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Ursula-F. Habenicht
R. John Aitken
Editors

Fertility Control

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Preface

The world's population is growing at an unsustainable rate. From a baseline figure of one billion in 1800, global population is predicted to exceed nine billion by 2050 and 87.8% of this growth will be localized in less developed countries. Such uneven population growth will yield a harvest of poverty, malnutrition, disease and environmental degradation that will affect us all. Amongst the complex mixture of political, social, cultural and technological changes needed to address this issue, the development of improved methods of fertility regulation will be critical. The inadequacy of current contraceptive technologies is indicated by recent data suggesting that the contraceptive needs of over 120 million couples go unmet every year. As a direct consequence of this deficit 38% of pregnancies are unplanned and more than 50% end in an abortion, generating a total of 46 million abortions per annum particularly among teenagers. If safe, effective contraceptives were available to every couple experiencing an unmet family planning need, 1.5 million lives would be saved each year (UNFPA 2003).

Progress in contraceptive technology should not only generate more effective methods of regulating fertility, but should also provide a range of methods to meet the changing needs of the world's population. Contraceptive practice was revolutionized in 1960 in the US and 1961 in Europe by the introduction of the oral contraceptive pill by Gregory Pincus, MC Chang and colleagues, based on fundamental hormone research conducted in Germany. While "the pill" continues to represent a highly acceptable and effective method of fertility regulation, we should not lose sight of the fact that this approach has its roots in the endocrinology of the 1920s and was designed to meet the clinical and social needs of the 1960s. During the past 50 years we have seen no radically new forms of family planning designed to meet the contraceptive needs of the twenty-first century. For example, fertility control in the future will have to be linked with the need to prevent the spread of sexually transmitted diseases (STD). Every year at least 340 million new cases of curable STD are notified, one third in young people under 25 years of age (World Health Organization 2001). Recent figures on AIDS indicate that this condition is continuing to spread at a rate of 2.7 million new cases a year, generating an

estimated annual death toll of 2 million (UNAIDS and World Health Organization (2009). Africa has been decimated by the disease and it is now rapidly gaining hold in SE Asia. Chlamydia is also spreading rapidly and is now one of the most commonly diagnosed bacterial sexually transmissible infections (Hocking et al. 2008). The spread of STDs is particularly marked in young women aged 15–25, for whom the risk of infection is approximately six times greater than their male counterparts. For these women, development of dual-purpose methods that simultaneously target pregnancy and STDs are desperately needed. Similarly, the contraceptive strategies we develop for the future should also recognize the increasing desire of men to actively participate in the family planning process. Furthermore, it should be emphasized that whereas in the past approximately 10 years of contraceptive protection was required in a lifetime, nowadays the average couple will require 30 years of contraception to meet their family planning needs, due to earlier onset of sexual activity, later time point for first birth and greater intervals between births. As a consequence we not only have to deal with the differing contraceptive needs demanded by diverse cultural and social environments, but also with the changing needs of individual women over their reproductive lifespan.

Given all the major improvements in healthcare that have been delivered by molecular medicine in the last half-century, it is remarkable that something that touches all of our lives should be so neglected. The major reasons for this state of affairs have been three fold. First, the specification for new contraceptive methods is extremely difficult to achieve. We want the new generation of contraceptive agents to combine absolute efficacy with the complete absence of adverse side effects. Because contraceptives are the only medicinal compounds that we give to perfectly healthy people, the risk-benefit equation is strongly driven the right i.e., all benefit, no risk. Developing pharmaceutical agents that meet such exacting standards will be hard. Secondly, the history of contraceptive development has been beset with the frustration of trying to project radically new methods of fertility control from an extremely narrow science base. It is extremely difficult to interfere with the reproductive system in a controlled, targeted manner, if we do not understand how the system works. Since the pharmaceutical industry is not primarily designed to make fundamental contributions to the science-base, this role has been left to public sector research institutions and, as a result, progress has been painfully slow. This situation has been exacerbated by the third factor, which is the low priority given to basic reproductive research by public sector funding agencies. Infertility is not seen as a life-threatening condition in the same way as cancer, multiple sclerosis or kidney failure, and governmental research priorities tend to reflect this perception, no matter how short-sighted.

