyte or zygote, although the evidence remains unsubstantial (9). Either way trophoblast precursor cells arise from pluripotent precursors in early development and subsequently become a self-renewing trophoblast stem (TS) population.

The continual proliferation and differentiation of TS cells by the implanting embryo is of critical importance for the maintenance of early pregnancy (10,11), both for rapid invasion of

the endometrium to establish a blood supply for the embryo and for production of hormone [human chorionic gonadotrophin (hCG)] to rescue the corpus luteum from regression and provide progesterone support to the uterus. But the process of human trophoblast development is poorly understood, because investigations have been severely constrained by obvious ethical and practical considerations. Moreover, while there are many surplus human preimplantation embryos generated worldwide with much research on embryo culture techniques, many aspects of the immediate postblastocyst stage remain unknown.

Usually a variety of trophoblast and placental cell lines are used for investigation, either from primary tissue samples (at term or earlier in gestation) or from tumor cells. Primary trophoblast progenitor cells or trophoblast cell lines derived from malignant choriocarcinoma, such as JAR or JEG3 cell lines, have been the most robust and consistent to study as models of trophoblast/placental development (12). But all these cell lines have a major shortcoming in that their phenotypes are already committed down the trophoblast lineage and therefore for the most part represent a later stage of development than that occurring at the peri-implantation stage. Moreover, while choriocarcinoma cell lines may well demonstrate some characteristics similar to those of primary trophoblast [such as the ability of hCG production; formation of syncytium by cell fusion; and invasion through extracellular matrix (ECM)], they can also behave differently to trophoblast with uncontrolled proliferation and invasive qualities that reflect their tumor origin (or transformation with virus), and hence they may fail to recapitulate the true physiology of trophoblast (12–14). On the other hand, investigations in vitro of trophoblast cells from primary human placenta facilitate investigations of trophoblast function in vivo such as syncytiotrophoblast formation. Yet only those cells derived from the first trimester placenta will normally spontaneously form extravillous trophoblast, a major component of the placenta, and such samples are not always widely available.

HUMAN EMBRYONIC STEM CELLS

In the past decade, the derivation of numerous human embryonic stem cell (hESC) lines has generated much excitement as a pluripotent sources to produce cell phenotypes to study human development in vitro and for therapeutic applications to treat degenerative diseases, so-called regenerative medicine (15). Many hESC lines have now been produced and comparative analysis undertaken so that key characteristics can be established and consistent standards adopted for research and clinical therapies (16). This embryonic stem cell (ESC) revolution has also created a new paradigm for studying the very early stages of human trophoblast and placental differentiation since it emerged that hESCs can spontaneously differentiate to trophoblast cells in culture (17–19). There are several advantages of using hESCs to study human trophoblast and implantation events. HESCs can be maintained indefinitely in the laboratory and therefore are a reliable and consistent starting source for trophoblast differentiation compared with primary tissue samples, which can vary considerably in proliferative capacity. The cell lines usually have a normal complement of chromosomes (although cell karyotype should be regularly monitored in culture for adaptive changes) (20) unlike choriocarcinoma and other tumorogenic or transformed cell lines, which show cancer-related features. Moreover, in principle at least, hESCs are the adapted self-renewing counterpart of the inner cell mass and therefore potentially provide a route to study the initial human embryonic trophectoderm transition and trophoblast differentiation that occur at implantation. Of course hESCs are themselves a highly controversial cell source, but in many countries the regulated use of these cells is seen as highly beneficial for medical research. The advances with hESCs have also been instrumental in the development of induced pluripotent stem (iPS) cells (21,22), which are set to circumvent most of the ethical dilemmas of hESCs.

Initially, the generation of trophoblast from hESCs seemed somewhat surprising as it appeared to be at odds with what happens in mouse ESCs (mESCS), which were first derived in the early 1980s (23,24). Trophoblast will rarely generate spontaneously from mESCs, although forced differentiation to a trophoblast cell type is possible if the gatekeeper gene of ESC pluripotency, Oct4, is conditionally downregulated (25,26), or there is forced expression of an early trophoblast-specific regulator, such as the transcription factor *Cdx2* (27). On the other hand, mouse TS cells can be isolated from pre- and postimplantation embryos, but these cells originate from trophectoderm lineage when cultured in conditioned medium supplemented

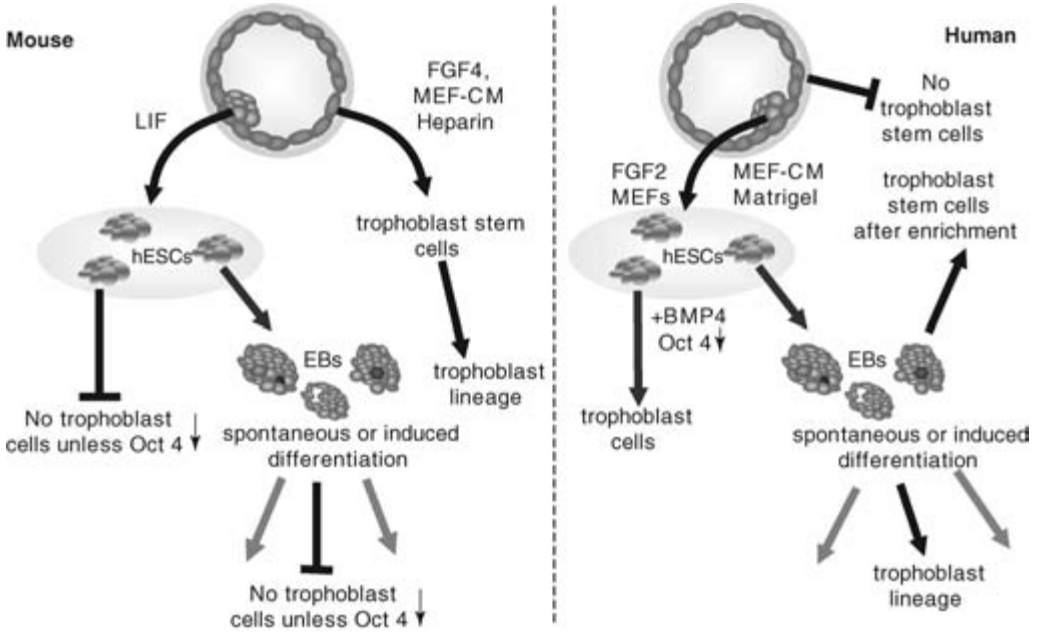
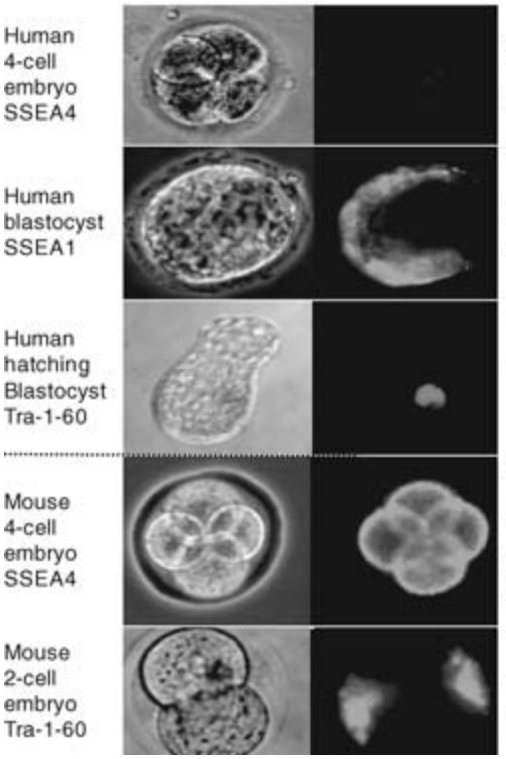


Figure 1 A schematic diagram summarizing differences in the differentiation of trophoblast from embryos and ESCs from the mouse and human and showing some of the inducing factors involved. *Abbreviations*: ESC, embryonic stem cell; EBs, embryoid bodies; LIF, Leukemia inhibiting factor; MEF, mouse embryonic fibroblasts; CM, conditioned medium.

with fibroblast growth factor 4 (FGF4). These murine TS cells can be maintained indefinitely with the capacity to differentiate along the trophoblast path when FGF4 is withdrawn (28). But so far it has not been possible to derive human TS cells equivalent to those in the mouse, although a TS phenotype in the rhesus monkey has been reported (29). These disparities seem to highlight some of the difficulties of extrapolating findings from the mouse model (Fig. 1).

A simple but perhaps significant illustration of differences between murine and hESCs can be observed in disparity in localization of stage-specific embryonic antigens (SSEAs) first generated to characterize early mouse embryo development (Fig. 2). These antigens are expressed on the plasma membrane of cells of the preimplantation embryo in a specific pattern, which is developmentally regulated during early embryogenesis and are widely used as markers to monitor the differentiation of both mESCs and hESCs and their malignant counterparts embryonic carcinoma (EC) cells. There are pronounced differences in the expression patterns of such markers between hESCs and mESCs (30), and this is reflected in preimplantation embryos as well (Fig. 2). The cause for this divergence is unknown, and the function of these carbohydrate antigens presents a conundrum. There is no doubt that their expression is closely regulated during early development and that change in expression correlates well with cell differentiation. In addition, the SSEA1/Le^x epitope may play a role in cell-cell adhesion between blastomeres at the morula stage. SSEA3 and SSEA4 antigens belong to the P blood group system, and the small number of individuals who lack the ability to synthesize the extended globoseries lipid structures that form the basis of these antigens (p^k and pp individuals) lack expression not only of the P blood group antigen but also SSEA3 and SSEA4 on their erythrocytes (31). Interestingly, it has been reported that women with pp and p^k phenotypes display a high rate of spontaneous early abortion and that abortion in such women is provoked by an immune response to an embryonic antigen; SSEA3 and SSEA4 are clearly obvious targets (32). The observation that about 1% of Caucasians lack expression of SSEA4 may be of more significance (though not SSEA3 or P antigen), which has been suggested equates to the Luke blood antigen. This begs the question of whether women with the Luke (–)/SSEA4(–) phenotype may also be subject to early abortions due to immune responses to early embryonic cells, like pp and p^k women. Clearly, to reconcile differences



	Stage of Development			
	2-8 cell	Morula	ICM	Trophoblast
HUMAN				
SSEA1	−	+	−	+
SSEA3	−	−	++	−
SSEA4	−	−	++	−
TRA-1-60	−	−	+++	−
TRA-1-81	−	−	++	−
CD9	+	+	++	−
MOUSE				
SSEA1	+	+	++	+
SSEA3	+	+	−	−
SSEA4	+	++	−	−
TRA-1-60	*	−	−	−
TRA-1-81	−	−	−	−
CD9	+	+	++	+/−

Figure 2 (*Left*) Comparative expression of some stage-specific antigen markers on human and mouse preimplantation embryos, which are also used to identify pluripotent ESCs and (*right*) a table of the distribution of markers at different stages of development for human and mouse embryos. Localization is quite different for the two species. *Abbreviation*: ESC, embryonic stem cell.

between species it will be important to give more consideration to the stem cell niche since these markers probably interact with the ECM milieu of early implantation. In this connection, it is of note that in a recent study mESCs retained the capacity to differentiate into trophoblast but only when given the correct extracellular signals of a collagen IV matrix (33).

TROPHOBLAST FORMATION FROM hESCs

From the earliest studies of derivation of hESCs and primate ESCs there were indications of spontaneous trophoblast differentiation, as indicated by expression of hCGβ and the occasional formation of multinucleated syncytial cells (34,35). However, more substantial evidence was acquired when methods of directing the generation of trophoblast phenotype from undifferentiated hESCs were developed. One approach is to drive differentiation to trophoblast directly from hESCs in monolayer culture. This can be achieved early on by induction with the inducer growth factor (IGF) bone morphogenic protein 4 (BMP4) (17) or alternatively by knockdown of genes associated with pluripotency such as Oct4 (36,37), Nanog (38), or Sox2 (39) followed by culture in medium without FGF2, which will sustain hESC self-renewal. Hence, it can be clearly demonstrated, using a range of expression markers, that such induction procedures convert hESCs directly to various trophoblast lineages, albeit with a fairly limited proliferative capacity, presumably because these cells rapidly underwent terminal differentiation. Furthermore, only a proportion of the cells in a culture will form trophoblast, thereby making it difficult often to interpret experimental results. Similar to the differentiation of other cell types, the efficiency of generation of trophoblast in vitro seems to vary depending on the hESC line used. Unlike mESCs, which are usually generated from specific inbred strains of mice (e.g., 127 strain), hESCs come from the outbred human

population. It is not known whether the genetic background or even the genetic sex (XX, XY) of a cell line may influence trophoblast formation, although this will be important to determine. Also, factors such as how the hESCs were initially derived and cultured may affect the differentiation capacity of the line. Recently, our laboratory employed controlled gene knockdown by inducible shRNA in hESCs, enabling gene downregulation in a time- and dose-dependent fashion. When this procedure was applied to Oct4 expression of the trophoblast-associated factors Cdx2, hCG and GCM1 increased with expression of Cdx2 and GCM1 at a rate controlled by the degree of Oct4 downregulation induced (Avery et al., unpublished). In the future these systems may facilitate directed differentiation but maintained at a controlled rate that also allows appropriate proliferation of the cell lineage.

Notwithstanding the drawbacks highlighted earlier, an advantage of monolayer culture of hESCs differentiation to trophoblast is the direct investigation of potential signaling pathways and mechanisms of trophoblast specification. In this respect, two-dimensional cultures grown in the presence of growth factors belonging to the BMP family, especially BMP4, seem to be the most effective. Cultures of hESCs supplemented with BMP4 between 10 and 100 ng/mL can lead to the appearance of larger, more flattened cells at the periphery of the colonies (17,40). Waves of differentiation have been described that progress inward toward the middle of hESC colonies at a rate that is apparently dependent on the BMP4 concentration (17). After five days of culture such colonies strongly expressed a range of genes associated with differentiated trophoblast, for example, *CGA*, *CGB*, *MMP9*, *KRT7*, and *IGFBP3*, and when dissociated and plated at a low density in the presence of BMP4, some cells fuse to form multinucleated cells resembling syncytiotrophoblasts (17).

The need for correct BMP4 activation to produce trophoblast has also been demonstrated with hESC clones lacking glycosyl-phosphatidyl-inositol–anchored proteins (GPI-APs) (41). These cells were generated by the induced gene deficiency of PIG-A (phosphatidyl-inositol-glycan class A), required for the first step of GPI synthesis. GPI-AP–deficient hESCs were capable of initiating cell differentiation but remained unable to form trophoblasts after culture supplementation with BMP4. The defect in trophoblast formation was due to the lack of GPI-anchored BMP coreceptors, resulting in the impairment of full BMP4 signaling. Thus, a GPI-AP–enhanced full activation of BMP signaling is necessary for human trophoblast formation.

The action of BMP4 to generate trophoblast is part of a complex signaling pathway operating in hESCs and possibly the blastocyst. A range of membrane receptor pathways maintain hESCs pluripotency (42), but it is becoming clear that Activin/Nodal signaling is a principal route (43), and its inhibition leads to loss of hESC self-renewal and trophoblast differentiation. Both Activin and Nodal belong to the transforming growth factor-β (TGF-β) superfamily that also includes BMP, and the specificity of the various ligands is controlled at multiple levels. Activin, Nodal, as well as TGF-β use one set of receptors (ALK4/5/7) and downstream signal molecules (SMAD2/3), while BMPs such as BMP4 utilize a different set of receptors (ALK1/2/3/6) and activate different SMAD transducers (SMAD1/5/8) and other targets (42,44). The two branches of this TGF-β/BMP signaling complex antagonize each other, because activated SMAD1/5/8 or SMAD2/3 need to compete for the common SMAD4, which is required for the activation of either branch. In the hESC colony, FGF2 upregulates TGF-β production in cells around the periphery, and it has been surmised that growth factor (IGF and others) from these cells creates the pluripotency stem cell niche (45). Trophoblast can be successfully derived by omitting FGF2 from some hESC cultures when 10 ng/mL BMP4 is used. Thus in conclusion, BMP4 activity seems to depend on inhibition of TGF-β/Activin/Nodal signaling, with trophoblast development from hESCs occurring when Activin/Nodal signaling is inhibited. Some of the potential signaling pathways involved in hESC maintenance and trophoblast differentiation are shown in Figure 3.

Of interest here is that rodent pluripotent stem cells can also be derived from the late epiblast layer of postimplantation mouse and rat embryos (46,47). These are called epiblast stem cells (EpiSC). Instead of being dependent on leukemia inhibitory factor (LIF) and BMPs for self-renewal, EpiSCs rely on Activin/Nodal signaling similar to hESCs. Significantly, EpiSCs readily differentiate to trophoblast in the presence of BMP4, suggesting a convergence of essential signaling pathways between these cells and hESCs.

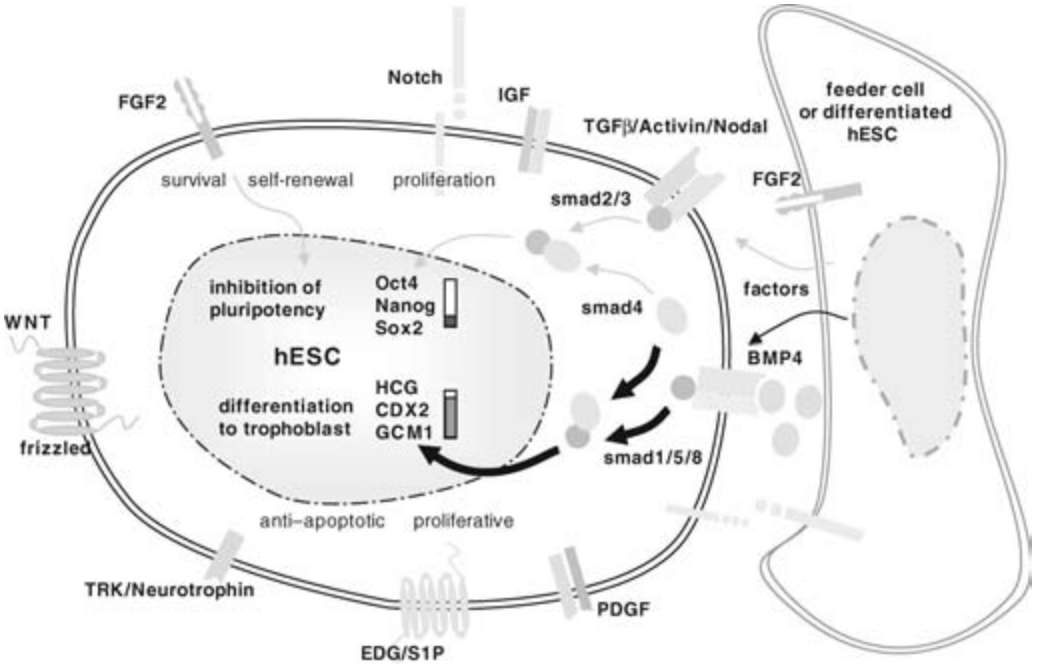


Figure 3 A schematic diagram showing some of the signaling pathways involved in hESC maintenance and differentiation to trophoblast. A plethora of signaling pathways exist in hESCs. BMP4 has been shown to be principal inducer of trophoblast and is part of the TGF-β superfamily, which includes Activin/Nodal. BMP4 uses one set of receptors (ALK1/2/3/6) and signaling SMAD transducers (SMAD1/5/8) and Activin/Nodal use another set of receptors (ALK4/5/7) and SMAD2/3, thereby targeting different genes. BMP4 induction and inhibition of Activin/Nodal signaling result in increased expression of trophoblast-associated genes and decrease in pluripotency gatekeeper genes. Two branches of the TGF-β signaling pathway antagonize each other, because activated SMAD1/5/8 or SMAD2/3 compete for the common SMAD4, which is required for the activation of either branch. Other signaling pathways, particularly FGF2 (bFGF), are involved in hESC survival, self-renewal, and proliferation to various extents. Differentiating hESCs at the periphery of a colony or in an embryoid body may signal through FGF2 to provide paracrine factors to maintain hESC niche in appropriate culture, but on further development may potentiate trophoblast differentiation. *Abbreviations*: hESC, human embryonic stem cell; BMP, bone morphogenic protein; FGF, fibroblast growth factor; WNT, wingless INT; TRK, trophomyocin receptor kinase; EDG/S1P, endothelial differentiation gene/Sphingosine-1-phosphate; PDGF, platelet derived growth factor; IGF, insulin-like growth factor.

BMP4 is clearly important for trophoblast differentiation, yet it is unlikely that this is exclusive and directed entirely toward trophoblast. Indeed BMP4 supplementation after one day leads to upregulation of genes more typical of endoderm and placental yolk sac such as α-fetoprotein (*AFP*), fibrinogen chains (*FGA, FGG*), and apolipoprotein A4 (*APOA4*) (40). Thus, while hESCs may serve as a model for studying extraembryonic tissue development, there is a likelihood that BMP4 treatment will lead to the appearance of more than one lineage of cells. For this reason researchers have also endeavored to devise other methods to generate more long-term proliferation and differentiation of trophoblast.

One method is to produce embryoid bodies (EBs). These are formed from the aggregation of hESCs in suspension, which then start to differentiate somewhat like an unregulated chaotic embryo, often forming a cystic (hollow) structure with multilayers of cells at the periphery. EBs will grow larger if prevented from adherence to a plastic culture surface (usually by culture in a bacteriological plastic petri dish) and are cultured in medium conducive for trophoblast development. The latter can merely lack factors that normally maintain pluripotency (i.e., FGF2, serum replacement medium) or be supplemented with factors that are beneficial for a more directed trophoblast induction (i.e., BMP4, conditioned medium, FGF4, serum). Undergoing spontaneous differentiation, EBs consist of cells of all three embryonic lineages, and a proportion of trophoblast cells are often (but not always) present around the periphery (18,19,48). Thus, compared with a flat monolayer of cells, EBs

have the advantage of developing in three dimensions and may therefore more closely mimic features of the blastocyst and trophectoderm during embryonic development. When EBs are generated in this way, they can be cultured further in various preparations of ECM, or in coculture with other cell types simulating conditions of the embryo implanting into endometrial stroma. These EBs then show trophoblast morphological features such as microvilli projections, very reminiscent of trophoblast villi of early placental development (19,49). Observations of this sort first led to the proposal of the use of hESC-derived EBs as a model system for investigation of human trophoblast differentiation and placental morphogenesis.