Hopefully contraception will not remain a neglected field for much longer. The political climate has recently changed to one that is more sympathetic to reproductive research. In the past decade we have also witnessed the birth of private–public partnerships in order to improve our fundamental understanding of the reproductive process through the creation of coordinated international networks. For example in 1997, the Rockefeller Foundation and Ernst Schering Research Foundation developed one of the first such networks to intensify research on the posttesticular

maturation of spermatozoa, utilizing new approaches in molecular pharmacology [the application of molecular pharmacology for posttesticular activity (AMPPA) network]. Hopefully this will be the predecessor of further targeted networks in the future. With the advent of such initiatives, as well as parallel developments in the fields of pharmacology and drug design, the scene is now set for dramatic improvements in the technologies we shall use to regulate our future fertility.

This volume could not have been produced at a more opportune moment. It brings together contributions from all corners of the globe on all aspects of reproductive biology pertinent to contraceptive development. It contains cutting edge assessments of the molecular mechanisms regulating male and female reproduction and the new opportunities for contraceptive development to emerge as a consequence of this knowledge. It also contains expert evaluations of the potential for product development in the contraceptive field. This book looks forward to a future where men and women will be able to choose from a range of novel, safe, effective, contraceptive methods tailored to their individual needs. Hopefully it will inspire a new generation of young scientists and clinicians to exploit recent gains in our understanding of reproductive mechanisms, to engineer such new approaches to the regulation of human fertility.

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Contents

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Part I
Female Reproduction

New Insights into Ovarian Function

JoAnne S. Richards and Stephanie A. Pangas

Contents

Abstract Infertility adversely affects many couples worldwide. Conversely, the exponential increase in world population threatens our planet and its resources. Therefore, a greater understanding of the fundamental cellular and molecular events that control the size of the primordial follicle pool and follicular development is of utmost importance to develop improved in vitro fertilization as well as to design novel approaches to regulate fertility. In this review we attempt to highlight some new advances in basic research of the mammalian ovary that have occurred in recent years focusing primarily on mouse models that have contributed to our understanding of ovarian follicle formation, development, and ovulation. We hope that these new insights into ovarian function will trigger more research and translation to clinically relevant problems.

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1 Introduction

Based on the theme provided by the Editors of this book *Fertility Control – Today and in the Future*, the mission of this chapter is to focus on new advances in basic research of the mammalian ovary that have occurred in recent years. This is a daunting task because of the vast number of novel studies and mouse models that have contributed to our understanding of ovarian follicle formation, development, and ovulation. Therefore, we will highlight those areas that seem to us to have provided the most impact. We hope that these personal choices are not overly biased and that any oversights and omissions are minimal.

Much of the reproductive lifespan of most mammals and women is determined ultimately on the size of the primordial follicle pool and the quality of eggs derived from them. However, oocytes within the pool of quiescent primordial follicles form during embryonic and postnatal ages, long before the onset of puberty. For this period of oogenesis, key questions still remain regarding the input of endogenous factors that impact the proliferation of oogonia, onset of meiosis, arrest of meiosis at metaphase I, the breakdown of oocyte nests, and finally, the formation of primordial follicles. Even more murky is knowledge regarding fundamental mechanisms that regulate primordial follicle activation, as well as specification and development of the somatic cells surrounding the oocyte (i.e., the granulosa and thecal cells), which are essential for subsequent oocyte development, ovulation, and fertilization. Modern technologies have opened many new and exciting approaches by which investigators can explore the molecular, cellular, and physiologic mechanisms controlling follicle formation and growth.

2 Novel Aspects of Gonadal Development, Primordial Follicle Formation, and Early Follicle Growth

The mammalian gonad first develops adjacent to the urogenital ridges as a thickening of the coelomic epithelium and is devoid of germ cells. Migrating primordial germ cells (PGCs) that were specified outside the embryo colonize the indifferent gonad, then undergo a period of proliferation. In females, the PGCs then enter meiosis and arrest in the first meiotic prophase. Many of the underlying signaling events that control ovary specification during this time are still being analyzed, but several key pathways have been identified. One of these is the WNT pathway. The WNT family is comprised of secreted glycoproteins that bind to, and signal through, the FRIZZED (FZD) receptors. Mice null for *Wnt4* exhibit