However, a significant disadvantage of EB preparations lies in the variability and the complexity in the differentiated cell composition of each EB. In our own laboratory, we have attempted to overcome this problem by purifying TS cells from EBs and thereafter use these cells for study. Trophoblast-containing EBs can be identified at an early stage of culture by measuring hCGβ in secretion. While this is not entirely a definitive marker (it is also secreted by some tumor cells), it is an easily measurable peptide hormone most associated with synctiotrophoblast. EBs were transferred singly to each well of a 96-well culture plate, and after three days of culture, hCGβ was detected in most wells, but only a small proportion (~4%) had concentration of hormone greater than 500 m IU/mL, a relatively high level for a small number of cells. The EBs in these wells were all of equivalent size and morphology to those expressing little hormone, indicating that the increase in production of hCGβ was most likely due to the proportion of trophoblast cells in the EB rather than a greater overall number of cells. Those EBs exhibiting high levels of hCGβ secretion were then subjected to several rounds of selective enrichment by growth in TS medium (conditioned with mouse embryonic fibroblasts and supplemented with FGF4), as described for the generation of murine TS cells by Tanaka and coworkers (28). This involved pooling the highest secreting EBs, carefully disaggregating the cells with a short digestion with a protease (trypsin or accutase), and then further culture for proliferation and formation of EBs, which are analyzed for hCGβ. By undertaking several rounds of this cloning method (Fig. 4), progenitor human trophoblast cell lines have been produced that proliferate for prolonged passage in culture, but with differentiation solely along trophoblast lineage, making them ideal for investigating trophoblast differentiation. The cells display markers of villous and extravillous

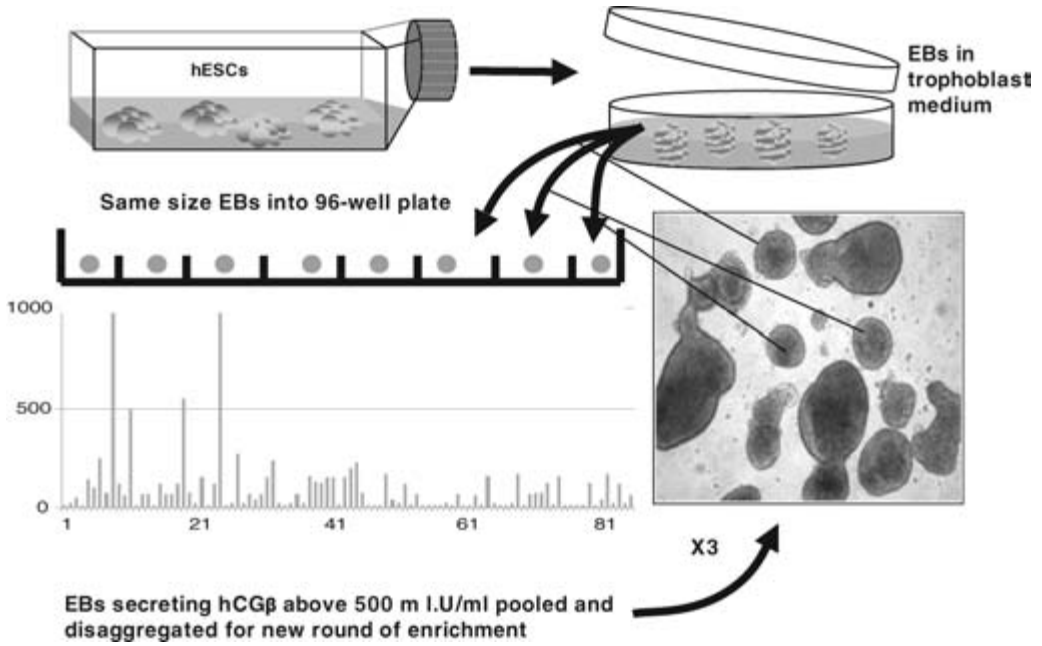


Figure 4 A diagram showing the enrichment process to generate CTBS cell lines from hESCs. This is via EB formation, ELISA measurement of hCGβ from single EBs, and then several rounds of purification. *Abbreviations*: CTBS, cytotrophoblast stem cells; EB, embryoid body.

cytotrophoblast and were termed "cytotrophoblast stem" (CTBS) cells (19). Some cells spontaneously form syncytia and begin to secrete hCG, and if not passaged frequently, terminally differentiate to an endovascular cell phenotype, which in situ is involved in maternal spiral artery remodeling and prevention of immune rejection (10,50).

In the mouse, TS cells can be derived from a cell niche that spans the entire extraembryonic and chorionic ectoderm (51), but it remains unclear whether a similar wide range of trophoblast development supports human CTBS cells. The direct developmental progression in vitro would possibly be by the differentiation of hESCs to the immediate trophectodermal stem cell phenotype of the preimplantation blastocyst and then to CTBS cells. However, the specific route of derivation still requires verification, as initial EB formation may induce various trophoblast cell types from which the cell lines were ultimately selected.

An alternative approach has been to isolate trophoblast cells from EBs based on selection for adhesive characteristic rather than for hCG production (52). Colonies of hESCs are cultured in a semisolid, methylcellulose-containing medium to form EBs adhering to plastic and from which a PECAM-1–positive cell layer can be removed by microdissection. The adherent cells also show markers of extravillous trophoblast with cells at the periphery of outgrowths secreting hCG and staining for the marker GB25, an antibody that recognizes syncytio- and cytotrophoblast. While this method is less laborious than the trophoblast enrichment procedure, the generation of trophoblast starts from hESCs rather than TS progenitor cells each time.

MODELS OF TROPHOBLAST FUNCTION USING hESC DERIVED CELLS

Up to now the simplest approach is to directly investigate the characteristics of differentiated trophoblast from hESCs, although, as mentioned, the presence of other cell types can often confound the results. Isolated TS cell populations therefore offer a better starting source of cells without the need for further isolation (28). For instance, cells generated in our laboratory by clonal enrichment display characteristics of CTBS cells consisting of single mononuclear cells, which do not form colonies. They express trophoblast-related mRNAs for Cdx2, HLA-G, CD9, and Ck7 and the majority of cells (~90%) express HLA-class I histocompatibility antigens (10) consistent with extravillous trophoblast. The expression of HLA-G is relatively weak in most cells, but a small proportion of cells show strong immunoreactivity, with some cells also expressing vimentin, a marker of interstitial cytotrophoblast (12). Following extended culture for one week or more in T25 flasks, the proportion of HLA-G^+ cells increases dramatically (>90%) and exhibits distinct endothelial cell morphology similar to cultures of differentiating cytotrophoblast from first trimester human placental tissue (53). Significantly, these cells coexpressed HLA-G and the platelet endothelial cell adhesion molecule 1 (PECAM-1), both markers of invasive endovascular (endothelial-like) cytotrophoblast (10). VE-cadherin and E-cadherin immunolocalization was weak or absent on endovascular cells but strong on a relatively small proportion (<5%) of multinucleated cells also present at this stage and most likely equivalent to the syncytial giant cells found in stroma of the developing placenta. Importantly, the endovascular trophoblast exhibited PECAM-1 expression, but neither vascular endothelial growth factor receptor 1 (flt-1) nor VE-cadherin were expressed, distinguishing these cells from a true endothelial phenotype. Such cells can be cultured and passaged extensively, suggesting the presence of a stem cell but not a residual hESC population, since cell cultures fail to display the required markers of hESCs and can never be induced to generate hESC colonies under permissive conditions. Other studies have shown that villous and extravillous phenotypes are generated, indicating that hESC-derived trophoblast show extensive differentiation, which can be further investigated (18,40).

Digital time-lapse video-microscopy has been used fruitfully to monitor cell-cell fusion and the formation of nonproliferative, syncytiotrophoblast (19). Previously there has been controversy as to whether syncytium may form from cell fusion or endonuclear duplication. Adherent trophoblast cells on plastic or on ECM such as Matrigel display progressive migration across the culture dish promoted by pseudopodial-like extensions. Cell fusion can be captured unequivocally when cells occasionally converge, with membrane fusion occurring at a single point and cytoplasm then rapidly mingling to form multinuclear syncytiotrophoblast cells, predominantly hCGβ and Ck7 positive but HLA-class 1 negative. When initially

plated out, single CTBS cells first fuse with each other, but this is quite infrequent; however, endonuclear duplication does not occur. Multinucleated cells seem to have a greater capacity for fusion with single cells (and sometimes other syncytium) perhaps because they are a larger target and less mobile. Trophoblast cell fusion was much more common in Matrigel, suggesting that ECM affects their adhesion/fusion properties. Interestingly, CTBS cells at confluence in culture started to exhibit morphology consistent with column formation while in low density the cells differentiated to endovascular cells with a characteristic endothelial-like pattern of cells on the plastic.

Another fairly simple model system is to follow EBs or trophoblast vesicles (derived from CTBS cells) in ECM during culture to mimic early placental formation (18,19,48). In this case, when EBs are transferred to Matrigel drops in a 3D culture, they begin to develop branchlike structures, with columns of cells projecting away from EBs into the surrounding ECM. These cultures can be continued for several weeks, and EBs form cystic structures with widespread differentiation of epithelial cells and the secretion of hCG, progesterone, and estradiol-17β increasing significantly by about 20 days and remaining elevated for a further four to five weeks (18). By comparison EBs maintained in suspension culture failed to demonstrate this elevation in hormone secretion, indicating an instructive role for the ECM in differentiation.

In contrast, we have followed EBs and CTBS vesicles plated onto Matrigel or primary endometrial cell preparation as a model of initial attachment and invasion of trophoblast at implantation (Fig. 5). Trophoblast vesicles cocultured on confluent layers of endometrial stromal and epithelial cells attached to both cell types and displayed erosion and migratory movements similar to that of blastocyst in coculture (19). This invasive capacity was less in

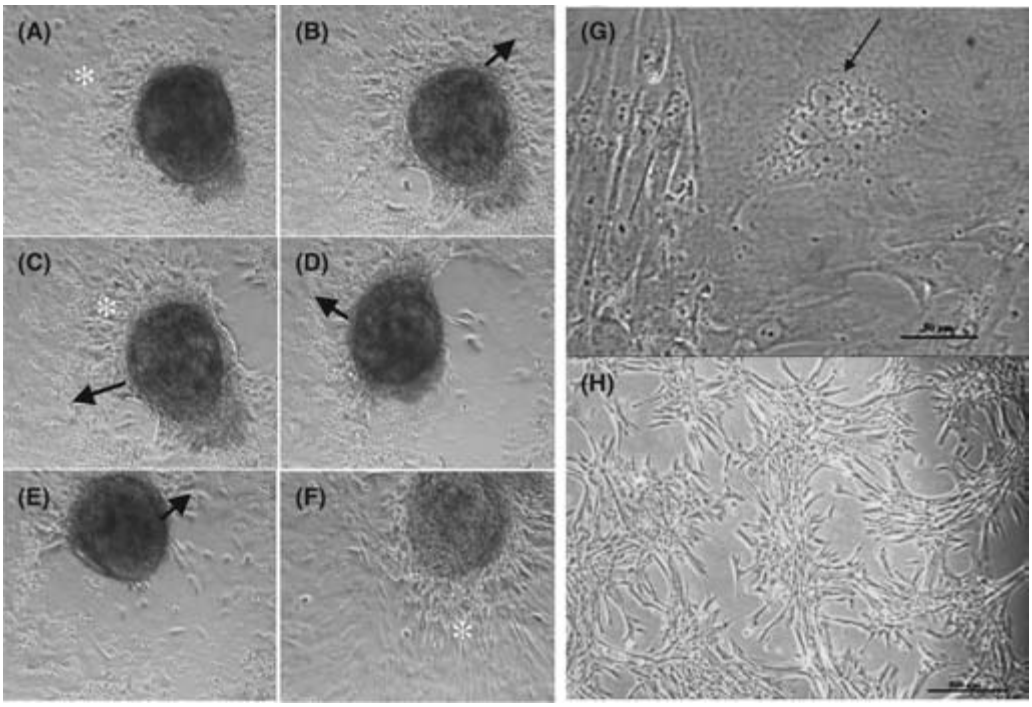


Figure 5 (**A–F**) Trophoblast vesicle (from hESC-derived CTBS cells) cocultured with primary human endometrium and time-lapse micrographs of adhesion and erosion of stroma over an approximately 48-hour period of culture. Trophoblast vesicles migrate around the stroma displaying an invasive behavior. Arrows indicate direction of migration and asterisk extravillous cytotrophoblast cells streaming from the vesicle. Clear areas of stromal erosion occur that correlate with high-matrix metalloprotease, MMP, activity (not shown). (**G**) Multinucleated syncytiotrophoblast cells are also generated; bar = 50 microns. (**H**) After prolonged culture there is differentiation of an endovascular trophoblast cell type; bar = 500 microns. *Abbreviations*: hESC, human embryonic stem cell; CTBS, cytotrophoblast stem cells.

Matrigel alone, although many cells on the periphery of EBs were multinucleated with up to 25 nuclei each. This difference in response of trophoblast between endometrium coculture and Matrigel may reflect maintenance of an invasive cytotrophoblast rather than differentiation to terminal syncytium. Epithelial matrix metalloproteinases, MMP2 and MMP9, enzymes previously suggested to play a role in embryo implantation, were expressed on the surface of CTBS vesicles and the expression and activity correlated with sites of erosion due to dissolution of underlying ECM or stroma. Correlated with EB formation the transcription factor NF-κβ subunit p65 also increases along with the transcriptional coactivator p300 (Kershaw et al., unpublished). This is of interest because exogenous tumor necrosis factor-α (TNF-α) increases invasion of primary cytotrophoblast from eight- to nine-week placenta investigations in first trimester trophoblastic cells, which in turn initiates NF-κβ activation, leading to MMP9 expression (54).

One difficulty of coculture systems is ready identification of origin and type of cell when the morphology of cells is quite similar. One way to overcome this problem is to constitutively express ubiquitously a marker such as enhanced green fluorescent protein, eGFP in an hESC line. When these cells differentiate to trophoblast, they continue to display green fluorescence, making them much easier to track under UV light (19).

In vitro culture systems of hESC-derived trophoblast have generally involved cell culture in 5% to 10% CO_2 in air and therefore in approximately 20% oxygen environment, however, early trophoblast invasion of endometrium occurs in hypoxic conditions (2–4% oxygen). CTBS cells proliferate under low oxygen conditions but differentiate at higher levels, mimicking the developmental transition they undergo as they invade the placental bed to establish the maternal-fetal circulation in vivo (55–57). Hypoxia-inducible factor-1 (HIF-1) is involved in this process to upregulate genes involved in the cellular oxygen deprivation and in CTBS differentiation. Therefore it is also important to develop hypoxic models of hESC-derived trophoblast. Certainly, trophoblast differentiation is accelerated from hESCs by about 24 hours in cultures at 20% oxygen relative to those under 4% oxygen. Moreover, while hCG is secreted abundantly in the medium of hESC cultured in high oxygen, it is almost undetectable when oxygen is 4% (58), perhaps indicating the retention of a CTBS rather than syncytial phenotype. MMP activity in EBs in matrix or stromal coculture is also increased in low oxygen conditions. Hence, it will be important in the future to develop a more complex physiological in vitro model, bringing together the microenvironment, the 3-D culture, and the stage-specific changes to simulate implantation.

Finally, we have noted that CTBS cells derived from hESCs can develop karyotypic mutation and aneuploidy in culture. Since these mutations were not present in the hESC lines or at initial isolation of CTBS cells, it is thought they arise during long-term passage. While cell lines do occasionally undergo adaptation in culture and acquire mutations, this feature might also recapitulate a mechanism occurring in vivo where high rates of aneuploidy exist among the normal human cytotrophoblast subpopulation that exit the cell cycle and invade the uterus (59), and aberrations in chromosome number are a normal part of differentiation rather than an anomaly. It is speculated that the accumulation of chromosome gains and losses could limit the proliferative and invasive potential of cytotrophoblast. In contrast, CTBS cells showing karyotypic mutations in vitro seemed to exhibit decreased proliferative capacity, but their invasive capacity is increased, as is often the case for adapted hESCs or tumor cells. Therefore, it will be important to investigate the mechanism of this process in the CTBS cells in vitro.

SUMMARY

Since hESC lines are the result of an altruistic donation of embryos for research from couples attending infertility clinics, it seems fitting that they are now being used to investigate problems of infertility (60). At present, the derivation of cells of the trophoblast lineage from hESCs is still in its infancy, with various cell types and cell lines being isolated and characterized. However, the initial results show great promise in that the differentiation process in vitro appears to recapitulate some of the early stages of trophoblast development and generates phenotypes with functional capacities, which appear at least comparable to those of first trimester placental tissue. Of course hESC-derived trophoblast studies will not replace the need for investigations with primary tissue, but they provide an experimental

approach, which is becoming more amenable to transgenic manipulation and with appropriate coculture systems provide a window to study human implantation events in vitro.

There are also some caveats. First, it should be emphasized that hESCs do not exist in nature but are an artifact of culture conditions. While they may approximate to the stage of the ICM cells they originate from, they have been adapted to self-renew in a way that does not occur in the blastocyst. Presently, we need to understand more fully this adaptation process and how the hESC niche can be created in vitro to duplicate the ICM/trophectoderm niche to provide a proper starting point to study embryogenesis. Secondly, epigenetic imprinting processes in hESCs can be different to those in the embryo, and we know that imprinting of genes plays a major role in trophoblast control and function (61). In this connection it remains uncertain to date whether trophoblast derived from hESCs can represent the initial trophoectoderm of the blastocyst. Finally, placental development involves waves of trophoblast progenitor cells, and while it is clear that trophoblast cells can differentiate from hESCs, their pathway of development need not necessarily correspond with the progression of differentiation that occurs in situ.

The advent of iPS cells (21,22) generated from human somatic cells such as skin fibroblasts now provides a much more practical and ethical route than somatic nuclear replacement to create cell lines from patients to study many diseases. Trophoblast differentiation occurs in iPS cells, and the efficiency of generating these cells is rapidly improving. Therefore, it should be feasible to create cell lines from infertile couples to assess the paternal and maternal contribution to implantation and placental dysfunction. Also one can speculate that in the future if trophoblast vesicles cultured from cell lines can closely mimic trophectoderm, then such cells may provide a reliable source to rescue embryos with defective trophoectoderm via ICM transplantation. Also by injecting hES cells into trophoblast vesicles it may be possible to create an "ES/trophectoderm embryo model" for experimental use, thereby further overcoming the restriction of obtaining and using human embryos directly for research.

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18 Embryo-Friendly Approaches to Human Embryonic Cell Derivation

Irina Klimanskaya

INTRODUCTION

The debates in the society about the ethics of using leftover IVF embryos for derivation of embryonic stem (ES) cell lines have been going on for years. ES cells, first derived in 1981 by Evans and Kaufman (1), have been studied extensively as a model of mammalian development and differentiation. Isolation of human ES (hES) cells from leftover IVF embryos in 1998 (2) has boosted the research in this field, leading to great hopes for cell therapies. ES cells, the progeny of the inner cell mass (ICM) of a blastocyst, remain pluripotent, maintain normal karyotype through multiple passages in culture, and can differentiate into derivatives of all three germ layers in vitro and in vivo (4,5). Among their differentiation derivatives that can be produced in vitro are such sought-after cells as cardiomyocytes, neurons, oligodendrocytes, retinal pigment epithelium, insulin-producing cells, and others (4,6–11). Such cells and tissues, if robustly produced from ES cells, would satisfy an unmet medical need for tissue and organ repair and could be generated to decrease the risk of immune rejection either through banking a variety of genetically diverse cell lines or via patient-specific technologies.

However, the need to use leftover IVF embryos for generation of ES cells as well as donation of oocytes, which are intended not for fertilization and pregnancy but to produce patient immune-compatible cells for regenerative medicine applications (10,12–14), have somewhat impeded hES cell research. These controversies have resulted in several bills passed by the legislators of different countries that put a number of restrictions on research in this field including limitation on the available government funds along with strict guidelines on oocyte and leftover embryo use (10,15–19).

Finding an alternative strategy to using embryos for sourcing cells and tissues for regenerative medicine applications would add vigor to ES cell research and satisfy its most zealous opponents. While currently most of hES cell lines were derived from IVF blastocysts, several approaches are being developed that could potentially allow creating ES cells without embryo destruction. Although some of these techniques have so far been mere proof of principle, there is a lot of potential that currently depends on the reproducibility and reliability of such technologies. At present, there are following possibilities to generate ES cells without embryo destruction: transdifferentiation or reprogramming (20–30) of somatic cells into another somatic or pluripotent cell type, deriving ES cells from activated oocytes, or parthenotes, which have no potential to develop into whole organisms (31–39), or making ES cells from one or two biopsied blastomeres [the same technique that is widely used for preimplantation diagnostics (PGD) of genetic diseases], which leaves the embryo alive (40–43). Although no naturally fertilized embryos are used for somatic cell nuclear transfer (SCNT), this procedure remains controversial, as an artificially created embryo can possibly develop to term, at least in animal experiments (44). Still, an approach called "altered nuclear transfer" (ANT) has been proposed to create genetically modified nonviable embryos for generation of ES cell (45). In addition to making new ES cell lines, another embryo-friendly way of deriving a variety of somatic cells would be to use existing ES cell lines for research or even therapy (if their safety can be ascertained) or adult stem or germ cells, which can differentiate into a broad range of cell types.