abnormal ovarian morphology in which structures similar to testicular chords are observed (Vainio et al. 1999), indicating that WNT4 might be a specific determinant of the female gonad. Mutations in the human *RSPO1* gene, a WNT pathway adapter molecule, indicate that this molecule is also a candidate female sex determining factor (Parma et al. 2006) and female mice null for *Rspo1* demonstrate partial sex reversal and oocyte loss (Tomizuka et al. 2008). Quite remarkably, mice null for both *Wnt4* and *Foxl2*, a forkhead box transcription factor, exhibit complete and functional sex reversal of the ovary to a testis in the XX genotype (Ottolenghi et al. 2007). These intriguing and novel results document unequivocally that there are organizers of ovarian vs. testis development. By contrast, XY male mice expressing stable beta catenin (CTNNB1), a downstream target of WNT signaling, using *Sf1-Cre* mice (Maatouk et al. 2008) or *Amhr2Cre* mice (Chang et al. 2008) exhibit partial male to female sex reversal with ovarian structures totally lacking germ cells or that exhibit seminiferous tubule demise and germ cell loss, respectively. Thus, proper WNT signaling, likely involving a critical role for *Rspo1* as well as a FZD receptor, is essential for normal gonad development.

Proper expression of CTNNB1 in the adult ovary is also essential for normal tissue maintenance because overexpression of a constitutively active form of *Ctnnb1* (*Ctnnb1*$^{flox(exon3)}$) can lead to abnormal follicle development and eventually to granulosa cell tumors (GCTs) (Boerboom et al. 2005). Moreover, the tumor phenotype can be enhanced when the tumor suppressor *Pten* is simultaneously disrupted in the *Ctnnb1*$^{flox(exon3)}$*; Amhr2-Cre* mouse strain. In these mice, abnormal lesions are observed in the embryonic gonad, aggressive tumors form before puberty, and the mice die within 6 weeks of age (Lague et al. 2008). Because FSH has been shown to phosphorylate and inactive GSK3β, a downstream component of the WNT/FZD signaling pathways that regulates CTNNB1, FSH also has the potential to enhance the transcriptional activation of CTNNB1 and its target genes but the physiological relevance of this pathway remains to be determined (Cross et al. 1995).

After their proliferative period, PGCs eventually divide to form syncytia of oocytes (termed germ cell nests or cysts) that are connected by intracellular bridges. These bridges are not essential for fertility in females (Greenbaum et al. 2006). Germ cells cysts break down during formation of primordial follicles, when individual oocytes become surrounded by somatic ("pre-granulosa") cells, putatively derived from the coelomic epithelium. The breakdown occurs prenatally in humans or shortly after birth in mice. Germ cell cyst breakdown is associated with massive germ cell loss, such that oocyte numbers are reduced from approximately six million in the fetal human ovary to one million at birth. These numbers further decline to puberty into adulthood (Faddy et al. 1992; Block 1952; Baker 1963). Inappropriate germ cell cyst breakdown may result in ovarian follicles with more than one oocyte, often called polyovular follicles or multiple oocyte follicles. Some inbred mouse strains are known to have increased incidence of polyovular follicles (Engle 1927; Jagiello and Ducayen 1973), and many mouse knockouts have been made that demonstrate this phenotype as well, included several in the TGFβ family. These include mice that overexpress the inhibin α subunit (McMullen et al. 2001),

mice conditionally null for ovarian activins (Pangas et al. 2007) or follistatin (Jorgez et al. 2004), and mice null for *Bmp15* (Yan et al. 2001). Exposure of neonatal mice to estrogen also increases polyovular follicle formation (Kipp et al. 2007; Iguchi et al. 1986, 1990; Iguchi and Takasugi 1986; Chen et al. 2007). This occurs in conjunction with loss of the activin β subunits (Kipp et al. 2007). These data along with the polyovular phenotype displayed in ovaries of activin βA conditional knockout (cKO) mice (see below) suggest a direct role for activin signaling in the appropriate organization of primordial follicles (Pangas et al. 2007). The effects of estrogen on primordial follicle formation have important implications regarding estrogen-like environmental contaminants that act as endocrine disruptors and may impact early follicle formation and eventually the ability to reproduce.