TRANSDIFFERENTIATION OR REPROGRAMMING

The old dogma stating that after a cell undergoes terminal differentiation, it is committed to this particular fate, or, in other words, that terminal differentiation is terminal, is now being reassessed as more and more studies appear that show the possibility of reprogramming (aside

from SCNT) the genome of an adult somatic—or "terminally" differentiated—cell. The prospect of turning one somatic cell type into another, differentiated or pluripotent, is very attractive, as this would allow one to create patient-specific cell types on demand without using embryos to create ES cells and developing technologies to differentiate them. The possible variations of this technology would be either direct transdifferentiation of a somatic cell to another somatic cell or to a pluripotent/multipotent cell that could then be differentiated into the desired cell type. The pioneering works of a Norwegian group led by Philipp Collas (21–23) used the bacterial toxin streptolysin-O to permeabilize the cellular membrane in the absence of calcium so that these permeabilized cells could be loaded with cellular extracts of another cell type. After the procedure, the cellular membrane was resealed in the presence of calcium, and the reprogramming of the recipient cell was achieved by the cytoplasmic "cocktail" of the donor cells. Such experiments produced putative pluripotent cells showing upregulation of pluripotency genes Oct-4, Sox2, Nanog, or Rex1, down-regulation of somatic cell gene expression over time, as well as acquisition of the ability to differentiate along the neural lineage and into adipocytes, osteoblasts, and endothelial cells.

A few studies have been carried out exploring the reprogramming ability of karyoplasts and cytoplasts, as well as whole cells in cell-cell fusion experiments. Tada and coauthors (46) showed that mouse thymocytes fused with ES cells began to express Oct-4 and such hybrids could contribute to all three germ layers in an embryo. Do and Scholer (47) showed the reprogramming ability of karyoplasts and mitomycin C–inactivated ES cells, which induced Oct-4 expression in cells of neurospheres after fusion with them, so the authors concluded that such Oct-4 reprogramming cues reside in the ES cell karyoplast. The main hurdle to using such reprogrammed cells, however, would be their polyploidy, as there is currently no technique to separate the chromosomes of somatic and ES cells. Another report (48) on successful reprogramming of human somatic cells contradicted these studies, as the authors used cytoplasts for reprogramming of the adult somatic cells, which acquired ES colony morphology, expressed Oct-4, and could be passaged. The authors did not, however, provide better characterization of the "cybrids," such as evidence of other markers of pluripotency, retention of such markers over passaging, differentiation, at least, in vitro into derivatives of three germ layers, or statistics on the efficacy of this method. Yet the major hindrance of this approach is the presence of all participants of the procedure in the mixed resulting cultures: ES cells, cybrids, and tetraploid cells coming from fusion with remaining nonenucleated ES cells, so development of a technique for isolation of reprogrammed cells is still in order.

These and other similar experiments have shown that reprogramming of adult somatic cells to the state of pluripotency by cues contained in other pluripotent cells is possible, but remained mostly a proof of principle until 2006, when a real breakthrough in this technology was announced by the work of Yamanaka group, possibly indicating the beginning of a new era in cell therapy. The group demonstrated that somatic cells can be reprogrammed to a pluripotent state by expression of several genes associated with pluripotency (49). Remarkably, this work was independently reproduced by several other groups within the next year, which resulted in developing rather robust procedures of generating "induced pluripotent stem," or iPS, cells from both human and mouse somatic cells (50–62). The transcription factors used in these, Oct-4, c-myc, klf-4, and Lin28, were delivered into the cells via retroviruses or lentiviruses, and the resulting iPS cells appeared similar to ES cells in many ways. They had ES cell morphology, expressed markers of pluripotency, differentiated into the derivatives of all three germ layers, and had normal karyotype.

However, the obvious safety questions were immediately raised because of the use of oncogenes and the need to use retro- or lentiviruses. One of these studies showed that the derivatives of iPS cells formed tumors in mice after transplantation, possibly due to reactivation of c-myc (52). Even if iPS cells could be created without genomic modification, for instance, using protein delivery or RNA transfection, or small molecules activating the target genes (63), it still needs to be determined how stable such transdifferentiated cell lines remain over time, whether they maintain normal karyotype, and how safe they are. Additionally, extensive in vivo studies of their differentiation derivatives would be needed to evaluate their functionality and safety in comparison with ES-derived and in vivo

counterparts. Nevertheless, the potential of this strategy of "turning lead into gold" can be compared to the derivation of ES cells in its possible impact on the regenerative medicine field.

DERIVATION OF ES CELLS FROM GENETICALLY MODIFIED EMBRYOS

SCNT is regarded as a powerful technology for creating patient-specific ES and, subsequently, somatic cells, but this approach is also not free from controversy: no genetically different human being is created, so some see it to sidestep the ethical issues (14); on the other hand, because cloned animals can be healthy and normal (44), opponents of cloning regard it as a new life being created and destroyed. A high-tech approach developed in the laboratory of Dr Rudolf Jaenisch at Massachusetts Institute of Technology (45) tried to address this controversy. The researchers used a combination of SCNT and gene-targeting techniques, which they called ANT, to create mouse blastocysts with a knocked-out cdx2 gene that resulted in abnormal trophoblast, so such embryos would be unable to implant and, therefore, develop. The defective blastocysts, nevertheless, generated pluripotent ES cells, so the authors proposed this technique as a way to produce ES cells without embryo destruction. This approach may need further investigation of human-specific trophoblast genes before this technology could be applied to human embryos. The other concern the authors raise is the use of retroviral vectors, which would bring up safety issues if applied to human embryos. Another rather ethical issue is that to avoid destroying human embryos for ES cell generation, defective human embryos are being created and destroyed, and this could raise concerns among those who see preimplantation embryos as human beings because the boundary between the destruction of nonviable embryos and refusal to support life may be too thin. Therefore, such technologies need to be approached with caution because they may result in creating even more controversy at the least.

ES CELLS VIA PARTHENOGENESIS

One attractive possibility to overcome the problem of immune compatibility is the generation of ES cells and their derivatives from activated nonfertilized oocytes, or parthenotes that would only carry maternal HLA genes and thus allow to reduce the variability and number of lines required for immune matching the patients. Due to genetic imprinting and deficiencies of maternal and paternal haploid gene sets, their combined action is required for normal development of an embryo, so parthenote mammalian embryos are unable to develop to birth. However, pluripotent cells were produced from primate (including human) parthenote embryos and seem to have the same phenotype, behavior, and differentiation potential as "normal" ES cells (31–39). From the ethical viewpoint, parthenote ES cells may be less controversial because no life is destroyed, and such embryos or ES cells can be seen as advanced derivatives of an ovarian teratoma, which is an in vivo analog of a parthenote. While in the recent years more progress has been made generating parthenote ES cell lines from human-activated oocytes, derivatives of such ES cell lines need to be extensively evaluated for safety and functionality in vivo.

PLURIPOTENT CELLS FROM THE ADULT ORGANISM

Embryonic germ (EG) cells derived from the primordial germ ridge could be an alternative to ES cells (64,65) due to their ability to retain pluripotency in culture and differentiate into derivatives of three germ layers, but by the same token—embryonic origin—their use for research or therapy is controversial. However, a group in Germany (66) has isolated multipotent adult germline cells (maGSCs) from mouse testes that meet all the criteria for ES cells, including germline transmission. If such cells were established from human testicular biopsies, they could become an excellent source of patient-specific cells for therapy without all the ethical problems surrounding ES cells. An interesting approach was developed by Primegene, Inc., a California cell therapy company (www.primegenbiotech.com), which claims to have developed a technique to reprogram adult male germ cells into a pluripotent state. Another California company, Moraga (www.moragabiotech.com), has announced that they isolated blastomere-like totipotent stem cells from somatic tissues, which could differentiate into all tissues of the body. If these announcements hold true and the techniques could be

reproducibly applied to isolation of pluripotent human cells, this could be a solution to the ES cell controversy.

SINGLE BLASTOMERE–DERIVED ES CELLS

In our lab we developed an approach to derive ES cells without embryo destruction using the single blastomere biopsy procedure (40,42,43), similar to what is routinely used for PGD. In IVF clinics such procedures have become routine, and one or two blastomeres are removed and analyzed without depriving the embryo of its developmental potential. Such biopsied embryos remain fully viable and have produced many babies (67). Multiple studies exploring the developmental potential of a single blastomere showed that it can contribute to all tissues and organs if aggregated with other blastomeres in a mouse embryo (68,69). However, the recent data have shown that there is possible predisposition of blastomeres to trophoblasts or ICM fate even at the two-cell stage (70–73) that depends on the spatial arrangement and order of their second cleavage divisions; therefore, the potential of blastomeres to form all tissues may depend on them being in the proper environment, that is, surrounded by other blastomeres. In earlier experiments single blastomeres from 129/Sv-*ROSA26:LacZ* mice were aggregated in microdepressions (similar to when making aggregation chimeras) with GFP-labeled mouse ES cells, which seemed to support their division. In a day or two, GFP-negative "buds" were observed on the sides of GFP-positive cell clumps, which were separated from GFP-positive cells under fluorescent microscope (40) either at this stage or later after such mixed clumps were plated on mitomycin C–inactivated mouse embryonic fibroblasts (MEF) and produced a mixed outgrowth. Using mechanical passaging controlled under the fluorescent microscope, GFP-negative cells were selected and mechanically passaged until a pure population of GFP-negative ES cells was obtained. Several ES cell lines were generated that had normal karyotype, expressed Oct-4, Nanog, and SSEA-1, stained positive for alkaline phosphatase activity, and showed germline transmission generating normal mouse pups in the second generation. The origin of these ES cells was confirmed by positive Lac-Z labeling and negative DNA PCR for GFP.

This work was later repeated (41,73,74) by different groups, generating ES cell lines from mouse single blastomeres and even polar bodies using different stage embryos with various efficiencies for different stage blastomeres, ranging from 5% to almost 79%.

Derivation of hES cell lines from single blastomeres appeared more challenging. A group in Singapore published a study (75) reporting unsuccessful attempts to derive hES cells from pairs of human blastomeres, and our own experiments showed that while derivation of such lines was possible, it had very low efficiency (42). In these experiments (42), the blastomeres were cultured for two days in blastocyst medium, which led to formation of "embryonic vesicles"—hollow spheroid structures —which were then plated on feeders in ES cell culture medium in proximity to GFP-labeled ES cells that presumably secreted some critical factors to support the progression of embryonic vesicles to ES cells. In our studies most of the embryonic vesicle outgrowths rapidly differentiated, possibly due to a commitment already made by the cells comprising these vesicles to trophectoderm, and only two lines from single blastomeres were established. It was shown earlier (76–79) that in preimplantation mouse embryos blastomere polarization is involved in specification of the polarized trophectoderm and nonpolarized ICM. Polarization of hES cells in culture can be a later event, determined by the microenvironment and possibly even reflecting a partial loss of ICM features but could be reversed by adding the component of the basement membrane, laminin, to the medium (80). To prevent the polarization of cells in blastomere outgrowths (and subsequent commitment to trophectoderm), laminin was added to the blastomere culture medium, and under these conditions no embryonic vesicles formed. Instead, dividing blastomeres formed cell clumps that were then transferred into microdrops with feeder cells as was previously described (42). The efficiency of hES cell derivation was significantly improved under these conditions, reaching the rate of 20%, comparable with those reported for hES cell derivation from blastocysts (43). In the first series of experiments (42) the embryos were not preserved, so our next goal was to create hES cell lines from single blastomeres without embryo destruction. Only one blastomere from each embryo was used, and the embryos were allowed to develop to

blastocyst stage, at which they were frozen (43), thus for each of the five lines created there is a live frozen blastocyst.

The cell lines established from single blastomeres appeared identical to conventional hES cells by multiple criteria. They had typical hES cell morphology and growth rates, expressed markers of pluripotency Oct-4, Nanog, SSEA-3/4, TRA 1–60/1–81, and alkaline phosphatase, differentiated into the derivatives of all three germ layers in vivo (teratomas in SCID mice) and in vitro, and maintained normal karyotype over multiple passages (43). Among the derivatives of single blastomere hES cell lines were hematopoietic and endothelial cells, and retinal pigment epithelium—cells of potential clinical value.

In conclusion, there are currently two most promising approaches to produce pluripotent cells without embryo destruction: reprogramming of somatic cells to "induced pluripotent cells"—iPS and derivation of hES cells from a single blastomere. Reprogramming of somatic cells to iPS cells has to be achieved without genomic modifications to ensure their safety. While this book was in production, several publications came out describing more safe alternative strategies to produce iPS cells, such as piggyBac transposition or transient transfection with subsequent removal of the reprogramming factors (81,82), using small molecules to complement reprogramming factors (83), or use of recombinant proteins (84,85). Still, comparative assessment of iPS and ES cells as well as of their derivatives is needed, and if a high degree of their similarity is confirmed, certain technologies of hES cell differentiation and isolation of derivatives could be transferred to iPS cells. Meanwhile, derivation of hES cells from a single blastomere is the only technology to date capable of generation of pluripotent hES cells and subsequently derivatives of clinical value without embryo destruction.

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19 Reprogramming of Somatic Cells: Generation of iPS from Adult Cells

Roberto Ensenat-Waser

INTRODUCTION

Definition of Stem Cells

All stem cells share two main properties, first, they can self-renew, that is, they can divide in a nearly unlimited way without loosing their phenotype and characteristics; second, they are capable of exiting their self-renewal and become specific mature cells with a different phenotype in a process called differentiation. This differentiation potential allows stem cells to generate cells from varied cell lineages. Stem cells isolated from different tissues and organs, and even from different developmental stages, show these properties, but differ in the way they express them. Thereby it is possible to isolate stem cells, which have a more limited differentiation potential, only to few cell types (multipotent) or even one or two (uni- and bipotent, respectively) or a broad one giving rise to all cell lineages found in an organism (pluripotent). Only the zygote can be considered a totipotent stem cell, as it is capable of generating all cell types found in the body, including those cells supporting the development of the embryo in the uterus (trophoblast, placenta, etc.) (1).

The degree of potency is generally dependent on the tissue from which the stem cell was isolated. Generally stem cells obtained from fully developed tissues, of newborn or adult organisms, known as somatic stem cells or adult stem cells, have both a limited self-renewal capability and a limited differentiation potential, restricted to the cell types found in the same tissue from which they were isolated, or with luck to some tissues of the same germ layer. Only few exceptional cultures of somatic stem cells have shown a pluripotent differentiation potential (2,3) In contrast, stem cells derived from early embryos [embryonic stem cells (ESCs)] or fetal tumors embryonic carcinoma (EC) are pluripotent and can be maintained in culture nearly forever without loosing their potential, but with the risk of acquiring mutations in their genome due to the long culture (4–6).

Stem Cells in Regenerative and Reproductive Medicine

It is because of their differentiation potential that stem cells are great candidates for their use in medical therapies that require reestablishment of cell populations, affected by degenerative disorders or cells depleted because of their risk for the patient, and could until now only be healed by transplantation of donated organs. As adult stem cells, even though they can be obtained from the patient to be treated (autologous treatment), can be in some cases difficult to isolate and have a limited expansion and differentiation capability, ESCs have become an obvious alternative as a source of cellular material for regenerative therapies. Actually ESCs have been shown to be capable of generating several cell types of clinical interest (7–11) and even germinal cells (12–14) for future use in reproductive medicine as described in previous chapters in this book.

Nevertheless the use of human embryonic stem cells (hESCs) in therapy has nowadays several limitations, coming from varied fields. From the scientific point of view, the main limitation is the fact that the generation of hESCs matching the patient's immunophenotype, by somatic cell nuclear transfer (SCNT, also known as therapeutic cloning), is until now highly inefficient, and even though thought to be possible as demonstrated by experiments in other species (15–18), recent events leave the applicability of SCNT in humans quite under questioning (19). Anyway, the limited number of donated oocytes also restricts this approach. This leaves as the only alternative the establishment of stem cell banks that should try to stock as many hESC lines as possible to match all HLAs (Histocampatibility Large Antigens), which even though being done might become a logistics problem, because of the number of lines

needed. Other limitations for the use of hESCs come from the field of ethics, moral and politics, because of the varied and sensitive thoughts on human embryos, and because of the lack of a common statement on the definition of human embryo itself, each country has developed a different legislation on the use and generation of hESCs, going from quite restrictive laws like in Germany to more open approaches, which might allow a more extensive use, like in Sweden or the United Kingdom. This situation not only affects the generation itself but also the transfer and experimentation of cell lines produced in one country to other countries.

Thereby and taking all together, there is a need to find either an alternative source of immunocompatible pluripotent stem cells, not coming from embryos, or a method to enlarge the differentiation and proliferation capability of adult somatic stem cells toward the cell lineage of interest for therapy. The way to achieve these goals is called reprogramming.

Definition of Reprogramming

During normal embryonic development and tissue regeneration the different stem and progenitor cells get programmed (differentiated) toward a final mature functional cell with a specific and fixed phenotype. On the basis of this normal developmental flow, reprogramming can be defined as the process through which the phenotype of a specific cell is changed to express the phenotype of a completely different cell. Originally reprogramming had been applied to generate cells of interest in research like hybridomas (20), or to study molecular pathways involved in development and embryogenesis.

Nowadays the aim of reprogramming has changed, and since the last few years reprogramming is considered as the process through which a somatic cell, or cell with a limited differentiation potential, is modified to provide it with a pluripotent phenotype, resembling the one of an ESC. In the year 2006, Takahashi and Yamanaka published a new method to obtain pluripotent cells from somatic cells (21). This method consisted in the overexpression of several factors induced by viral infection. The pluripotent cells obtained by this method were named induced pluripotent stem (iPS) cells. Since then many scientists name any somatic cell reprogrammed to a pluripotent phenotype also as iPS.

Reprogramming Approaches

There are different approaches to increase or change the differentiation potential of somatic cells, and they can be arranged on behalf of the proceeding applied in four different groups (Fig. 1): (*i*) nuclear transfer techniques, (*ii*) cell fusion approaches, (*iii*) factor or protein-induced

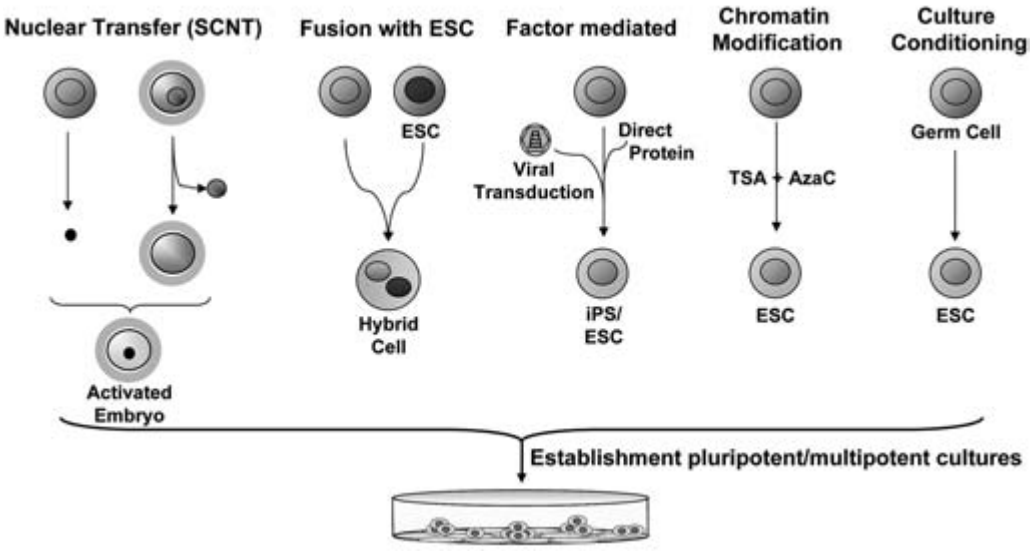

Figure 1 Scheme showing the techniques nowadays available for the reprogramming of somatic cells to obtain pluripotent cells. Some of these approaches can also be modified for the generation of lineage-specific cells.

reprogramming, (*iv*) reprogramming by epigenetic modification, and (*v*) culture-induced reprogramming.

Nuclear Transfer

In SCNT, also know as cloning, the nucleus of a somatic cell is transferred into an enucleated oocyte. After this transfer, the factors included in the ooplasm are capable of modifying the epigenetic state of the chromatin of the somatic nucleus, allowing the expression of genes involved in embryonic development, originally blocked in the somatic cell. This oocyte can then be activated to enter into the normal embryonic development, pass through the different stages, and be capable of giving rise to newborn progeny if implanted in the uterus. This technology has been successfully applied for the generation of cloned animals of several species (16–18,22), and several reports suggest that it works also with human cells, at least until blastocyst stage (23,24). This apparent limitation would not be such in reprogramming purposes, as it might be possible to isolate ESCs from embryos generated by cloning as seen in other species (25), even though it is nowadays not clear how feasible this can be in the human system.

It is also known that the reprogramming capability of oocytes, which resides in the cytoplasm, jumps over the species barrier (26,27) and might open the possibility of using oocytes of animal origin for the generation of hESC lines by SCNT, to overcome the shortage of human-donated oocytes. Such trials have been recently approved in the United Kingdom. Anyway, SCNT and its techniques and characteristics are described with better and deeper detail in other chapters of this book.