In theory, increasing the size of the primordial follicle pool may be one way to extend reproductive lifespan and prevent diseases associated with menopause or reproductive senescence, such as increased cardiovascular disease and osteoporosis. For example, adult female mice null for the proapoptotic gene *Bax* have increases in primordial follicle numbers, an extended period of folliculogenesis, and decreases in age-related health defects (i.e., *Bax* knockout mice demonstrate decreased bone and muscle loss, adiposity, alopecia, and some behavioral changes, amongst other measured parameters) (Perez et al. 1999, 2007), although some of these changes may not be directly related to ovarian function. However, recent studies have suggested that *Bax* deficient ovaries have an increase in follicular endowment that is due to increased embryonic oogonia proliferation and not a rescue of oocytes from apoptosis (Greenfeld et al. 2007). The factor(s) that govern oogonia proliferation and germ cell survival during germ cell cyst breakdown during embryogenesis and gonadogenesis are not known, and thus remain a key research focus.

3 Transcription Factors That Regulate Early Postnatal Follicle Growth

Recent studies have identified a number of transcription factors whose expression, at least in adult tissues, appears to be restricted to germ cells or oocytes, and which are necessary for early folliculogenesis (Pangas and Rajkovic 2006). These transcription factors control, in part, the coordinated expression of genes necessary for early follicle growth, including growth and differentiation factor 9 (*Gdf9*) (see below) and the zona pellucida genes (*Zp1-3*). Factor in the germline alpha (FIGLA) was the first of these transcription factors to be identified (Liang et al. 1997), and mice null for *Figla* are sterile and primordial follicles do not form in the ovary (Soyal et al. 2000). *Figla* encodes a basic helix–loop–helix (bHLH) transcription factor that regulates expression of the zona pellucida genes, which encode the egg coat (Liang et al. 1997). Subsequent to the discovery of *Figla*, several other

germ-line expressed bHLH transcription factors have been identified, including spermatogenesis and oogenesis bHLH transcription factors 1 and 2 (*Sohlh1* and *Sohlh2*). SOHLH1 and SOHLH2 are approximately 47% identical in the bHLH sequence, have a similar expression pattern in oocytes, and mice null for either gene have a similar female phenotype: postnatal oocyte loss leading to female sterility (Choi et al. 2008; Pangas et al. 2006a). Gene expression changes are similar in the mutant mice, with alterations in expression of genes known to be critical in folliculogenesis. Both knockout mouse models have deficiencies in ovarian expression of several homeobox transcription factors, *Lhx8*, *Pou5f1* (Oct4), and *Nobox*; in *Figla* and the zona pellucida genes *Zp1* and *Zp3*, in growth factor *Gdf9* and the kit ligand receptor, *Kit*. In addition, deletion of *Sohlh2* results in a more than 90% decrease in *Sohlh1*, while deletion of *Sohlh1* causes a 60% reduction in *Sohlh2* (Choi et al. 2008), i.e., *Sohlh2* mutant ovaries lack both *Sohlh1* and *Sohlh2* (are in effect doubly mutant), while *Sohlh1* ovaries are hypomorphic for *Sohlh2*. It is possible then that SOHLH2 regulates *Sohlh1* expression and much of the phenotype in both mouse models may be a direct consequence of loss of *Sohlh1*. Additional gene expression changes can be attributed to loss of *Nobox* (newborn ovary homeobox gene) expression. NOBOX has been shown to directly regulate expression of *Gdf9* and *Pou5f1* (Choi and Rajkovic 2006), and deletion of *Nobox* causes female sterility and postnatal oocyte loss (Rajkovic et al. 2004). Currently, it is unclear how these transcriptional networks intersect to control oocyte development, and which genes are direct targets of the various oocyte-expressed homeobox and bHLH transcription factors.

While deletion of oocyte-expressed genes is a straightforward approach with little to no embryonic or adult phenotypic consequences beyond those due to reproductive dysfunction, many genes expressed in oocytes are also expressed in other adult or embryonic tissues. This makes it necessary to develop conditional mouse models to study their intraovarian function, most commonly by using the Cre/lox site-specific recombination system. Generation of oocyte-specific gene deletion in mice has been facilitated by a number of mouse lines with oocyte-restricted promoters to express Cre recombinase [reviewed in (Pangas and Matzuk 2008)]. In particular, Cre recombinase expression from the *Zp3* promoter has been widely used (Lewandoski et al. 1997). More recently, the *Gdf9* promoter, which has a slightly earlier oocyte expression pattern than the *Zp3* promoter, has been used to express Cre recombinase in oocytes (Lan et al. 2004). Various oocyte conditional knockouts and knockdowns with female reproductive phenotypes include *Pten* (see below) (Reddy et al. 2008), *Cpeb* (cytoplasmic polyadenylation element binding protein) (Racki and Richter 2006), *Gcnf* (*Nr6a1*; an orphan nuclear receptor) (Lan et al. 2003), *Pig-a* (phosphatidyllinositol glycan class-A) (Alfieri et al. 2003), and *Pou5f1* (POU-type homeodomain-containing DNA-binding protein, Oct4) (Kehler et al. 2004). Mouse models to study somatic cell function during primordial, primary, and secondary follicle formation are lacking, in part due to the paucity of mouse lines that direct efficient expression of Cre recombinase to the somatic cell compartments during primordial and primary cell stages (see below). However, some models mice expressing *Sf1-Cre* (Maatouk et al. 2008), *Amhr2-Cre*