Fusion with ESCs

Fusion of different cell types is a technique used since decades to generate hybrid cells, which share the properties of the parental cells fused together. The fusion of the cells takes place through the fusion of the cytoplasmic membranes of the parental cells mediated by an electric impulse [electrofusion (28)] or by polyethylenglycol [PEG (29)], which changes the fluidity of the membranes. Because of this fusion, the cytoplasms of both cells get mixed, and the nuclei coexist in the same hybrid cell. After the first round of division takes place, both nuclei get finally integrated into one tetraploid, and the chromatin gets its final state, allowing the expression of a mixed phenotype, were genes expressed in either parental cell populations keep their balanced expression level (30).

This technique was used to establish the hybridoma lines, where a B cell, which has limited expansion potential, expressing the antibody of interest is fused to a myeloid cell line, to get a cell line expressing the desired antibody (20). The same approach has been used to provide fibroblast with phenotypes of pluripotent cells, like teratocarcinoma cells (31) and ESCs from both mouse and human (28,29,32,33). As a result of the fusion with a pluripotent cell, the fibroblast shows a pluripotent phenotype, being capable of differentiating into all three germ layers including the germ line and giving rise to viable offspring if injected into blastocysts. Thereby, on the basis of previous knowledge it should be possible to get a hybrid cell with the pluripotency of an ESC and the immune properties of the somatic cell, and thereby after differentiation get a specific progeny useful for the treatment of the patient.

As expected, hybrids of ESCs and somatic cells show the pluripotency of the stem cell but few properties of the somatic partner; furthermore recent results claim that there is a complete erasure of the somatic memory, understood as a loss of any leftover of the somatic phenotype, at least in fusions between teratocarcinoma stem cells (P19 and F9) and neural stem cells (34). Thereby it is still unclear whether or not the hybrid cell will have the immunophenotype of the somatic cell of the patient.

Surprisingly and in contrast to what happens in SCNT, the pluripotent-reprogramming activity of ESCs does not reside in the cytoplasm, but in the nuclei of the cell. A recent study showed that after fractionation of an ESC into cytoplasm-containing vesicles, named cytoplasts and karyoplasts, vesicles containing mainly the nucleus and a small cytoplasmic fraction, only the last ones could reprogram somatic cells through PEG-mediated fusion. So it must be the

transcription factors included in the nucleus, which might drive the reprogramming process, at least in such settings (35).

The major limitation to the therapeutic use of fusion hybrids is the risk of using tetraploid cells in therapy, which might increase the tumor potential of cells that because of their pluripotent origin have tumor potential per se. Few reports have described segregation of DNA in hybrids between ESC and somatic cells, but this return to a diploid state mainly took place during the undifferentiated expansion of the reprogrammed cells and generally affected the somatic chromosomes by removing them out, so thereby the chances to get a fully differentiated cell with the HLAs of the donor, and so fully compatible, gets dramatically reduced (36,37). Anyway, further studies need to be performed to fully analyze and understand how reprogramming works and whether or not there is any real leftover of the somatic phenotype.

Reprogramming by Factors and Proteins

In parallel to the reprogramming by fusion with ESCs, the group of Dr Phillipe Collas established a method of reprogramming based on the idea, by that time not yet demonstrated, that the reprogramming of cells was mediated exclusively by proteins. The starting hypothesis was that the phenotype is determined by the proteome of cells, and thereby, if the proteome is transferred to another cell, this last one will reprogram and express the phenotype of the donor cell. To do so, native cell extracts of the cell type to be obtained have to be generated, including all cytoplasmic and nuclear proteins and assuring that they are fully cell and RNA-DNA free, to avoid no carry-over of material, which could lead to a false positive.

In initial experiments, fibroblasts were used as recipient cells to be reprogrammed, because of their phenotype. After permeabilization with Streptolysin O, an agent, which transiently generates pores in the membrane of the cell, the fibroblasts were incubated with native protein extracts of different cell types. After a short period of culture, they could demonstrate that a significant percentage of those fibroblasts had reprogrammed and were expressing the phenotype of the original cell. In this way cells could be reprogrammed into T cells (38), insulin-producing cells (39), or cardiomyocytes (40). This approach has been also successfully used for the differentiation of ESCs into specific cell types like pneumocytes (41).

The obvious next step was to reprogram different human cell lines with extracts from embryonic [mouse D3 ESC (42)] and carcinoma stem cells [human NCCIT (43)]. In the published results they could show that it is possible to induce a genetic and epigenetic change toward a pluripotent phenotype. In such way and after two weeks of culture the original cells acquired (*i*) the colony morphology expected for NCCIT cells and could be expanded; (*ii*) a pluripotent RNA expression profile, with several pluripotency-associated genes (*Nanog, Rex1* and *Stella*) and (*iii*) an epigenetic state resembling that of human carcinoma stem cells, as seen from the methylation status of the *Oct-4* and *Nanog* promoters, and ChIP (chromatin immunoprecipitation) analysis (44,45). Furthermore, the pluripotent cells generated by this kind of reprogramming were induced to differentiate and generate neural and mesoderm (adipocytes, osteoblasts, and endothelial cells) lineages (43). Even recently, mouse fibroblasts reprogrammed with ESC extracts could be used for the generation of neural and cardiac cells and transplanted into mice with either ischemic hindlimb or acute myocardial infarction (AMI) (42). Opening the future to a possible application of extract-reprogrammed somatic cells.

Anyway to achieve such results in future, even more for reproductive medicine, the step of generating human ESC-like cells through this method is still missing, because the differentiation potential of carcinoma stem cells is more limited than that of ESC (46–48), and a full pluripotency is needed to achieve the optimal differentiation. Additionally, two main problems should be solved before thinking in human therapy; on one hand, the limitation of permeabilizing the cells in an effective and reversible way without toxic or pathogen-derived substances and second, the problem of standardizing and validating the cell extracts, that is, which proteins are really needed and which not, also which proteins might be harmful under specific conditions. Thereby it is needed to purify the protocol and define the factors, which are really in charge of the reprogramming, so that the list of proteins gets reduced, and also a method to introduce those factors in the cells.

In the last few years, to solve the problem of bringing in reprogramming factors two main directions have been taken: (*i*) the use of transducible factors and (*ii*) reprogramming by viral transduction.

Factor and Protein Transduction

Protein transduction domains (PTDs) are small peptides (20 amino acids) enriched in basic residues that allow the translocation of the protein, in which they are present, through the cytoplasm membrane. The exact mechanism by which this translocation occurs is still under discussion (49). Once in the cytoplasm, the proteins can exert their original function directly in the cytoplasm or even after migrating into the nucleus. Such kinds of sequences have been found in the Antennapedia (Antp) protein of drosophila, in the transactivation of transcription (TAT) protein of HIV (50) or in VP22 of Herpes simplex virus (51) or even in mammalian proteins like Pdx1 (52). Other viral proteins also include PTDs in their sequences.

The approach in this case would be to modify the amino acid sequence of the transcription factor to include a PTD, generating a stock with all the factors that might be needed to achieve a proper reprogramming. Then a cocktail including the desired factors could be added to the culture media of the cells to be reprogrammed, and it will be just a matter of time to get the results and analyze them. A good thing of this setup is that it is possible to control the concentration of factors added to the medium. Obviously it is not possible to know exactly how much of that is then taken up by the cell, but at least it would be easier to try to get a standardized protocol. Until now such a system has only been used to label cells (53) or to modify ESCs with a transducible Cre recombinase (54,55). Probably in the next few years the sufficient pool of factors will be generated to be able to attempt such an experiment; unfortunately the production of PTD-containing proteins is quite sensitive, as the insertion of the domain might interfere in the function and transducibility, and only optimal selected clones might be used. For a more massive transduction, with a higher amount of proteins, which might take too long for all to be modified, a protein carrier could be used. Such a reagent is being commercialized, and it allows the transduction of complete cell extracts to cell in culture (56,57).

Reprogramming by Viral Infection: Viral iPS Cells

iPS from mouse cells. The problem of identifying the factors contained in the ESC proteome involved in the reprogramming toward a pluripotent phenotype was approached in 2006 in a new and trendy manner by Takahashi and Yamanaka (21). They hypothesized that the factors involved in the pluripotent phenotype should be the ones that were controlling and maintaining ESCs undifferentiated. Thereby they took microarray analyses data and filtered the genes list to get a short list of 24 genes that because of their expression level and significance in ESCs seemed to be relevant. Overexpression constructs were generated for retroviral or lentiviral infection and combinations of each of the 24 factors were used to infect primary mouse embryonic fibroblasts (PMEFs). To assess the reprogramming efficiency, fibroblasts expressing the neomycin resistance gene under the control of the *Fbx15* (a factor expressed exclusively in undifferentiated mouse ESC) promoter were used. These cells allow the identification of pluripotent cells by their resistance to neomycin. Thereby, they were able to assess the reprogramming efficiency based on the number of ESC-like (iPS) neomycin-resistant colonies obtained after infection and 10 to 16 days of culture in the presence of mouse ESC medium with G-418.

By infection with all 24 factors and beginning the selection seven days later, they were able to obtain 22 resistant colonies from which, after additional seven days of culture, 5 were ESC-like colonies. To find the minimal necessary factors, infections were done removing one factor each time from the complete mix. They could then reduce the list to 10 factors by removing 1 of the 24 factors mixed each time, infecting the cells and calculating the reprogramming efficiency. Actually the use of only 10 factors for the infection rendered higher amount of reprogrammed colonies than the 24-factor combination. By analysis of the reprogramming efficiency results, three factors were identified as key for the generation of ESC-like colonies, which are *Oct4, Klf4*, and *Sox2*. Additionally, *c-Myc* was found to increase

the amount of ESC-like reprogrammed colonies and was consequently added to the factor mix. Reduction of this four-factor cocktail to three or less was not successful in generating ESC-like colonies when infecting fibroblasts. Anyway, it has been recently published that infection of neural stem cells (NSCs) with a combination of three or even two factors (*Oct4* and *Klf4*) is enough to render reprogrammed pluripotent colonies, though in a less efficient way.

Analysis of the gene expression profile, methylation status, and differentiation potential demonstrated that iPS cells were pluripotent cells, with a genetic profile by microarray closer to ESCs rather than to PMEFs. iPS cells expressed pluripotency markers like *Oct4*, *Nanog*, *Sox2*, *ESG1*, several members of the *Dppa* family, and *Rex1.* The expression levels of many of those genes were below the level measured in mouse ESC cells, which suggests a limited pluripotency or reprogramming. In addition to this, the methylation profile and ChIP analysis of iPS cells showed that many promoters related to pluripotency in ESCs were still not completely reprogrammed and expressed. Thereby iPS cells in mouse are similar to ESCs but not exactly the same. Anyway, besides those differences, iPS cells are pluripotent as demonstrated by teratoma formation assays after injection in immunosuppressed mice. The in vivo pluripotency could not be fully confirmed as iPS cells injected into blastocysts could generate derivatives from all three germ layers but could not colonize the germ cell compartment, rendering incomplete chimeras.

Several groups hypothesized that the reason behind the limited germ line contribution was that iPS cells had been only selected for their *Fbx15* expression. It is true that *Fbx15* is a pluripotency-related gene, but other genes have a more direct and determining role in ESC pluripotency. Thereby in a second round of experiments, iPS cells were selected on behalf of their *Nanog* or *Oct4* expression by selection constructs expressing eGFP (enhanced Green Fluorescent Protein) and an antibiotic resistance cassette (neomycin or puromycin) under the control of the *Nanog* or *Oct4* promoters. As any of both the transcription factors are genes expressed and determined in fully pluripotent cells, iPS cells derived from fibroblasts expressing these factors should be fully pluripotent and thereby have a better germ line contribution. Reprogrammed iPS cells selected in this way had more pluripotent phenotype as suggested by their contribution in tetraploid aggregation studies (58) and confirmed by their methylation and expression profile, which was much closer to wild-type embryo-derived ESCs. Surprisingly only some of the iPS lines established by this system had germ line contribution after chimera generation, and still the majority of iPS cell lines could not fully colonize the germ line (59,60).

Similar results of reprogramming were achieved if *Sox2*, *Klf4*, and *c-Myc* were substituted by other members of the same family. For example, many iPS-reprogrammed colonies could be obtained and expanded if *Sox2* was replaced by *Sox1* or *Sox3*, *Klf4* by *Klf2*, and *c-Myc* by *n-Myc* or *l-Myc*. Fewer colonies and thereby lower reprogramming efficiencies were measured if *Sox15*, *Klf1*, or *Klf5* were used in the viral infection. In contrast to this, *Oct4* could not be replaced by any of these homologues, and so the reprogramming potential was limited to *Oct4* and not present in either *Oct1* or *Oct6.* Surprisingly *c-Myc* could be completely depleted from the viral mix, without fully impairing the appearance of reprogrammed iPS colonies (61,62). Moreover a recent study demonstrates that mouse somatic stem cells can be reprogrammed using only two factors (63). Later on more will be commented on this study. All those data suggest that the factor mix needed is not only dependent on the specific factor but probably on protein interactions and other stochastic events that might take place as a result of the infection procedure.

To improve the chances of getting iPS cells into therapeutic applications, the selection of the reprogrammed cells should be done without the assistance of any reporter construct. This requires not only a good timing and control of the culture itself (Fig. 2) but also a good knowledge of the morphology and growth characteristics of mouse and human ESCs, to be capable of recognizing the possibly reprogrammed colonies. This is even more important in human cells where transfection with reporter constructs might be more difficult than in the mouse system and also undesired. Different reports have shown that this unassisted selection is feasible (61,64) but might take longer to properly identify the reprogrammed colonies. The efficiency is at least as good as when using reporter constructs because (*i*) cultures are left until

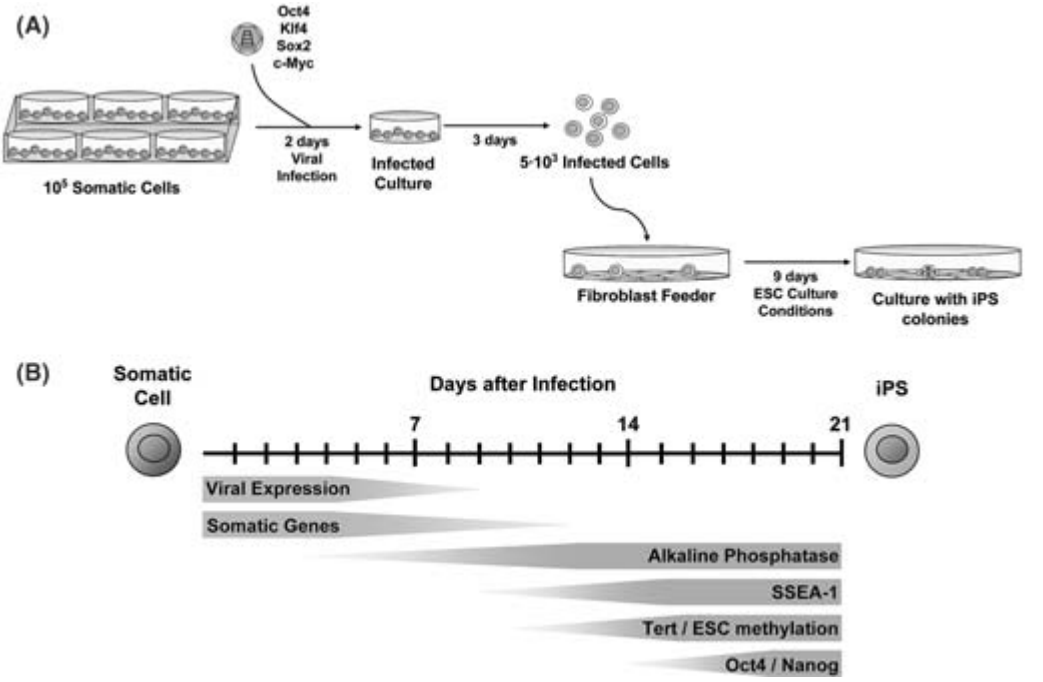

Figure 2 (**A**) Diagram with the experimental setting for the reprogramming of somatic cells by viral infection. (**B**) Scheme showing the gene expression kinetics during the reprogramming process. The minimal days of viral expression required are shown, together with the down-regulation of somatic genes (like *Thy*1), the up-regulation of ESC-specific markers (AP, SSEA-1, and *Tert* and methylation pattern), and finally the establishment of an ESC-like phenotype (*Oct*4 and *Nanog*). *Abbreviations*: ESC, embryonic stem cells; AP, alkaline phosphatase; SSEA, the stage-specific embryonic antigens.

ESC-like colonies arise, which might allow cells to reprogram to a higher extend before getting isolated (eGFP expression might be earlier than the real full reprogramming) and (*ii*) less colonies are analyzed and the work is focused only on those colonies resembling ESCs. iPS cells generated by this method are also pluripotent and can be used in directed differentiation protocols to generate mature cells for regenerative medicine (65). Even though these unmodified iPS cells were pluripotent and could generate teratomas and chimeras, the question about the germ line contribution was so far not fully assessed. An important observation regarding the safety of iPS cells is that with the protocols published until now, a significant number of chimeric mice generated from iPS cells developed tumors after birth. This might be because of a possible residual activity of the reprogramming viruses, but further analyses have to be done to properly assess this point.

So it is clear that even though iPS cells might be potent enough for generating cell types needed in regenerative medicine, such as cardiomyocytes, neurons, hematopoietic cells, etc., therapies or approaches requiring the generation of germinal cells might not find an optimal starting material in these cells, keeping in mind that even ESCs, which are fully pluripotent, are difficult to differentiate into fully functional gametes.

Generating iPS from human cells. After having established the protocol for mouse fibroblasts, from both embryonic and adult (tail tip) origin, the next step was to apply the approach on human fibroblasts. The first group to report the generation of human iPS (hiPS) cells was again the group of Yamanaka (66) closely followed by the group of Daley (67) and Thomson (68). As the first starting material, cell lines of human foreskin fibroblasts were used. All three groups were able to establish stable cultures of hiPS cells but with minor differences in protocol details. As an example, Yamanaka and colleagues used the same factor combination as previously applied for mouse cells (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*). Furthermore, a later

publication by the same group showed that *c-Myc* could be dispensable, like in the mouse system (62). Anyway, the viral overexpression constructs were adapted for the human system, with the only limitation of a reduced infection rat, which dropped from the 50% in mouse fibroblasts to a 20% in human foreskin fibroblasts. Additionally, the kinetics of iPS cells' appearance was much slower, having the first ESC-like colonies after 25 days of culture.

In contrast to this, the group of Daley and others demonstrated that in their hands *Oct4*, *Sox2*, *Klf4*, and *Myc* were not sufficient to induce reprogramming of human fetal and adult fibroblasts. To get the appropriate reprogramming and proliferation, they added the human telomerase (*TERT*) cDNA and the SV40 large T viral protein to the basic factor combination (67). To enhance the survival of single reprogram cells, the ROCK inhibitor Y27632 was added to the culture media. Using the complete mix, they were also able to isolate iPS lines not only from fetal fibroblasts but also from other adult human cells, both fibroblasts and mesenchymal stem cells (MSCs), even though with a lower efficiency.

Finally, Thomson's group developed its own factor mixed for viral infection based on microarray data on the profile of human pluripotent stem cells. In their mix, *Oct4* and *Sox2* were common to the other protocols, but included as new factors were *Nanog* and *Lin28* (68). This combination of transcription factors has been recently retested and improved by the data published by two independent groups (69,70), were a transient expression system was used. Similar to the previous two protocols, this group was able to isolate and expand reprogrammed iPS cells with the same kinetics. All generated lines fulfilled the requirements for a pluripotent human ESC line. Cells were positive for alkaline phosphatase (AP), the stage-specific embryonic antigens (SSEA) 3 and 4, TRA-1-80, and TRA-1-61 and expressed several pluripotency markers in level similar to human ESCs. Additionally, microarray analysis of the undifferentiated hiPS cells and hESCs revealed that the expression profile was similar between both cells types. Even the methylation profile of hiPS cells resembled that of hESCs.

The differentiation potential of hiPS cells was initially assessed in vivo by teratoma formation after injection in immunosuppressed mice (all lines gave rise to teratomas with cells compromising all three germ layers). Unfortunately the definitive experiments to demonstrate pluripotency, tetraploid embryo aggregation, or chimera formation with germ line contribution cannot be performed with human cells because of obvious ethical reasons. Anyway, it has been recently suggested that as an alternative to confirm the full pluripotency of human pluripotent stem cells, those cells should be able to generate haploid functional germ cells after in vitro differentiation. The limitation of this test is that up to now there is no optimal differentiation protocol for human ESCs, capable of inducing both germ line differentiation, full meiosis and, even less, functional gametes.

Starting Cellular Material for Viral Reprogramming

Once clarified that iPS generation from fibroblast of both mouse and human is possible and more or less efficient, the question arises as to which might be the best cell type to be reprogrammed. Two main characteristics have to be taken into account: first, the accessibility of the cellular material; that is, to get to a therapeutic use, cells should be taken from the patient in the as less invasive way as possible. Second, it is important to isolate a cell genetically stable, which means that cells that might accumulate mutations because of their environment or biology should be avoided, otherwise there could be a high risk of generating transformed iPS cells. And finally, to reduce the amount of factors needed or maybe even to accelerate the reprogramming process, a cell phenotypically more flexible should be used.

In relation to these questions, several somatic cell types have been used and tested for their iPS generation potential, for both human and mouse. Initially all fibroblasts used for the iPS reprogramming were either PMEFs or tail tip–isolated fibroblasts in the mouse system (21) or obtained from commercially available cell lines in human [from newborn and adult donors (66)]. The first observation is that embryonic, fetal, or newborn cells render iPS cells with both a slightly higher efficiency and to a higher extend than more mature cells. Even though in mouse cells those differences are not so strong that the factor mix might have to be changed, several reports detail for human cells that even the mix used for reprogramming has to be modified, adding more factors to ensure a successful reprogramming of adult-derived cells like skin dermal or synovial fibroblasts.