(Chang et al. 2008; Boerboom et al. 2005), and *Cyp19-Cre* (Fan et al. 2008a,b, 2009) have been useful.

Members of the forkhead family such as *Foxl2* and *Foxo3* also impact early follicle growth. Targeted disruption of *Foxl2* in mice leads to abnormal follicle development and premature ovarian failure (Uda et al. 2004a), and in humans is also associated with the craniofacial disease, blepharophimosis, ptosis, and epicanthus inversus syndrome (BPES) (Crisponi et al. 2001). *Foxl2* is expressed in the early stages of gonadal development and has been shown to direct ovarian and oppose testis development. Specifically, genes that increase in early postnatal *Foxl2* null ovaries include *Dax1* (*NrOb1*) and *Wnt4*; genes that decrease include *Nr5a2*, *Cyp19*, *Fst*, and *Apoa1*. Additional genes regulated in the ovary by FOXL2 at later stages of follicle development include *Inhbb*, *Nr5a2*, *Srebf1*, *Pgc1a*, *Cyp11a1*, and *Star* (Pisarska et al. 2004; Uda et al. 2004b; Moumne et al. 2008). These results indicate that FOXL2 likely impacts not only embryonic ovarian formation but also specific basic metabolic aspects required for somatic cell proliferation and differentiation. Reduced levels of *Foxl2* have also been linked to aggressive progression of ovarian GCTs (Kalfa et al. 2008), indicating that FOXL2 may regulate multiple effects in granulosa cells that are context and stage specific. Another striking, recently published, ovarian phenotype occurs in mice in which the *Foxo3* gene has been disrupted (Castrillon et al. 2003). These mice exhibit premature ovarian failure due to inappropriate oocyte activation and the premature entry of primordial follicles into the growing pool. Upon exhaustion of the primordial pool, the ovaries become devoid of growing follicles and the mice are infertile. In line with these studies, forced overexpression of *Foxo3* selectively in oocytes reduces the number of follicles growing (Liu et al. 2007). Because the activity of FOXO3 is negatively regulated by the PI3kinase (PI3K) pathway, investigators also generated mice in which the *Pten* gene was conditionally disrupted in oocytes (Reddy et al. 2008). Because PTEN is a negative regulator of PI3K, its removal enhances PI3K activity leading to increased phosphorylation of downstream targets including AKT and FOXO3. As a consequence, the activity and levels of FOXO3 are dramatically reduced, leading to premature oocyte activation and release of primordial follicles into the growing pool thereby generating an ovarian phenotype identical to that of the FOXO3 null mice. Although microarray data have been generated from the *Foxo3* null ovaries, the specific targets of FOXO3 in the oocyte that impact the surrounding somatic cells remain to be defined (Gallardo et al. 2007).

In contrast to FOXO3, which is expressed in and impacts oocyte functions, FOXO1 is expressed preferentially and at high levels in granulosa cells of growing follicles. Because *Foxo1* null mice are embryonic lethal (Hosaka et al. 2004), an analysis of the role of this transcription factor in the ovary has been precluded. However, mice in which *Foxo1*, *Foxo3*, and *Foxo4* alleles have been engineered to contain loxP sites ("floxed" alleles) for conditional deletion provide the opportunity to determine the cell specific disruption of these genes individually or collectively in the ovary (Paik et al. 2007; Tothova et al. 2007). These studies are now in progress and suggest that disruption of *Foxo1* impairs fertility. Although the mechanisms