Anyway, skin fibroblasts are cells, which are exposed to many environmental factors like chemicals, UV, radiation, etc. Those factors influence not only the growth of the cells, but can also alter their DNA, even giving rise to skin tumors. Thus fibroblasts of the skin of the typical patient requiring regenerative medicine (of middle or old age) might carry many point mutations, which might become a great risk if other potential oncogenes, like *c-Myc*, are introduced when generating the iPS cells. Thereby other sources, involving cells that are better protected from external factors, have been tested, for example, stomach and liver cells (71), *Pdx1* expressing pancreatic cells (72) or even B cells (73). These experiments suggest that all kinds of somatic cells can be reprogrammed by viral infection with an efficiency either similar to or even better than fibroblast cultures and furthermore that even purifying cultures to get more homogeneous populations does not help to increase the efficiency. This is a partial answer to a still present question about whether the low reprogramming efficiency is due to the fact that only a small population of cells among the culture might be susceptible to reprogramming.

In this direction and assuming that only those cells with a higher plasticity or even stem-like cells are the cells that will reprogram under these conditions. Some groups have begun to use adult and somatic stem cells as starting material for the reprogramming. One observation that reinforces this hypothesis is the fact that NSCs and hematopoietic stem cells (HSCs) share not only signaling cascades but also transcription factors with ESCs. On the basis of this observation, such kind of somatic stem cells should be a more suitable and easier reprogrammable material, and in addition to that, somatic stem cells are located in controlled niches where they are optimally protected against any external influence, keeping a highly stable and unmodified genetic identity. Experiments done with mouse NSCs have shown that using these cells in viral infections reduces the time needed to detected and isolate ESC-like colonies to less than one week (in contrast to the 14 days with other cell types). Furthermore, as NSCs express both *Myc* and *Sox2* in levels similar to ESCs, those two factors could be removed from the infection cocktail without impairing the generation of iPS cells (63). These results confirm the hypothesis at least in the mouse system. Unfortunately, in human somatic stem cells this point could not be confirmed, as experiments done with human MSCs showed not only no improvement but a reduction of the reprogramming efficiency, needing additional factors (67). Anyway, this last result might not be in contradiction with the previous results as MSC expression profile and machinery has less overlap with that of ESCs. Other human somatic stem cells might need to be tested, as isolation of some of them might not need an invasive isolation.

In Vitro Differentiation of iPS Cells

The objective of iPS cells is to be an alternative to hESCs in regenerative and reproductive medicine. Their strong point is that they can be generated directly from patient cells, giving rise to immune-compatible cells, nearly an autologous transplant. In addition to that, the process for generating the iPS cells seems to be more efficient than the establishment of patient-specific hESC lines by SCNT. Forgetting any weak point in iPS generation and to fully demonstrate this possible use in therapy, several studies have been performed. Their main objective has been to show that iPS cells can be differentiated in a controlled manner in vitro like ESCs and that their products can be used in animal disease models.

This point has been confirmed for mouse iPS cells by their use in several directed differentiation protocols previously applied in mouse ESCs. Using those protocols, mouse iPS cells were able to generate functional hematopoietic (65,74), cardiac (74), and neural (75) lineages, which could be later transplanted into animal models of sickle anemia or Parkinson's disease (65,75). In the sickle anemia model, tail tip fibroblasts of the mice were isolated, and iPS cells generated from them. Obviously as the iPS cells were carrying a genetic defect causing the disease, gene therapy had to be applied before the directed differentiation into HSCs. The mutation was targeted by homologous recombination and, after that, HSCs were generated from the iPS cells and transplanted into the original donor mouse. These animals recovered all normal blood cell levels and correct hemoglobin proteins (65).

In the Parkinson's disease rat model, mouse fibroblast–derived iPS cells were differentiated into dopamine neurons. Those differentiated cells were transplanted into the

striatum of the rats, and response and evolution of the rat was followed for four weeks. In addition to the improvement of the rat behavior, histological analysis of the graft area showed that implanted neurons could integrate and establish new and long connections with the surrounding tissue (75). Both studies also analyzed the tumor potential of the iPS-derived differentiated progeny. IPS-derived HSCs did not show any tumor formation, at least up to 12 weeks after transplantation. IPS-derived neurons showed clear tumors even after four weeks of transplantation. The authors suggest that the tumor formation is not due to the viral transgenes, as no expression of those could be detected in the tumors. They rather claim that this is a result of residual undifferentiated iPS cells, which might then spontaneously differentiate in vivo. This hypothesis is supported by the fact that neural cells depleted of *Nanog* or SSEA-1-positive cells did not form tumors at least up to 12 weeks after transplantation. Anyway, tumor formation studies should be carried for a longer period of time as the therapeutic objective is a transplanted tissue, which should last for at least one year in the body, and previously published studies demonstrated that residual undifferentiated or transformed cells, even at low number need up to three months to generate a recognizable tumor (76).

In the human system there are no published records about transplantation of differentiated cells into wild-type animals or disease models. hiPS cells have been spontaneously differentiated in embryoid body cultures and showed generation of cells from different germ layers, including beating structures, cartilage, and neural and gut structures (66–68,77). Until now only HSC-like cells have been generated from hiPS cells, rendering cultures of myeloid and erythroid colonies (67). Also trophoblast has been generated under BMP-4 culture conditions (69). But as none of those differentiated cultures has ever been transplanted, the tumor formation risk has so far not been assessed.

As summary of these results, iPS cells seem to be a good substitute of ESCs in regenerative and reproductive medicine, as long as all the limitations in their generation and differentiation have been overcome.

Mechanism of Viral iPS Generation

Viral infections, the effect of the viral integration in the neighboring genes in the DNA and how the factors interact to activate and fulfill the reprogramming program, are black boxes, which limit the understanding of the iPS establishment and so the possibility to improve the available protocols. Because of this, several groups are putting emphasis in dissecting the pathways and steps taking place in this phenomenon, by microarray, ChIP, and chromatin analysis. Unluckily, the fact that reprogramming takes place only in few cells and that the cells which get reprogrammed, are still unknown limits mechanist studies to a few time points or populations to be compared. Those are the total initial unmodified population and the single isolated and expanded iPS colony. Taking into account that each iPS line has its specific origin, even coming from the same starting population, studies to get a general idea of the processes should take many lines at a time.

Until now the only studies done were those where iPS cells were compared with the original somatic population and to undifferentiated ESCs. Comparing these populations, several conclusions could be taken. First, that the reprogramming involves a complete reorganization of the chromatin structure and state. This includes complete demethylation of the promoters of genes involved in pluripotency like *Oct4* and *Nanog*, mediated by DNA methyltransferases (Dnmt1 mainly) and the methylation of lineage-specific gene promoter by Dnmt3l (de novo methyl transferase). The restructuring takes place also at the histone level with dramatic changes in the acetylation and methylation pattern of the histones. Also the bivalent chromatin domains get modified into an ESC-like state (59). Second, the over-expression of the different viral constructs is needed only at initial steps of reprogramming. Actually the viral transgenes get inactivated through the process of reprogramming by methylation, similar to what happens with many viral constructs in ESCs. So thereby if the endogenous genes do not get reactivated and express the required factors, the reprogramming will never take place. In fact, this might be the reason many iPS colonies do not persist in culture, or even though looking like ESCs, they do not reprogram to the pluripotent phenotype. And third, there might be a precise timing for the whole process, as it has been

shown that the time point were the cell selection begins clearly influences the success in the isolation of reprogrammed pluripotent cells.

To overcome the limitation of analyzing only initial and final steps in the iPS viral reprogramming and to minimize the negative effect of not knowing the cells, which cells might be infected, recently a system with inducible constructs was established (78,79). By this system, fibroblasts were infected with the four factors' expression vectors controlled by doxycycline (Dox), which led to an overexpression of all four factors when Dox was present in the media. iPS cells were generated, and single iPS lines were used to generate chimeric and, later, homozygous transgenic mice, carrying the four factors' inducible system in all its cells.

The benefit of this approach is that the viral infection is not further needed and that all cells have the same integration sites and number of copies as the original iPS cell line used, reducing the cell to cell differences and rendering a population of cells where all have the same chances to be reprogrammed and also in a synchronously way. This enhances the statistical value of the comparison analysis. Obviously the limitation of this system is that it represents only one integration site combination, as the transgenic mouse was generated from only one mouse, and so analysis of several strains of these iPS-derived mice might be needed.

Nevertheless, using those mouse fibroblasts carrying the inducible constructs, this group was able to precisely follow some of the events taking place during reprogramming, as they could isolate cells, which were at different stages of the process, just by depleting Dox. The first result coming out of this system is that there is a minimal overexpression time needed. There has to be at least nine days of factor overexpression to achieve a proper reprogramming. Once that time has been reached, further overexpression does not improve the final reprogramming yield, as reprogrammed iPS cells will appear always at day 16 after Dox activation (78). This time sequence has been also tested in other somatic cells expressing the reprogramming factors with the inducible system, like pancreatic β-cells (72), and B-cells (73). In the very first days (after 3 days) after induction of the expression of the viral constructs, somatic lineage specific markers get downregulated, whereas AP (marker also of pluripotent cells) gets upregulated by the same time. AP expression reaches a maximal peak of 80% od AP-positive cells in the culture by day 25 after the induction. Five to six days later (by day 8 after induction), 5% of the induced cells become also SSEA-1 positive. The percentage of SSEA-1-positive cells increases up to 12% of the total population by day 14 after infection and then gets stabilized. Finally, if the culture is kept, some of the SSEA-1-positive cells will also express *Oct4* and *Nanog* and will generate ESC-like colonies and an iPS cell line (Fig. 2). Several studies have shown that only the SSEA-1-positive population is capable of generating iPS cells, clearly defining the steps followed by the cells from the somatic compartment up to the pluripotent iPS compartment, at least in the mouse model (78,79).

Using these markers and stopping the induction at the appropriate days, it is possible to isolate by cell sorter cellular populations belonging to those different stages. RNA and chromatin can then be isolated, and the different stages of reprogramming compared by microarray and other molecular biology techniques (80). Microarray data showed that there was a gradient of gene expression from the somatic cells ending at the iPS cells. In the intermediate stages of reprogramming, genes specific for somatic cells, iPS cells, and ESCs were expressed with an intermediate level or completely absent. In contrast to this, there was a cluster of genes, which was downregulated in somatic cells, iPS cells, and ESCs, but was clearly upregulated in partially reprogrammed cells. These represent genes probably involved in the reprogramming process but not with pluripotency itself. In concordance with these results by microarray, data coming from promoter methylation analysis and histone methylation studies confirmed that partially reprogrammed somatic cells had an epigenetic profile, which was between the one of somatic cells and those seen in both ESCs and iPS cells.

Interestingly, in fully reprogrammed iPS cells, the methylation analysis of Histone 3 in the *Ddx*4 (*Mvh* or *Vasa*) promoter region showed that this histone had a high methylation degree on Lysine 27, which induces a clear inhibition of the gene expression. In contrast, not reprogrammed fibroblasts, which do not express *Ddx4*, did not show this kind of histone methylation pattern. At a DNA methylation level, iPS cells showed a clear unmethylated pattern for those genes involved in pluripotency, like *Oct4*, *Dppa5*, *Rex1*, or *Nanog* nut and also

in some germ line–related genes like *Gdf3*; other germ line–specific genes like *Stella* or *Cyct* showed a complete methylated pattern. On the basis of these results, it is possible to conclude that iPS cells have an epigenetic restriction for germ line markers, which might be responsible for the low germ line contribution in chimeric mice and the probably limited germ line differentiation potential of these cells. This phenomenon could be a major impediment for the use of iPS cells on reproductive medicine.

Technical Limitations

Besides the obvious decision about which cell type might be the best one to be reprogrammed (more critical if older patients have to be treated) and about which factor combination to use to get the best reprogramming, there are several technical limitations to be considered to launch the large-scale viral iPS generation needed for a therapeutic use.

The first limitation is the use of virus itself. Technically iPS generation by viral transduction is a very inefficient reprogramming system. It has been published that efficiency is not higher than 0.16% of the properly infected population, but might be even lower depending on the way it is calculated. One of the main limitations for the efficient reprogramming by viral transfection is the efficiency of infection itself. Even though it has been described that fibroblasts can be infected with a success rate of nearly 80% (72), the infection rate in other somatic cells might lie below 50%. Taking into account that there has to be a simultaneous infection with at least four different viruses at the same time, the maximal theoretic infection rate could be 6.25%. That means that only 6.25% of all cells might have all overexpression constructs at the same time. It has to be clarified that the efficiency of the reprogramming is normally assessed using only the percentage of cells infected with all four viruses as starting population. As a result of that, the described 0.16% of reprogramming rate on the successfully infected cells becomes a real reprogramming rate of less than the 0.01% of the initial total cell population. This limited infection efficiency is even of higher importance in the human system, as human cells were infected only with 20% efficiency in contrast to the 50% ~80% of mouse cells. Together with the observation that human cells might need two extra factors leaves the infection efficiency of all factors together at around 0.0064%.

The second factor to be considered, besides the infection itself, is the amount of active transgene copies of each factor needed to get a reprogramming effect. This is still an unclear point, as the different publications and unpublished data show that at least one copy of each construct is needed, but normally more than three are randomly integrated into the cell genome (58). Real-time polymerase chain reaction (PCR) data can give an approximate idea of how much mRNA (messenger ribonucleic acid) of the exogenous factors might be needed. Unfortunately this kind of measurements does not directly correlate with the final real protein amount of each transcription factor, and it is normally being used as relative quantification using ESCs as reference. Until now there has been no report measuring the amount of each factor's mRNA needed to get the reprogramming event. Such a data would be very helpful to look for and apply alternative strategies to get reprogrammed cells.

A third limitation comes from the fact that, even with the help of SSEA-1 selection to select the intermediately reprogrammed cells (a setting that has not been tested in human cells yet), many iPS colonies might have to be analyzed to find those with the better differentiation potential. Furthermore, if iPS cells have a restriction toward germ cell commitment, generation of gametes or other cell types of the germ line might require additional modification or treatment of the selected iPS lines, to open their potential. Such treatments or modification might negatively influence the quality of the cellular product. The last consideration is related to the previous one, assuming that iPS cells have no risk of reactivating the exogenous viral constructs and that the tumor risk depends only on residual undifferentiated iPS cells, it will be important not only to have an efficient iPS generation system with human cells but also a system to discard all the intermediately reprogrammed and undifferentiated iPS cells remaining after differentiation. Similar to the mouse model, surface molecules specific of hESCs could be used, but it still remains a question whether depleting cells will fully remove the tumor potential of iPS-derived differentiated cells.

Medical Limitations of Viral iPS

In addition to the more technical limitations and the critical points of view inherent to the medical use of human pluripotent stem cells, there are some more medical-oriented questions, which might limit the therapeutic use of viral iPS cells. The first and main one is the use of viruses for cell modification, and even more using oncogenes (*Myc*) to induce pluripotency. Biological safety rules similar to those applied for gene therapy might be needed to ensure that the integration sites did not transform the cells during the generation or after differentiation of the iPS cells. Furthermore, it will be important to check whether the functionality of the derived differentiated cells equals that of tissue-derived cells. From a practical point of view, if a quarantine period is required by drug safety organizations like FDA, the treatment of a patient might be critically delayed. This also relates to the question of what happens if the patient carrying a genetic disease would have to be compensated. Reports showed that it is possible in the mouse system, once the iPS cells have been generated, to reestablish a gene by homologous recombination, but for that extra viruses will be needed, increasing again the risk of malignization.

Another big limitation from a more reproductive point of view points to the limited germ line contribution of those cells. It has been already shown that mouse viral iPS cells have limited germ line contribution based on an impaired epigenetic status of germ line–related genes. Taking into account that hiPS could not be tested to this extend and that hESC *per se* are more difficult to differentiate into germ cells, it is logical to think that probably hiPS might have an even more limited potential in that direction, dramatically reducing the possible future appliance in reproductive medicine.

Thereby other approaches have to be studied and tested to find a proper way of reprogramming somatic cells. In this direction recently two reports showed the improvement of the reprogramming efficiency of viral infection by the addition of chemical compounds during the iPS generation, mainly signaling cascade inhibitors (81) and demethylating agents (80). Unfortunately none of these studies went further away and tried to substitute the viral infection by other reprogramming systems, but at least they demonstrate that other alternatives are still open.

Reprogramming by Epigenetic Modification

The phenotype of any cell type is defined by its gene and protein expression profile. This gene repertoire is fine-tuned by transcription factor but initially controlled by the status of the chromatin. The status of the chromatin is controlled by the chemical status of the histones, which can be methylated, phosphorylated, and acetylated, and the DNA itself, which can be methylated. In summary, gene expression is inhibited when histones are hypoacetylated and DNA hypermethylated, and as a result, the chromatin is condensed. Thereby to achieve a significant gene expression, the chromatin structure has to be opened so that histones get hyperacetylated and loose their connection with the DNA, and the DNA itself gets hypomethylated, allowing enzymes and factors to bind. This is a reversible process in vivo, and it is controlled by two sets of enzymes, on the histone side by histone acetyltransferases (HAT) and deacetylases (HDAC) and at a DNA level by DNA methyltransferases (DNMT).

If gene expression is controlled by these chromatin-modifying enzymes, any change or influence on their activity should result in the random activation or inhibition of genes. Thereby agents inhibiting HDACs and DNMTs, like Trichostatin A (TSA) and 5-Aza-cytidine (AzaC), respectively, will render a chromatin in a artificially open state, allowing the reexpression of genes, which were normally silenced (82,83). This approach has been used for the inhibition of tumor cells in clinical trials (84,85) and for the differentiation of pluripotent cells (86), as genes involved in commitment and growth arrest could be upregulated. On the basis of this idea and knowing that some somatic stem cells (NSCs and HSCs) have a common expression profile with pluripotent stem cells, the use of these chemicals might allow inducing expression of pluripotency-related gene and so enlarge the differentiation potential of tissue-specific stem cells.

Such an approach was successfully applied for enlarging the differentiation potential of HSCs in vitro (87,88). These studies showed that TSA/AzaC treatment on cultured or cord

blood–isolated HSCs, even though restricting the growth rate, rendered homogenous cultures of highly undifferentiated HSCs, which could, after transplantation into irradiated mice, fully restore the bone marrow. Stimulated by these results, the group of Müller treated neurospheres (which contain NSCs) derived from mice with TSA/AzaC. After treatment, neurospheres were transplanted into irradiated mice and were able to repopulate the bone marrow and reconstitute the blood system, to such a degree that it was even transplantable to secondary recipients. Thereby NSCs from the neurospheres gained hematopoietic potential. A deeper look into the genetic profile during reprogramming of neurospheres by TSA/AzaC revealed that several pluripotency genes (*Oct4*, *Nanog*, and *Dppa* family members, etc.) had been transiently activated and that even germ line–specific genes like *Mvh* (*Vasa or Ddx4*) and *Stella* (*Dppa3*) had been also reactivated, whereas neural lineage-specific genes were dramatically down regulated (89). The authors hypothesized that TSA/AzaC removes the lineage-specific restriction, rendering cells, which might not be fully pluripotent but at least could move their differentiation potential between different somatic stem cells. In this case, HSCs, germ cells [primordial germ cells (PGCs)], and NSCs, through this system and in the appropriate environment, generate cells from germ layers different from the one of its own origin.

The limitations of this system are mainly the amount of cells needed for the reprogramming, as chromatin-modifying agents induce a lot of apoptosis, which implies the use of a big initial cell population and that probably the epigenetic modification itself might not be sufficient to induce the phenotype change required for generating pluripotent or germ stem cells.

Culture-Mediated Reprogramming

Last but not least and related to the last comment about the influence of the environment, several groups have been trying to expand the differentiation potential of somatic stem cells by changing the culture conditions. Previous studies had shown that under specific conditions and in the presence of actively diving cells, non-replicating cells gained proliferation capability (90). Thereby it was hypothesized that cells with a genetic profile similar to pluripotent cells but without pluripotency might be under controlled culture conditions modified to become pluripotent cells.

Thereby several groups isolated PGCs and more developed germ cells from fetal and adult tissues and cultured them in ESC-like condition to see whether they gained pluripotency. In 2004 it was published that germ line progenitor from mouse fetal testis could be expanded in vitro, which became ESC-like cells, showing all pluripotency markers, sharing a similar methylation profile, and giving rise to teratomas after injection in severe combined immunodeficiency (SCID) mice and generating chimeras with germ line contribution. Furthermore those cells could be differentiated like ESCs and generated in vitro representatives of all three germ layers (91). Later studies demonstrated that germ cells from newborn and adult testis could also be reprogrammed into ESC-like cells and could describe markers of those cells able to suffer this transformation (92–94). All such cells could show in vitro differentiation potential and could generate both teratomas and chimeric mice. Unfortunately, these last studies could not show if these pluripotent cells could generate viable chimeras after tetraploid embryo aggregation. Gene expression analysis of human germ cells show that they also share many pluripotency markers like what happens in mouse testis (95). On the basis of the positive results in the mouse testis reprogramming and the genetic profile in human germ cells, several groups are trying to establish pluripotent stem cell cultures from human testicle biopsies. So far, even data have been presented in international meetings, there is no published report describing this kind of culture.

Nevertheless it is unclear what would be the use of this kind of cells in therapy, because first, they could be only obtained from male patients (testicle derived); second, until now, unpublished data, suggest that big pieces of tissue are needed for the isolation of a few number of expanding pluripotent cells; and finally, this kind of system looks more like a controlled transformation. So it would important to assess the risk of further changes that might spontaneously occur in the culture. Anyway, getting cells from testis might not have any sense

at all in a reproductive medicine setting. It may be useful just to generate germ cells where a genetic disease might be compensated.

CONCLUSIONS

Even though many different systems are nowadays available to generate cells with an enlarged differentiation potential, or even to guide the reprogramming directly toward the desired cell type, further work has to be done, linking the knowledge and needs of the different fields involved to get a proper final protocol satisfying all needs.

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20 Somatic Nuclear Transfer to In Vitro–Matured Human Germinal Vesicle Oocytes

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INTRODUCTION

Somatic cell nuclear transfer (SCNT) involves the transfer of somatic cell nuclei into enucleated oocytes. In almost all species, preimplantation development is successful after SCNT, giving blastocyst formation percentages almost comparable to in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) embryos (reviewed in Ref. 1). Concomitantly, the different steps in the SCNT process (e.g., cell cycle stage and type of recipient oocyte and donor cell, method used for nuclear transfer (NT) and artificial activation, culture environment) are well described and optimized in several animal models like the mouse, but not in human (for reviews see Refs. 1–4). Embryonic development after SCNT in nonhuman primates has been shown to be compromised (5–8), similarly as in human (reviewed in Ref. 9). Moreover, attempts at reproductive cloning in the nonhuman primate have resulted in little success, which has been attributed to removal of important microtubule proteins during enucleation of the recipient oocytes (6,7,10).

Because human in vivo–matured oocytes are scarcely available, alternative and more abundant sources, such as in vitro–matured germinal vesicle (GV) or metaphase I (MI) oocytes, failed, and abnormally fertilized oocytes are also being used to verify whether these oocytes support preimplantation development of human SCNT embryos (11–15). Working with these oocytes of inferior quality will require an efficient or adapted technology of human SCNT since current experiments have demonstrated that these oocytes do not support well embryonic development after SCNT (11–15). Still recent reports in mice have shown that by adaptation of the SCNT process, these oocytes might be used for SCNT (16,17).

The long-term objective of SCNT involves the creation of human embryonic stem cell (hESC) lines for future stem cell therapies, also called therapeutic cloning (reviewed in Ref. 18). The main advantage of using this technology would be that the derived stem cell lines can be made patient specific, avoiding the need for life-long immunosuppressive drugs. With respect to infertility, landmark investigations have demonstrated that mouse embryonic stem cell (ESC) can differentiate to primordial germ cells and subsequently form early gametes and blastocysts (19–21). Recently, ESC-derived sperm cells have even generated live offspring (22). Preliminary data indicate that hESC most likely display a similar developmental capacity (23–25), and a few progenitor germ cells could be observed in differentiating hESC.

Using SCNT technology for human reproductive purposes (reproductive cloning) should be totally banned at this moment, since the technology of animal cloning to date has continued to show problems during pregnancy, fetal abnormalities, and early death of newborn animals (reviewed in Ref. 26). This makes it quite clear that for the foreseeable future it would be foolhardy to attempt cloning human beings, even apart from the many very strong ethical objections. Therefore, the hoax reports of human reproductive cloning attempts must end because it triangles the therapeutic purpose from the reproductive.

Until now, no hESC lines have been established from SCNT embryos, mainly due to the current low efficiency of preimplantation development after SCNT and the difficulties in procurement of human oocytes for this type of research due to ethical and regulatory guidelines. Though the recent breakthrough of the successful establishment of primate ESC from SCNT embryos is encouraging, all the more this success has been attributed to a new technological optimization of the SCNT process, namely, the noninvasive removal of the chromosomes of the recipient oocytes (see sect. "Enucleation Methods and Possible Consequences") (10). Some technical breakthroughs seem requested to establish hESC lines

from SCNT embryos. The optimization of parthenogenetic human embryo development derived from in vivo– or in vitro–matured oocytes demands further attention and optimization since they form the most strict control group of human-reconstructed SCNT embryos and would also provide patient-specific ESC (reviewed in Ref. 27).

STATE OF THE ART OF HUMAN SOMATIC CELL NUCLEAR TRANSFER

In recent years, the number of reports about human SCNT has only scantily increased (Table 1). Cibelli et al. were the first to declare that they had produced human SCNT embryos using donated in vivo–matured oocytes and cumulus cells and fibroblasts as donor somatic cells, although embryonic developmental potential was limited to early stages (28). The first successful production of human blastocysts was reported by Lu and colleagues using a reverse-order SCNT technology (29). This rather unknown report described a new method of enucleation (see sect. "Enucleation Methods and Possible Consequences") and a blastocyst formation rate of 7% using fetal fibroblasts and cumulus cells as donor nuclei and in vivo–matured oocytes donated by patients undergoing fertility treatment. A major shortcoming in that report was the lack of any molecular proof of the clonal origin of the obtained SCNT blastocysts.

In 2004 and 2005, the Korean group under Hwang falsely reported the derivation of pluripotent patient-specific ESC lines from cloned human blastocysts. A final report from the investigation committee of the Seoul National University concluded that these authors of two papers published in *Science* had engaged in research misconduct and that the papers contained fabricated data (30). Therefore, the editors of *Science* retracted these two papers and advised the scientific community that the results reported in them are deemed to be invalid (31). Stojkovic et al. claimed to be the first group that succeeded in the creation of a human-cloned blastocyst in 2005, though undifferentiated hESC were used rather than an adult somatic cell as donor cell (12). Still, this report showed the feasibility of human oocytes to support development of reconstructed nuclear transfer embryos to the blastocyst stage and emphasized that the time from oocyte collection to NT seemed to be crucial as only one blastocyst was obtained when the recipient in vivo–matured oocytes were enucleated within one hour after collection. Another report described the use of failed fertilized human oocytes 20 to 22 hours after IVF as recipients for SCNT experiments (11). Developmental potential of the SCNT-reconstructed embryos was severely compromised, with only one embryo reaching the six-cell stage, which could be attributed to extensive chromosomal abnormalities as determined by fluorescent in situ hybridization (FISH) analysis.

Regretfully, Zavos and Illmensee reported in 2006 the creation of a four-cell SCNT embryo that was transferred back to the patient (32). The success of this report is dubious as blind enucleation was performed to remove the metaphase–spindle complex of the recipient oocytes, and fortunately no pregnancy was established. Another ambiguous article published in *Transplantation Proceedings* the same year, a rather unknown journal in the field of SCNT and ESC, described the successful production and transplantation of hESC derived from SCNT embryos (33). No data on the number of used recipient oocytes and subsequent SCNT embryonic development were given, nor did the authors report how they successfully enucleated the recipient oocytes giving rise to one blastocyst at day 6. The inner cell mass (ICM) cells of this blastocyst were further cocultured with donor bone marrow cells for only 48 hours and infused into the periphery of the recipient. This reported short-time interval of obtaining putative hESC and the above-mentioned major shortcomings make this article not to be considered seriously from a scientific point of view and too premature from an ethical point of view, since the risks of teratoma formation after transplantation of hESC into severe combined immunodeficiency (SCID) mice is well described.

In 2007, two similar articles reported on the use of failed fertilized oocytes versus in vivo– and in vitro–matured oocytes as recipients in human SCNT (13,14). The first study demonstrated cleavage failure following SCNT, with failed fertilized oocytes collected 48 hours after insemination and fibroblast-like cells as donor nuclei. Although cleavage occurred following SCNT using in vivo–matured oocyte recipients, subsequent embryonic development was severely compromised, with no embryonic development observed beyond the six-cell stage. Immunocytochemical analysis further revealed that many of the aged oocytes displayed

Table 1 State of the Art and Used Methodology of Human SCNT

Recipient oocytes	Donor cells	Enucleation method + NT procedure	Activation method	Efficiency of SCNT reconstruction	No. activated	No. cleaved	Stage of final development	Reference
In vivo matured (n = 19 reconstructed)	Fibroblasts (n = 11) Cumulus (n = 8)	–1 μg/mL Hoechst 5 ng/mL cyt B 20 min –Piezo injection	5 μM ionomycin (4 min) + 2 mM 6-DMAP (3 hr)	NR 11 reconstructed from fibroblasts 8 reconstructed from cumulus	7/11 4/8	0 3/8	0 3 embryos cleaved to 4–6 cells	28
In vivo matured (n = NR)	Fetal fibroblasts / cumulus cells	–Reverse-order technology –5 μM Hoechst (15 min), 10 μg/mL cyt B (40–60 min) –Injection	5 μM A23187 ionophore (10 min) + 2 mM 6-DMAP (4 hr)	NR –52 reconstructed from reverse order –23 reconstructed from invasive enucleation	–81–97% –NR	41/52 14/23	4 blastocysts[a] 5 eight cell	29
Failed fertilized (n = 7) In vivo matured (n = 18) In vitro matured (n = 2)	Human embryonic stem cells	–DNA staining + UV light +10% FCS + 5 μg/mL cyt B –Electrofusion	10 μM A23187 ionophore (5 min) + 2 mM 6-DMAP (4hr)	NR	NR	0/7 3/18 0/2	0 1 blastocyst 0	12
Failed fertilized (n = 39)	Fibroblasts	–DNA staining –Piezo injection	Electrical pulses	30/39	14/30	2/14	1 6 cell	11
In vivo matured (n = 3)	Fibroblasts	–Blind enucleation (5 g/mL cyt B) –Electrofusion	7% ethanol (5 min) + 10 μg/mL cycloheximide + 5 μg/mL cyt B (5 hr)	NR	NR	1/3	1 4 cell	32
In vivo matured (n = NR)	Cumulus cells	–Enucleation method NR –Injection	Electrical pulses	NR	NR	NR	1 blastocyst	33
Failed fertilized (n = 48) In vivo matured: –n = 9 –n = 14 –n = 25	Fibroblast like Fibroblast like ESC Fibroblasts	–5 μg/mL Hoechst + 7.5 μg/mL cyt B –Electrofusion	5 μM ionomycin (5 min) + 2 mM 6-DMAP (4 hr)	6/48 6/9 8/14 22/25	NR NR NR NR	0/48 1/6 1/8 7/22	0 0 0 0	13
In vitro matured (n = 61)	Cumulus cells	–1 μg/mL Hoechst + 1 μg/mL cyt B	10 μM A23187 ionophore (7 min, 2x)	36/61	22/36	13/22	2 morula	14

(*Continued*)

In vivo matured (n = 54) Failed fertilized (n = 45)		–Injection	+ 2 mM 6-DMAP (3 hr)	42/54 26/45	27/42 13/26	17/27 1/13	1 morula 0	
In vivo matured (n = 21)	Fibroblasts	–6 μg/mL Hoechst + 5 μg/mL cyt B (45 min) –Electrofusion	–10 μM A23187 ionophore (4 min) + 2mM 6-DMAP (4 hr) (n = 17 oocytes) –10 μM A23187 ionophore (4 min) 10 μg/mL cycloheximide + 2.5 μg/mL cyt D (3 hr) (n = 4 oocytes)	12/17 2/4	12/12 2/2	9/12 1/2	5 blastocysts 0	34
–In vitro matured (n = 27) Failed/abnormally fertilized after ICSI (n = 49) Failed/abnormally fertilized after IVF (n = 30) –In vitro matured (n = 53) Failed/abnormally fertilized after ICSI (n = 18) Failed/abnormally fertilized after IVF (n = 12)	Human embryonic stem cells	–Invasive enucleation: 5 μg/mL Hoechst + 7.5 μg/mL cyt B (5–15min) Injection –Noninvasive: Oosight Imaging system in 7.5 μg/mL cyt B Electrofusion	10 μM ionomycin (5 min) + 2 mM 6-DMAP (4 hr)	20/27 34/49 24/30 11/53 5/18 6/12	NR	6/20 7/34 5/24 5/11 1/5 2/6	2 embryos beyond 4-cell 4 embryos beyond 4-cell 1 embryo beyond 4-cell 3 embryos beyond 4-cell 0 1 embryo beyond 4-cell	15

[a]Two blastocysts derived from cumulus cells, two blastocysts derived from fetal fibroblasts.
Abbreviations: NR, not reported; IVF, in vitro fertilization.

aberrant expressions of important spindle microtubule proteins [nuclear mitotic apparatus protein (NuMA) and EG5] and possessed abnormal disrupted and tetrapolar spindles, which could be an indication of the inferior quality of these oocytes to serve as recipients in SCNT. Still the failure of cleavage using freshly collected in vivo–matured oocytes beyond the six-cell stage suggested that the used SCNT protocol needed profound further optimization. The second study investigated whether in vitro–matured GV oocytes collected from stimulated cycles could support human SCNT development in comparison with in vivo–matured oocytes and failed fertilized oocytes after ICSI and using cumulus cells as somatic donor cells (14). Comparable embryonic development up to the morula stage was achieved in both in vitro– and in vivo–matured oocyte recipient groups as opposed to the near failure of cleavage when failed fertilized oocytes were used as recipients. The importance of the short-time interval between oocyte collection and the start of SCNT was further confirmed when using in vivo–matured oocytes, and fluorescent analysis revealed that arrested SCNT embryos showed a high degree of micro- and multinucleation in their blastomeres.

The first proof of successfully reconstructed human SCNT blastocysts was reported earlier this year by French et al (34). These authors succeeded in obtaining five blastocysts using 21 in vivo–matured oocytes donated by young patients (20–24 years) undergoing fertility treatment and fibroblast cells as somatic cells. Interestingly, only one blastocyst was confirmed by DNA and mitochondrial DNA (mtDNA) fingerprinting to be of unambiguous clonal origin, while two other blastocysts revealed to be generated by the SCNT technology, but this could not be supported by the mtDNA analyses. This achievement is very much applauded by the scientists performing SCNT and ESC research, but still the underlying reasons, biological and/or technical, as to why this study succeeded in blastocyst formation have to be revealed yet. Presumably the quality of the recipient oocytes and the used SCNT technology are the key parameters that contributed to this achievement, as was also suggested by the authors. The final report until now confirmed that embryonic development was restricted to early stages using different sources of inferior recipients (abnormally fertilized, failed fertilized, and in vitro–matured oocytes) (15).

All these studies demonstrate that the current protocols used for human SCNT demand further optimization to improve the efficiency and indicate that SCNT methodologies proven successful in animal models cannot be simply translated to the human species thus far. First of all the preimplantation efficiency warrants improvement to minimize the use of the scarcely available human oocytes and to succeed in the derivation of hESC.

CHOICE OF RECIPIENT OOCYTES FOR HUMAN SCNT

There is a general consensus that the quality of the used recipient oocytes in SCNT research is a key factor determining the success rate of embryonic developmental potential, as is also the case after any assisted reproduction technology (2,3). Metaphase II (MII) oocytes have become the recipient cell of choice in animal SCNT experiments, as it was suggested that high levels of maturation/meiosis/mitosis-promoting factor (MPF) activity are a necessity for efficient reprogramming of the inserted donor somatic cell (reviewed in Ref. 35). MII oocytes can be easily recovered in vivo from superovulated small animals, but in large animals, this becomes arduous and more expensive. Improvement of the human SCNT preimplantation developmental potential has been thwarted by the very limited availability of human oocytes for this purpose. Potential sources of human oocytes to perform this research include the following:

1. In vivo–matured donated oocytes predominantly sourced from patients undergoing fertility treatment: The quality of these oocytes will likely be influenced by the age of the donor without female infertility and with proven established pregnancies.
2. Spare immature oocytes donated from IVF or ICSI cycles: Approximately 10% of the oocytes collected after stimulation are at the GV or MI stage and when enough MII oocytes are available for the fertility treatment, the former oocytes are mostly discarded due to inferior quality (36). Improvement in the field of in vitro maturation (IVM) protocols may contribute to increase developmental potential using these oocytes as recipients.

3. Donated failed fertilized oocytes after IVF or ICSI: Clear distinction should be made between failed fertilized oocytes after IVF or ICSI, because the latter have been reported to mostly contain a sperm cell inside (37), the failure of fertilization mainly being due to an activation deficiency, while IVF failure is mainly due to failure of sperm penetration. This source of oocytes might be valuable to test some key technology parameters such as enucleation or artificial activation, or might serve as a suitable control when conducting SCNT experiments such as the creation of parthenogenetic embryos.
4. Abnormally fertilized zygotes after IVF or ICSI: Zygotes showing one or three or more pronuclei (PN) are not used for embryo transfer because of chromosome abnormalities that arise in these embryos (38).

Arrested MII stage oocyte recipients clearly support the development of NT embryos much more effectively than zygote-stage recipients (39,40). Few reports have profoundly compared the capacity of different sources of oocytes to support embryonic development or studied whether they are different in reprogramming capacity of the somatic donor cell (reviewed in Ref. 41). Maturation conditions have been shown to influence the embryonic development in bovine and mouse SCNT experiments (42). A direct comparison of the efficiency of using in vivo– versus in vitro–matured GV oocytes was conducted in mouse SCNT experiments (43). These data demonstrated that the use of in vitro–matured GV oocytes for SCNT is not ideal, but this can be circumvented by the appropriate choice of IVM medium and the use of a serial NT technique to an in vivo–matured zygotic cytoplast, which allows developmental rates equal to the use of in vivo–matured oocytes. This study was later confirmed by Bai et al. who found that when the PN of a mouse SCNT zygote derived from in vitro–matured GV oocytes was transferred into the cytoplast derived from an IVF zygote, blastocyst formation rates also significantly increased (44). Additionally, studies of pigs, sheep, and bovine have shown that GV-matured cytoplasm is less capable of supporting SCNT development compared with in vivo–matured cytoplasm both in terms of blastocyst rates and postimplantation developmental potential (42,45).

In human, several reports indicate that the used culture systems adequately support nuclear maturation but fail to produce human oocytes with cytoplasmic competency, thereby resulting in embryos with reduced developmental capacity (46–48). Cytoplasmic maturation is a complex process and encompasses a wide array of metabolic and structural modifications, ensuring the occurrence of normal fertilization, inducing meiotic to mitotic cell cycle progression, and activating pathways required for genetic and epigenetic programs of embryonic development (46,49). Barnes et al. were the first to report successful development to the blastocyst stage of human oocytes matured in vitro and cultured in sequential culture medium designed specifically to optimize blastocyst development (50). Hwu et al. demonstrated that embryos derived from in vitro–matured oocytes arrest at the 2- to 16-cell stage of development when cultured in human tubal fluid (HTF) culture medium alone; however, in coculture, 30% blastocyst formation was obtained (51). Cobo et al. similarly reported high rates of development of embryos derived from in vitro–matured oocytes to the blastocyst stage (49%) when embryos were cocultured with endometrial epithelial cells (52). Further, it was demonstrated that both maturation and early embryonic development rates of GV stage–denuded oocytes can be influenced by the type of IVM medium whereby a newly designed medium gave a maturation and blastocyst rate of 75.7% and 12.9%, respectively, compared with 55.7% and 0%, respectively, in tissue culture medium (TCM) supplemented medium (53). De Vos et al. already reported on the influence of IVM media on subsequent embryonic developmental potential and further demonstrated that rapidly in vitro–matured human oocytes (GV oocytes matured after 24 hours vs. 48 hours and MI matured after 6 hours vs. 24 hours) resulted in higher blastocyst formation after ICSI (36). These findings indicated that by optimization of IVM conditions or by adequate culture environment optimization, development of in vitro–matured human oocytes could be increased, which might be beneficial for human SCNT experiments when in vitro–matured oocytes are used as recipients. One study has shown no difference in embryonic development between in vitro– and in vivo–matured oocytes after human SCNT, indicating that other technology parameters might also influence this efficiency (14).

It was stressed that for human and nonhuman primates, the best results were obtained when freshly collected oocytes were immediately used for SCNT (6,12,14). The time interval from oocyte collection to SCNT seemed to be crucial, and only in vivo–matured oocytes that were enucleated within one hour proved to be successful for subsequent development to later stages (12). Even more, it was suggested that the best source of recipient oocytes for human SCNT might be oocytes that were still in prometaphase II or telophase I stage that had just extruded the first polar body (PB) (6,12). Detailed observation of in vitro–matured GV or MI oocytes would allow the use of prometaphase II oocytes immediately after first PB extrusion.

Hall et al. have shown that aged, failed fertilized oocytes used for human SCNT resulted in very poor development (13), as also shown by other reports (11,12,14,15). Most of these oocytes possessed abnormal meiotic spindles that could reflect their inferior quality, and aberrantly expressed key microtubule markers such as NUMA, HSET, and EG-5, as also reported in nonhuman primate SCNT (6). Aberrant mRNA expression of oocyte-specific markers was also present in these aged human oocytes. Interestingly, human SCNT embryos reconstructed from in vivo–matured fresh oocytes also displayed aberrant spindles, resulting in aneuploidy and failed to develop past early cleavage divisions, as also confirmed in other human and nonhuman primate SCNT experiments (6,11). Encouraging findings that may be relevant for the use of failed fertilized oocytes for human SCNT were recently published (16). Oocytes that had failed to fertilize after IVF-termed "aged, fertilization failure" (AFF) oocytes were used as recipient eggs for mouse SCNT. While the efficiency of NT and subsequent preimplantation development was significantly lower for the AFF oocytes in comparison with in vivo–matured freshly used oocytes, they found that embryos that did reach the blastocyst stage could be used to derive ESC at a success rate equivalent to that of their fresh counterparts. The authors stated that although aged mouse oocytes bear the same common abnormalities as aged human oocytes, such as aberrant spindles and transcriptional profiles, most of these aberrations are believed to occur in the spindle rather than the cytoplasm, as associated developmental arrests could not be circumvented when spindles of aged oocytes were transferred into fresh oocytes (54). Another study in mice not only confirmed these results of the compromised blastocyst formation rate when using aged oocytes as oocyte recipients but also showed that a serial NT could increase the blastocyst formation rates of the SCNT embryos reconstructed from aged oocytes up to the level of fresh oocytes, which is in contradiction with Wakayama's suggestions that only the spindle was inferior and not the cytoplasm (55). It would be interesting to test this second NT in human SCNT experiments in the future since it now has proven extensively its usefulness when inferior oocyte recipients are used in SCNT experiments at least in mice (43,44,55). A possible manner would be to use failed fertilized or in vitro–matured oocytes, which are the most abundant source of human oocytes for research in a first step to form a PN after SCNT, followed by transfer of this PN to an in vivo–matured and abnormally fertilized zygotic cytoplast.

Alternatively, abnormally fertilized oocytes might be suitable as direct recipients, as reported using a modified protocol of mouse SCNT technology (17). In the past, several animal studies had shown the incapability of zygotes to reprogram somatic cells in SCNT experiments (40). This was mainly attributed to a loss of activity in the zygote cytoplasm after fertilization, which is crucial for NT and/or reprogramming. Egli et al. suggested that these factors, which are required either for reprogramming or to support embryonic development, become sequestered in the PN of zygotes and are aberrantly removed after enucleation. They reasoned that the loss of these crucial factors could be minimized or eliminated if NT is conducted when both the recipient zygote and the donor cell are arrested at the mitotic cell division. Synchronizing abnormally fertilized zygote recipients temporarily in a mitotic state using nocodazole caused spindle disruption and pronuclear envelope breakdown followed by condensation of the chromosomes. To allow again spindle polymerization and concomitant noninvasive visualization of the chromosome position, the proteasome inhibitor MG-132 was used. When transferring, somatic cell nuclei also arrested with nocodazole in these modified zygotic recipients; successful embryonic development was achieved and gave rise to both ESC lines and full-term offspring. This method would also allow the use of abnormally fertilized oocytes, which is an abundant source to serve as human recipient oocytes. Whether this intriguing hypothesis can be translated to human SCNT has yet to be investigated.

ENUCLEATION METHODS AND POSSIBLE CONSEQUENCES

Removal of the spindle-chromosome complex of the recipient oocytes is one of the most important key steps in the SCNT process. Several methods exist, but no definitive proof or comparisons have yet been reported as to which one is the most efficient for human SCNT. Reported protocols of the used method of enucleation in specific supplement conditions are unfortunately often not presented in a detailed way. To decrease oocyte lysis, a microfilament inhibitor (cytochalasin B) is mostly used (56). This induces relaxation of the cytoplasm, allowing for mechanical removal of the spindle-oocyte complex. French et al. emphasized that an increased incubation time of 45 minutes attributed to their overall cloning efficiency in human SCNT (34). Different concentrations of cytochalasin B have been reported in human and nonhuman primate SCNT in the presence or absence of increased concentrations of serum (Table 1). The concentration of cytochalasin may be important when inferior-quality oocytes are used as recipients, as they possess a more fragile oolemma compared with their in vivo–matured counterparts, leading to higher damages after intensive micromanipulation and concomitant decreased efficiency (13,14).

Removal of oocyte chromosomes has been commonly accomplished by aspiration or extrusion of the spindle-chromosome complex containing the metaphase plate. Other enucleation methods, not used for nonhuman primates or human SCNT, include centrifugation, inactivation, or destruction by ultraviolet (UV) or laser irradiation or noninvasive chemically induced extrusion (57). Successful enucleation needs to be confirmed in nonhuman primate and human SCNT by incubating the oocyte in Hoechst 33342 followed by UV irradiation since the spindle complex is not visible with normal inverted-stage microscopy (14). Using this procedure, very little cytoplasm surrounding the spindle can be removed (Fig. 1). Although excitable fluorochromes and UV light are required for complete and accurate enucleation, they also pose the risk of damaging the maternal cytoplast and causing hampered development (14,58,59).

Blind enucleation, involving the aspiration or squeezing-out method of a region of cytoplasm close to the first PB where the spindle normally resides, is not 100% reliable and thus requires additional confirmation with Hoechst staining and UV irradiation. The main disadvantage of this method is that more surrounding cytoplasm with possible important factors has to be removed, especially in bigger oocytes as in human and nonhuman primate oocytes. This blind enucleation cannot be successfully performed in human oocytes since the first PB is not a reliable indicator of spindle-chromosome complex location as shown by recent noninvasive studies in human oocytes using polarization microscopy (60). The so-called handmade cloning method is based on the principle of blind enucleation and involves removal of the zona pellucida followed by bisection of the nuclear material using a very thin blade without the need for micromanipulation (61). Although the simplicity of this method is a major advantage, more than necessary cytoplasm is removed together with possible factors important for later development.

The use of a new, developed, computer-assisted polarization microscopy system (Oosight Imaging System, CRi Inc., Woburn, Massachusetts, U.S.) allows reliable noninvasive spindle imaging in mammalian oocytes (Fig. 2) (60). The polarized light microscope observes birefringence, which is an optical property that results from high molecular order in some

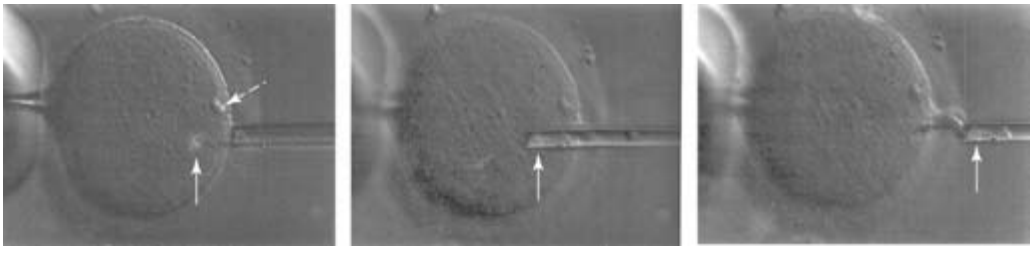

Figure 1 Enucleation of a human MII oocyte using invasive visualization of chromosomes by Hoechst staining and UV irradiation and aspiration with a blunt pipette through a hole made by partial zona dissection close to the first polar body: (*broken arrow*) polar body chromosomes; (*arrow*) oocyte chromosomes.

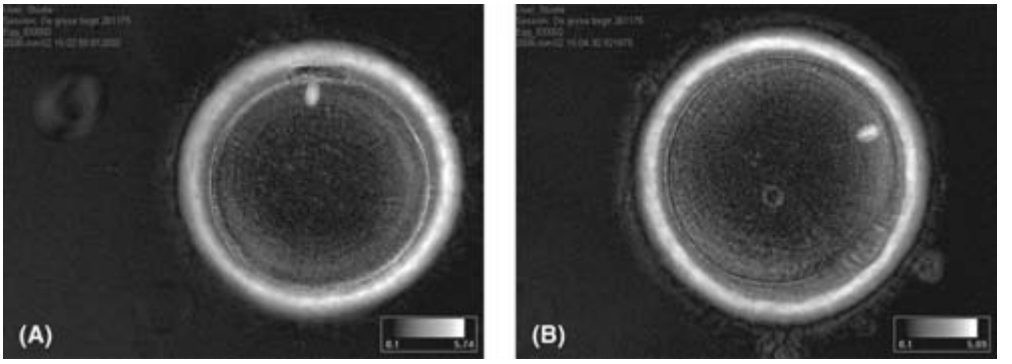

Figure 2 Noninvasive visualization of spindle and zona in human MII oocytes using polarized microscopy (Oosight Imaging System, CRi, Inc., Woburn, Massachusetts, U.S.). (**A**) Spindle located adjacent to the first polar body. (**B**) Spindle located at 3 o'clock relative to the first polar body.

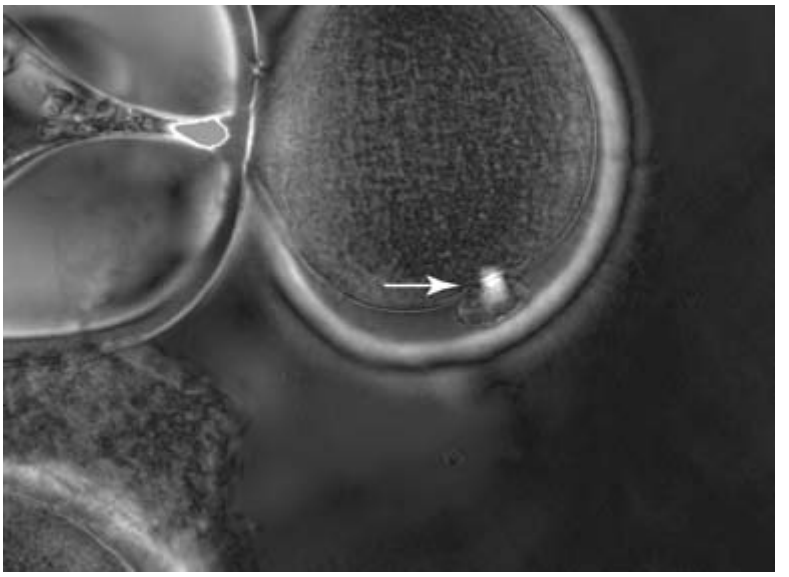

Figure 3 Noninvasive imaging of an in vitro–matured oocyte using polarized microscopy: MII oocyte with spindle still connected to the first polar body (*arrow*).

biological structures such as mitotic spindles and the different layers of the zona pellucida. The percentage of visible spindles in human oocytes varies between laboratories, but generally in more than 80% of human MII oocytes a spindle can be visualized using this polarized light microscopy (60). Additionally spindles can also be observed in in vitro–matured human MI or GV oocytes whereby distinction can be made between the final stages of maturation, for example, telophase I spindles with the spindle of the oocyte still connected to the first PB (Fig. 3). So far, this Oosight imaging system has been used in one human NT experiment, but development was poor after the use of failed or abnormally fertilized zygotes and in vitro–matured oocytes (15). Interestingly, using donor cells that express human Oct-4-GFP, it was demonstrated that only embryos originating from noninvasive enucleation expressed GFP in opposite of NT embryos derived from invasive enucleation using Hoechst and UV irradiation. The only blastocyst achievement after human SCNT used the conventional aspiration and squeezing technique based on Hoechst staining and UV irradiation to remove the oocyte chromosomes (34). On the other hand, the successful derivation of nonhuman primate ESC has

been attributed to the noninvasive enucleation method using this Oosight imaging system to remove the spindle–chromosome complex with a minimum of surrounding cytoplasm (10). Blastocyst formation was significantly increased from 1% to 16% when the use of Hoechst and UV irradiation was omitted. Still, the advantages of this system need further prospective confirmation.

An alternative method of enucleation in sheep comprehends the so-called reverse-order NT technique, whereby the donor nucleus is first transferred into a nonenucleated recipient oocyte, and the oocyte chromosomes are removed after artificial activation, when it reaches the anaphase-telophase II stage (62). A significantly increased developmental potential to the blastocyst stage was observed using this new noninvasive enucleation technology. Apparently this technology was used by Lu et al. in human SCNT, albeit modified, as instead of removing the telophase II-spindle complex connected to the second PB, the female PN was aspirated, identified as the one closest to the second PB (29). Direct comparison was made between this modified enucleation method and the conventional method of Hoechst staining and UV irradiation. Embryonic development of human SCNT embryos to the eight-cell stage was significantly better using this modified technology, and only blastocysts could be obtained in this group (Table 1). This original method is of huge potential interest for the progress in human SCNT, since it allows a new noninvasive way of depleting the oocyte chromosomes with little accompanying cytoplasm, and possibly without the aberrant removal of important spindle proteins, which have been shown to be concentrated around the metaphase spindle. Although the authors performed some preliminary tests showing this method was 100% reliable as confirmed by FISH analysis, it remains to be shown using DNA fingerprinting that parthenogenetic embryos indeed were not produced in this way.

Great concern has been devoted to the possible removal of important spindle proteins and proteins associated with the chromosomes and kinetochores during the process of enucleation, because this could result in abnormal mitotic spindles in reconstructed SCNT embryos with concomitant chaotic distribution of chromosomes (5,6). These include proteins such as spindlin, centrosomal and motor proteins, protein kinases such as polo-like and aurora-A and aurora-B, protein phosphatases, and other microtubule-associated proteins. Although some of these proteins exist in ooplasmic reservoirs or may be replenished after removal of the spindle-chromosome complex, it is possible that other proteins may be irretrievably depleted by spindle removal (5,6,9). γ-Tubuline is localized in the microtubule-organizing center (MTOC) and is needed for normal mitotic organization and spindle function and constructs the centrioles in somatic cells (5,6). NUMA1 is concentrated at the spindle poles during mitosis. In primate SCNT experiments, enucleation of the spindle-chromosome complex resulted in depletion of NUMA1 and HSET (kinesin), both of which are important for correct pole formation of the mitotic spindles (5,6). Consequently, disarrayed microtubule patterns and misaligned chromosomes on multipolar spindles after SCNT in nonhuman primate embryos were reported (6). By using the squeezing method of enucleation in prometaphase-II oocytes through a small hole in the zona pellucida, instead of aspirating, the spindle-chromosome complex, less microtubule- and centrosome-associated molecules, and adhering cytoplasm are discarded (6). Further analysis of this possible aberrant removal of spindle proteins during human SCNT remains to be done.

NUCLEAR TRANSFER AND ARTIFICIAL ACTIVATION PROCEDURE

The next step of SCNT is to insert a somatic nucleus into an enucleated oocyte, which is mostly done by electrofusion or direct mechanical or piezo injection, depending on the cell type (2,3). Given the reported fragile oolemma of failed fertilized and in vitro–matured human oocytes and concomitant lower survival rate after enucleation and NT compared with their in vivo–matured counterparts (14), piezo-driven micromanipulation or electrofusion might be better suitable when these sources of oocytes are used to increase the survival rate.

Thus far only cumulus cells and fibroblasts were used as donor cell nuclei in human SCNT experiments using all three above-mentioned methods (Table 1). Simerly et al. reported higher reconstruction and developmental efficiencies when using fibroblast cells compared with cumulus cells as somatic cells in nonhuman primate SCNT experiments, and only blastocysts were obtained with fibroblasts (6).

The next crucial step in the SCNT technology is the artificial activation of the reconstructed oocytes. Mature oocytes remain arrested at the MII stage and contain a high level of MPF factor (4). Cytostatic factor, which stabilizes MPF activity, is also kept high in MII oocytes. Inducing artificial activation can be obtained by a wide range of various chemical, mechanical, or physical stimuli that elicit one or several calcium transients in the oocyte (2,3,37). Regardless of the method of choice, the ultimate challenge of artificial activation protocols is to mimic as closely as possible the activation mechanism that normally occurs when the sperm fertilizes the oocyte. Although the precise signaling pathway initiated by the sperm remains unclear, literature now generally accepts the sperm factor hypothesis (63). This hypothesis proposes that the sperm introduces a protein factor into the ooplasm after gamete fusion, and that this sperm factor, identified as phospholipase C (PLC)ζ, initiates Ca^{2+} release in the oocyte, leading to the appropriate pattern of waves of Ca^{2+}. Most critically it was demonstrated that PLCζ is an effective mimic of the sperm factor because microinjection of complementary RNA (cRNA) encoding for both human and mouse PLCζ into mouse oocytes causes Ca^{2+} oscillations identical to those seen after fertilization (63,64).

Several reports have confirmed that although Ca-oscillations fulfil a critical role in the oocyte activation mechanism, they can also affect long-term developmental events (65). A popular and effective oocyte activation method for human oocytes involves inducing an elevation in intracellular Ca^{2+} with a calcium ionophore while maintaining low levels of MPF using a protein or kinase synthesis inhibitor for several hours after the initial Ca^{2+} elevation (14). In human SCNT experiments, artificial activation is commonly performed using Ca ionophore in combination with 6-DMAP, a kinase inhibitor (Table 1). Ionophores induce a single Ca^{2+} rise in the oocyte, so this method does not allow mimicking Ca-oscillations as normally observed after release of the sperm factor (37). However it remains to be elucidated whether mimicking the normal Ca-pattern is beneficial for SCNT experiments since the need of high kinase (MPF) levels might be necessary for efficient reprogramming (35).

Interestingly, bovine oocytes activated with ionomycin and 6-DMAP display some alterations in the DNA content (ploidy abnormalities), reflecting an abnormal pattern of karyokinesis during the first cell cycle (66). Moreover, 6-DMAP inhibits phosphorylation of ribosomal protein S6 and activation of the 70-kDa S6 kinase in somatic cells, and it drastically affects cytoskeletal components, leading to the formation of micronuclei-containing chromosomes. Using this activation method, the presence of micro- and multinucleated blastomeres was frequently observed in human SCNT embryos (14). It should be considered that these inhibitors are not specifically interfering with one kinase, but with several involved in other cell functions, whose inhibition may be deleterious in subsequent embryonic development activation (66).

Three different methods were compared to induce activation in nonhuman primate SCNT (6). When simultaneous fibroblast fusion and cytoplast activation was performed, significantly more expanded blastocysts were produced compared with either chemical activation using ionomycin and 6-DMAP or injection of sperm extract after two hours of incubation post-NT, although the number of oocytes used in that study was limited. French et al. could only obtain blastocyst formation after human SCNT when a combination of ionophore and 6-DMAP was used to induce activation of the reconstructed oocyte as opposed to the use of ionophore and cycloheximide, which failed to give rise to blastocyst formation (34). Additional information on which artificial activation method to be used to improve the success rate of human SCNT comes from the creation of human parthenogenetic embryos (see sect. "Parthenogenetic Development of Human Oocytes").

INFLUENCE OF CULTURE MEDIA ON SCNT

Efficient SCNT requires silencing of the donor cell gene expression program and the initiation of the embryonic gene expression program (67). Failure to silence the donor cell program could lead to altered embryonic phenotypes and to aberrant gene expressions in the early SCNT embryo of gene products, which are related to the donor cell, or to hybrid expressions, one from the embryo driven by maternal mRNAs and one from the somatic cell (35). Indeed, it has been shown in mouse SCNT that different culture conditions may influence the blastocyst

formation rate and, remarkably, control normally fertilized embryos, or parthenogenetic embryos react in a different way to altered culture conditions (35,68,69). One possible explanation could be that reprogramming of the donor nucleus is not limited to the early time interval between NT and the beginning of activation, but that this process is progressively continuing during preimplantation development (35). Indications for this came from the fact that mouse SCNT embryos did not develop efficiently in standard embryo culture conditions like CZB or KSOM (potassium simplex optimized medium), culture media that are specifically designed for in vitro mouse embryo culture (68,69). This was supported by reports showing that mouse SCNT embryos thrive better in somatic cell–like culture conditions, such as α-MEM medium (35). Depending on the used type of donor somatic cell, different culture media may be required, as SCNT embryos using myoblast donor nuclei fail to develop in standard embryo culture media (70). They rather require somatic cell–like culture conditions, which differ from those required by normally fertilized or parthenogenetic control embryos (35,68–70). Human SCNT embryos have been cultured in standard commercially available culture media, and no comparison of different culture conditions has been performed so far.

PARTHENOGENETIC DEVELOPMENT OF HUMAN OOCYTES

Alternative methods for the creation of patient-specific ESC include reprogramming of somatic cells by fusion with ESC and direct induction of pluripotency in somatic cells (reviewed in Ref. 18). The creation of induced pluripotent cells (iPS) was achieved recently by several groups (see chap. 19) using four different transcription factors to directly render somatic cells pluripotent. Another method is the creation of parthenogenetic embryos and deriving female diploid ESC from them (reviewed in Ref. 27). Parthenogenetic embryos are created by artificially activating unfertilized MII oocytes without the use of the male counterpart and preferentially maintaining a diploid karyotype in the reconstructed embryo by preventing second PB extrusion after activation. From literature human oocytes can be successfully artificially activated by various activation stimuli, but mostly parthenogenetic embryos became arrested at the six- to eight-cell stage as opposed to what is observed in other species (27). Most studies were predominantly performed using aged, failed fertilized human oocytes after IVF or ICSI exposed to various activation stimuli (27). These oocytes might be compromised and not the ideal material to optimize protocols for human parthenogenetic activation. Indeed, being aged and failed fertilized following ICSI, they frequently contain a sperm in the ooplasm (37) and thus cannot be considered as true parthenotes.

The production of parthenogenetic blastocysts and the successful derivation of ESC (71) that showed all the characteristics of traditional human ESC from these embryos in nonhuman primate species has stimulated new interest in human parthenogenesis as a potential source to create patient-specific stem cells for use as future cell therapies. Several reports have demonstrated recently that blastocyst formation can be successfully achieved using donated in vivo–matured oocytes for parthenogenetic human reconstruction (Table 2) (28,72–76). These accomplishments confirmed earlier experiments reported by Cibelli et al. who reported a blastocyst formation rate of 27%, although none of the obtained blastocysts possessed a visible ICM (28). The number of oocytes showing successful activation with the formation of one PN and without extrusion of the second PB ranged from 67% to 91%. One study did not report the activation rate (72), while another observed pseudo-PN (75), although ionomycin and 6-DMAP were used, which normally results in the formation of one big PN without the extrusion of a PB. Detailed assessment of the activation rate defined by the number of observed PN and the number of PBs should be reported to compare different activation stimuli in a more accurate manner. Cleavage potential of reconstructed human parthenogenetic embryos ranged from 62% to 91%, but again this was not reported in all studies. A huge variation in blastocyst formation rates was observed (ranging from none to 52%) (28,72–76). This may be attributed to the low number of available oocytes in some reports, the variable age of donors, the used culture environment, or the inefficient or different assessment of the obtained blastocysts. Revazova et al. emphasized that the use of low oxygen in their study was critical for the high percentage of blastocyst formation they attained (52%); however, more than half of their

Table 2 State of the Art of Human Parthenogenetic Development Using In Vivo–Matured Oocytes

No. of donors (mean age, yr)	Used activation method	No. of used MII oocytes	No. of activated oocytes (% of total MII oocytes)	No. of cleaved (% of total MII oocytes)	No. of blastocysts (% of total MII oocytes)	Culture environment[a]	Reference
3 (28)	5 μM ionomycin (4 min) + 2 mM 6-DMAP (3 hr)	22	20 (91)	NR	6 (27)[b]	G1.2/G2.2 Cook cleavage/ cook blastocyst	28
2 (age NR)	a) 5 μM ionophore A23187 (5 min) + 1 mM 6-DMAP (3hr)	14	NR	NR	4 (29)	IVC-ONE IVC-THREE	72
	b) 5 μM ionophore A23187 (5 min) + 10 μg/mL puromycin (2 hr)	5	NR	NR	0		
	c) Sham ICSI + 50 μM ionophore A23187 (15 min)	6	NR	NR	1 (17)		
5 (NR, all over 31)	5 μM ionomycin (5 min) + 1 mM 6-DMAP (4 hr)	44	40 (91)	40 (91)	23 (52)[c]	Universal IVF/ Sequential BlastAssist (Medicult) 5% O_2	73
38 (35.2)	5 μM ionomycin (5 min) + 2 mM 6-DMAP (3 hr)	104	70 (67)	64 (62)	9 (8.6)	G1/G2	74
10 (NR)	Electrical activation + 5 μM ionomycin (5 min) + 2 mM 6-DMAP (5 hr)	19	16 (84)	NR	4 (21)	Quinn's Advantage Cleavage/ blastocyst 5% O_2	75
5 (32.2)	10 μM ionomycin (6 min) + 2 mM 6-DMAP (3 hr)	36[d]	31 (86)	30 (83)	5 (14)[e]	KSOM	76

[a]The used sequential culture media were used in 6% CO_2, 20% O_2 at 37°C, unless stated otherwise in the table.
[b]Six embryos showed blastocoele cavity; however, none displayed an ICM.
[c]Of the 23 obtained blastocysts, 11 displayed a visible ICM, while 12 did not have a visible ICM.
[d]Frozen, thawed oocytes were used.
[e]It was reported that five embryos showed cavitation of poor morphology.
Abbreviations: NR, not reported; IVF, in vitro fertilization; KSOM, potassium simplex optimized medium; IVC, in vitro care.

blastocysts did not display a visible ICM (73). Seven parthenogenetic ESC lines were successfully derived in this study, which showed a normal characterization profile with exception of one ESC line with an abnormal karyotype. It was also suggested that this strikingly high efficiency might be due to superior quality of the used oocytes since four of the five donors became pregnant after their infertility treatment. Interestingly, no ESC line could be isolated from the one patient where a pregnancy was not established. Another report also succeeded in the creation of two ESC lines from parthenogenetic origin, although one putative ESC line failed to give rise to teratoma formation when injected into SCID mice and displayed chromosomal abnormalities during long-term in vitro culture (75).

As shown in Table 2, a lot of variation exists in all the crucial steps of this technology, making it difficult to compare these studies. Revealing the crucial factors in the process of human parthenogenesis might benefit the progress of human SCNT. It has to be stressed that all the successful above-mentioned studies used in vivo–matured donated oocytes to achieve parthenogenetic blastocysts. Due to the limited availability of this source, alternative sources such as in vitro–matured oocytes and failed fertilized oocytes might be less successful to support parthenogenetic development, as until now no one has succeeded in the creation of parthenogenetic blastocysts from in vitro–matured oocytes, probably due to their inferior quality. Artificial activation methods like the microinjection of human cRNA encoding PLCζ may be a good alternative, since the Ca-pattern occurring after this injection is closely mimicking the normal pattern observed after fertilization with sperm (63,64).

CONCLUSIONS

In summary, major achievements in the optimization of human SCNT so far include the short-time interval between collection and start of SCNT. In vitro–matured oocytes that just extruded the first PB might be ideal in terms of freshness, but have been shown to possess limited developmental potential after SCNT. Alternative methods of enucleation, for example, noninvasive imaging of the spindle in human oocytes or the reverse-order technique involve other technological key parameters that need to be further elaborated in human SCNT. From nonhuman primate experiments it has been stressed that SCNT embryos showed major spindle defects, but that blastocyst formation could be dramatically increased when noninvasive enucleation was performed. Valuable information comes from recent mouse SCNT experiments, where in vitro–matured or aged oocytes were used as recipients in human SCNT. The inferior quality of in vitro–matured oocytes can be circumvented using a serial NT to an in vivo–matured zygotic cytoplast, while aged oocytes might be a valuable source when the SCNT methodology is adapted. It may be speculated that technical rather than biological barriers still form the major bottleneck in the progress of human SCNT. Still, when using in vivo–matured oocytes following a certain SCNT protocol, blastocyst achievement strongly depends on the quality and timing of donated oocytes.

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21 Derivation and Banking of Human Embryonic Stem Cells for Potential Clinical Use

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INTRODUCTION

Regenerative medicine promises to revolutionize medical treatments for a vast spectrum of degenerative processes and tissue injuries arising as a result of disease, aging, or external insults. Health conditions that are candidates for regenerative medicine-based treatments include some of the top health problems faced by aging populations in developed countries such as heart disease, diabetes, and Parkinson's disease. Its potential relies on the use of stem cells and their ability to give rise to new, differentiated cells that can replace and/or repair injured or diseased tissue.

POTENTIAL USE OF HUMAN EMBRYONIC STEM CELLS IN THERAPY

Embryonic stem cells are the earliest stem cell population that can be derived from a developing embryo and thus are biologically predisposed to be the most pluripotent stem cells that can be obtained (apart from the preimplantation embryo itself whose survival in culture is limited to a couple of days). This is reflected in their open chromatin structure and ability to spontaneously give rise to cells of all three germ layers (i.e., teratoma formation). Indeed, they originate from the inner cell mass (ICM) of the blastocyst and share with this cell population the ability to give rise to all cells of the human body. Unlike stem cell populations, which arise later in development, human embryonic stem cells (hESC) have an unlimited life span due to the robust telomerase activity.

Because of their undisputed totipotency in the absence of any genetic manipulation [while ICM cells give rise only to embryonic tissues, hESC also differentiate into trophectoderm (1), an extraembryonic tissue] and their unlimited proliferative capacity, hESC represent ideal candidates for a cell source in future stem cell–based therapies (Table 1). A number of animal studies demonstrate their potential in regenerative medicine applications. Recent results show that differentiated hESC can improve cardiac function, blood vessel formation, and regeneration of damaged heart muscle (2–5). Hemangioblast precursor cells derived from hESC repaired vasculature and restored blood flow in animal models of diabetes- or ischemia/reperfusion injury–caused retinal damage, myocardial infarction (50% reduction in mortality rate), and hind limb ischemia (6). In an animal model of acute liver injury, the hESC-derived definitive endoderm cells differentiated into hepatocytes and successfully repopulated the damaged liver (7). hESC-derived glucose-responsive insulin-secreting cells protect mice against diabetes (8,9). hESC-derived cells delayed development of Sandhoff disease (10), reversed functional deficit in an animal model of Parkinson's disease (5), and restored lost limb function in rats that had stroke-related brain damage (11). hESC-derived oligodendrocyte progenitor cell transplants remyelinated and restored locomotion in an animal model of the spinal cord injury (12,13). hESC-derived retinal cells rescued visual function in an animal model of retinal disease (14). Clinical trials involving hESC are scheduled to start soon in the United States for the treatment of spinal cord injury, myocardial infarction, and macular degeneration. Additional trials are planned for treatment of diabetes and Parkinson's disease.

Therapeutic application of embryonic stem cells in humans awaits results of the first clinical trials. However, before it becomes a reality, a number of issues need to be resolved. These include decisions regarding which cells qualify for use as a source material, how they need to be derived and stored to fulfill safety requirements, and how are immunocompatibility concerns addressed. In this chapter we will tackle some of these questions with the idea not to

Table 1 Comparative Advantages of hESC

	CBSC	ASC	iPS	ESC
Unlimited proliferation	No	No	Yes	Yes
Pluripotency	No	No	Yes	Yes
Unmodified	Yes	Yes	No	Yes

Table 1 compares main characteristics of major types of human stem cells that are currently being evaluated for therapeutic use.
Abbreviations: CBSC, cord blood stem cells; ASC, adult stem cells; iPS, induced pluripotent cells; ESC, embryonic stem cells.

present the final solutions, but rather point to potential directions that may be taken in addressing these issues and how chosen paths can influence quality and availability of embryonic stem cell treatments in the future.

PERSONAL VERSUS UNIVERSAL EMBRYONIC STEM CELLS

There are two major models for producing, banking, and using stem cells for therapy: (*i*) production of personal (custom) stem cells that are close or ideal genetic match for the recipient and (*ii*) derivation of "universal" stem cell lines that match as closely as possible the recipient's major histocompatibility complex [MHC, HLA (human leukocyte antigen)] profile to prevent immune rejection or are made immunologically compatible with the entire population. In the case of embryonic stem cells, the first option is limited to the IVF patient population, while the second option may be applicable to the whole population as long as appropriate immunological match can be identified.

In general, embryonic stem cells stored for personal or family use have an important advantage over potential universal cell lines due to the significantly lower number of population doublings they need to endure before being prepared for the therapeutic use. Indeed, it is well established that the amount of mutations increases with the number of division cycles cells undergo, and the cell expansion needed to treat significant number of patients will dramatically increase the chance of accumulating mutations. In addition, extensive cell culture manipulations required for such a large-scale cell production will significantly increase the risk of epigenetic changes in cell population, which may affect differentiation and functionality of the final cell product used in therapy (i.e., partially or terminally differentiated cells).

In the case where embryonic stem cells are derived from the supernumerary whole embryos that remained after IVF procedure, they are not identical to any of the family members who may potentially need to use them in the future. Because they are related to both parents and children, the chances of immunological match are greatly increased in the same way as for the organ donation between close relatives. The level of match required will most likely depend on the site and type of the cell therapy (15). Therefore, the likelihood of using the embryonic stem cell line will neither be the same for all family members nor for all potential therapy sites. Immunoprivileged sites such as parts of the central nervous system will likely show minimal immunorejection, and therefore, for example, treatment of spinal cord injury would most probably be available to all family members, whereas treatment of the infracted heart may require much more restrictive match and will limit the use of cells for some family members.

New advancement in embryonic stem cell derivation overcomes this problem and allows for production of personal embryonic stem cells. One of the key advantages of using personal embryonic stem cells is that they are ideal genetic match to recipient and thus will not put a patient at risk of immunorejection. This unique option would be available only to IVF babies since their personal embryonic stem cells will be derived from a blastomere extracted from the same embryo from which they originate. At the same time these cell lines can also be used for genetically related family members with the same constrains as outlined for hESC derived from the supernumerary embryos.

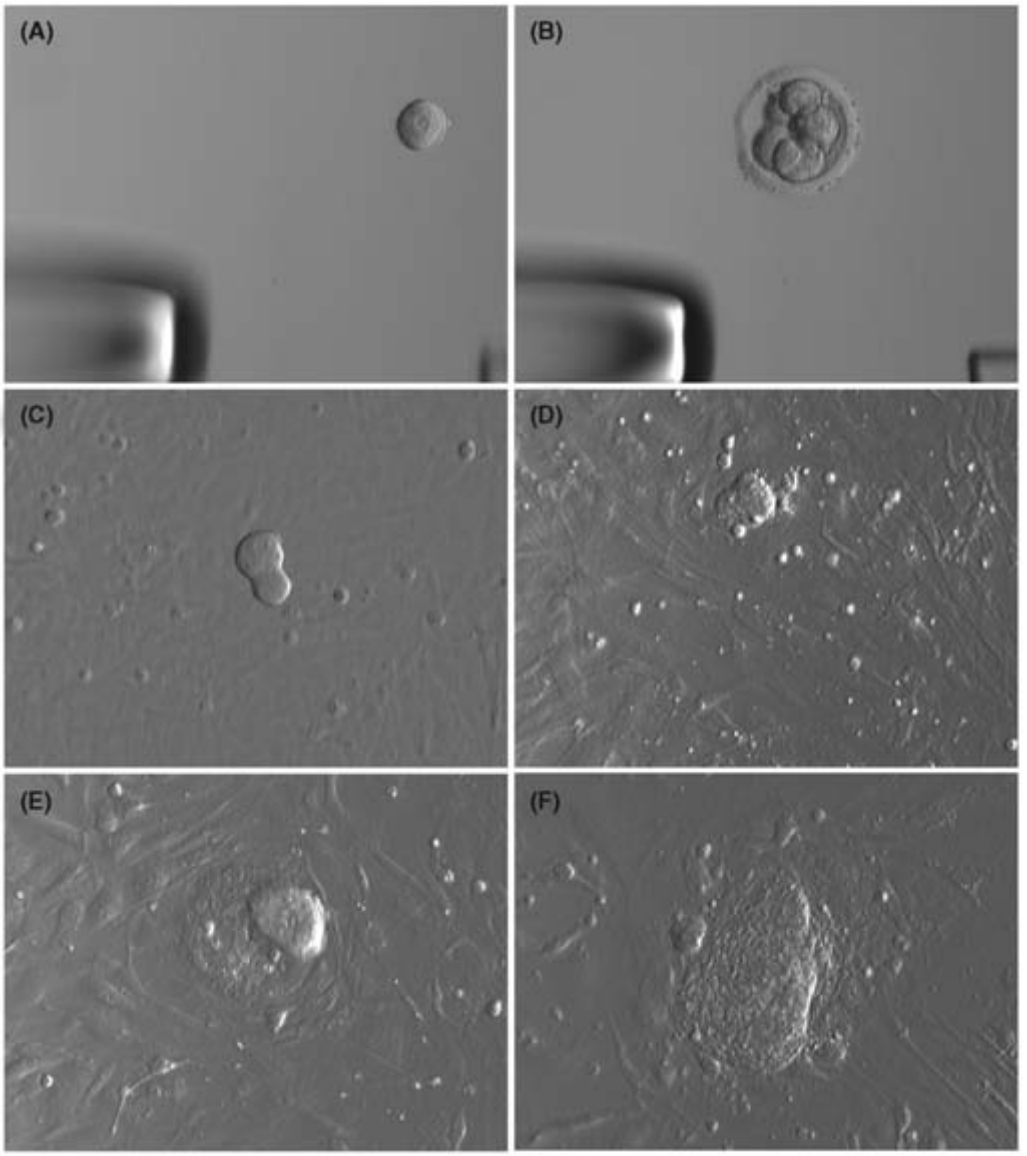

Figure 1 Derivation of hESC from extracted blastomere. Using micromanipulator-equipped microscope, blastomere is extracted from the embryo through the laser-drilled hole in the zona pellucida (**A**). The embryo is left to continue development (**B**), while extracted blastomere is placed onto feeders to allow its proliferation (**C**) and formation of the initial cell outgrowth (**D, E**). Outgrowth develops into hESC-like colony (**F**) that exhibits morphology of traditionally derived hESC.

The procedure of blastomere extraction without harming embryo development (Fig. 1), a novel technique that provides basis for this option, is not going to be discussed here, since it is the topic of another chapter of this volume (see chap. 18). What needs to be stressed here is that while this technique holds great potential in terms of offering the IVF babies the best possible source of identical stem cells, which they may utilize throughout their lives, it is limited to this population and thus may be used just as one of multiple options in stem cell therapy.

Unlike personal, universal embryonic stem cells would be available to all members of the general population who are immunocompatible with the used cell line. Indeed, the major obstacle for cell, tissue, and organ transplantation is the rejection due to the activation of the innate and adaptive immune systems (reviewed in Ref. 16). As discussed earlier, the level of

the match required would depend on the cell therapy site, and while it may not be as critical for immunoprivileged sites to develop lines with appropriate match, it will certainly be required for recipients of stem cell therapy for treatment of the injuries of the heart, liver, and other nonprivileged organs. One way to address this issue is to produce and bank sufficient number of hESC lines that will cover major combinations of immunological determinants available in population. Another option is to use genetic manipulation to reduce/eliminate expression of MHCs in hESC and their progeny. The main disadvantage of the first approach is that there is a requirement for a large number of hESC lines which will be necessary to sufficiently cover the whole population (15). The main disadvantage of the second approach is an introduction of additional risks associated with genetic manipulation of the cells used in therapy. Finally, there have been suggestions that the number of hESC lines needed to cover the whole population would be significantly reduced if cell lines would be derived from embryos that would belong to O-type blood group and be homozygous for all the major histocompatibility loci. This could be achieved by the derivation of hESC lines from either parthenogenetic embryos or from selected gamete donors. While theoretically this seems an attractive idea, the safety concerns associated with the use of parthenogenetic lines and the difficulty and ethical issues related to obtaining/generating such embryos put applicability of this approach to question. Additionally, this would not eliminate immunocompatibility issues related to minor histocompatibility antigens, which are expressed by hESC and their derivatives. An interesting approach proposed by Grinnemo et al. (16) is induction of immunotolerance in prospective recipients by costimulation blockade and injection of hESC-derived hematopoietic cells to establish mixed chimerism within the recipient. The efficacy of this approach remains to be evaluated in a clinical setting.

REQUIREMENTS FOR CLINICAL IMPLEMENTATION OF EMBRYONIC STEM CELLS

First hESC were derived under conditions that are not optimized for their use as a source of cells for clinical therapy. Specifically, hESC were exposed to animal constituents in every step of derivation process, which poses a risk, both in terms of transmitting infections (17) and also the acquisition of xenogeneic antigens, which might augment immune rejection (18). For example, immunosurgery with antibodies and complement of animal origin was used to remove trophectoderm during derivation, fetal calf serum (FCS) was a constituent of the culture medium, and hESC were cultured on mouse fibroblast feeder cells (19,20). Since then, a number of research groups have been involved in an effort to minimize the use of animal products during derivation and cell culturing. Mechanical isolation of the ICM or use of the whole embryo for derivation, culturing on human skin or placental pathogen-free fibroblast feeder cells, and utilizing a culture medium with serum replacement instead of FCS, all reduced the exposure of derived hESC to animal products (21–23).

More recently, attempts have been made to derive hESC lines under feeder-free and xeno-free conditions. First hESC derivation performed under defined xeno-free conditions used chemically defined medium that contained basic fibroblast growth factor (bFGF), LiCl, γ-aminobutyric acid (GABA), transforming growth factor (TGF)-β, and pipeolic acid and a combination of human laminin, collagen IV, fibronectin, and vitronectin matrix (24). However, the problem with this feeder-free derivation system was that one of derived lines was chromosomally abnormal from the beginning, and the other gained an extra chromosome between four and seven months of culture, suggesting that the culture conditions used were promoting genetic instability. More recently, a modification of this method has been published that produced two more hESC under xeno-free and chemically defined conditions (25). However, questions about the efficiency of this method to generate genetically stable hESC lines still remain. Therefore, additional research is necessary to establish conditions that will allow efficient derivation of genetically stable hESC under completely defined conditions. Moreover, it is not clear whether elimination of feeders is a necessary step in producing clinical-grade hESC, in particular in case when feeder cells are of human origin, have been derived under xeno-free conditions, and have tested negative for a battery of potential pathogens.

An additional requirement for the development of clinical-grade hESC is derivation under controlled conditions and in compliance with standards known as current good tissue practices (cGTP) and/or good manufacturing practices (GMPs). Exact requirements have not yet been defined, and regulatory agencies in different countries have somewhat varied approaches in terms of acceptable processes for obtaining clinical grade cells (for detailed discussion of the topic in the context of the U.K. regulations, see Ref. 26). Differences in acceptable procedures for hESC derivation and expansion also arise from the lack of clarity whether hESC should be treated as a raw material, intermediate products, or medicinal products. This ambiguity stems from the notion that hESC are going to be used as a source material for obtaining differentiated cells that will be applied in therapy and will not likely ever be directly transferred into patients.

Each embryo is unique, and therefore consistency of starting material is the first obstacle for creating rigid protocols (standard operating procedures) for derivation and early propagation of hESC lines. During derivation, consistency in the conditions and materials used is achievable and highly desirable. However, some flexibility in timing and amount of mechanical manipulation during initial establishment of the first hESC colonies is absolutely necessary to optimize the derivation efficiency and not likely to affect the end product. Once an hESC line is established, cell expansion can follow a more rigid protocol with frequent quality controls in place to ensure the lack of spontaneous differentiation and karyotypic abnormalities. Naturally, at the time of banking established hESC have to demonstrate all major characteristics of hESC lines, including expression of appropriate pluripotency markers, ability to differentiate into all three germ layers, and form teratomas in immunocompromised mice, stable karyotype, and absence of potential pathogens. Equally important is demonstrating stability after cryopreservation (Fig. 2) by testing plating efficiency, survival, proliferation, differentiation, and karyotype. This should be a necessary prerequisite in qualifying newly derived hESC for clinical use (26). While in general these criteria have been accepted by majority of the scientific community, regulatory agencies currently differ in their approach, and stringency of requirements for qualifying hESC for clinical use may vary from country to country. For example, questions remain regarding the acceptable tests to determine pluripotency of hESC lines, the list of pathogens that need to be tested for, ability to use hESC derived on mouse feeders, etc.

Figure 2 Cryopreservation of hESC. Once hESC cell line is established and characterized, limited culturing is performed to obtain sufficient number of vials that are cryopreserved in liquid nitrogen. Additional qualification of the hESC line is performed after thawing random vials from each freeze batch.

CONCLUSIONS

Since the first hESC lines have been derived nearly 10 years ago, enormous progress has been made in areas of hESC derivation, differentiation, and therapeutic application even though multiple issues remain regarding the use of hESC as a source material for treatments in regenerative medicine. A number of highly qualified investigators and clinicians around the world are involved in this ground-breaking area of regenerative medicine, and the amount and quality of data coming out of their laboratories on daily basis give reason for optimism that their current efforts will result in development of clinical applications in the not-so-distant future. Clarification of regulatory issues related to the use of hESC as a source material for cell therapy and establishment of international standards is necessary to streamline scientific efforts toward therapeutic applications.

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