remain to be determined, FOXO1 may impact specific genes controlling proliferation (Park et al. 2005), differentiation (Park et al. 2005), or metabolic pathways (Liu et al. 2008a) in granulosa cells based on the expression of FOXO1 mutants in these cells. Specifically, expression of a constitutively active nuclear form of FOXO1 (FOXOA3 in which three serines have been substituted for alanines) in granulosa cells not only suppresses expression of *Ccnd2*, *Cyp19*, *Fshr*, and *Lhcgr* but also acts as a potent negative regulator of essentially all genes in the cholesterol biosynthetic pathway (Park et al. 2005; Liu et al. 2008a). The negative effects of FOXO1 appear to be mediated in part by the ability of the FOXO1 mutants to interact with other transcription factors including nuclear receptors, SP1 and SMADs (van der Vos and Coffer 2008; Rudd et al. 2007), and to reduce expression and activity of *Srebf1* and *Srebf2* in granulosa cells (Liu et al. 2008a). Because these two transcription factors regulate essentially all genes in the cholesterol pathway and some involved in fatty acid synthesis as well, reduction of these transcription factors impacts multiple genes that coordinate cholesterol and fatty acid biosynthesis. Likewise in liver (Zhang et al. 2006; Matsumoto et al. 2006) and pancreatic beta cells (Buteau et al. 2007), FOXO1 appears to play a major role in cholesterol and glucose homeostasis. Thus, drugs given to regulate cholesterol levels in humans or patients with diabetes will likely and potently impact the function of ovarian cells as well.

4 Oocyte-Derived Growth Factors That Mediate Somatic Cell Function and Follicle Growth

Early follicle growth (i.e., after primordial follicle activation but before antrum formation) is considered to be largely driven by ovarian-derived growth regulatory factors independent of pituitary-derived follicle stimulating hormone (Kumar et al. 1997). The first of these intraovarian factors to be identified was oocyte-derived GDF9, a member of the transforming growth factor β superfamily (McPherron and Lee 1993; McGrath et al. 1995). In the mouse, *Gdf9* is first expressed in oocyte cysts and primordial follicles of newborn ovaries (Rajkovic et al. 2004), although the protein is undetected until follicle stage 3a (a class of primary follicles) and subsequently increases in level in all other follicles (Elvin et al. 1999a). Consistent with this, mice with a genetic disruption of *Gdf9* are infertile and demonstrate abnormal follicle development, with an arrest at the primary follicle stage (Dong et al. 1996). However, the primary follicles that form have abnormal oocytes and somatic cells. While granulosa cells initially organize around the oocyte, they are defective in their proliferation. In addition, the thecal cell layer fails to organize. These defects occur in concert with inappropriate and accelerated oocyte growth, leading to abnormally large and defective oocytes (Carabatsos et al. 1998). Primary follicle stage arrest can be partially rescued by removing expression of the inhibin alpha (*Inha*) gene, which is inappropriately upregulated in granulosa cells of *Gdf9*

knockout (KO) ovaries (Elvin et al. 1999b), suggesting that suppression of *Inha* expression in granulosa cells is an important step in early folliculogenesis to allow normal granulosa cells to grow and differentiate. Follicles from double mutant *Inha Gdf9* homozygous null mice are able to form multilayer follicles, but then arrest prior to antrum formation and do not develop a functional thecal cell layer (Wu et al. 2004). These data further highlight the importance of the TGFβ family in multiple stages of follicle development, though many of these functions are still not understood.

Other members of the TGFβ family that influence follicle physiology and growth include BMP15 and activin. Similar to GDF9, BMP15 is an oocyte-derived growth factor (Dube et al. 1998) that functions by regulating granulosa cell proliferation and differentiation. Mice with homozygous null mutations in *Bmp15* are subfertile on some genetic backgrounds, while mice deficient for both *Gdf9* and *Bmp15* phenocopy the *Gdf9* homozygous null mouse model (Yan et al. 2001). However, removal of one copy of *Gdf9* in a *Bmp15* null background results in additional decreases in fertility compared with $Bmp15^{-/-}$ (Yan et al. 2001). It appears that BMP15 is not critical for early follicle development in mice, or alternatively, its loss may be compensated for at these early stages by GDF9. However, BMP15 appears to influence the development of the granulosa cell layer most closely associated with the oocyte, collectively called the cumulus cell layer. Studies on double mutant $Gdf9^{+/-}$ $Bmp15^{-/-}$ mice demonstrate that cumulus cells cannot appropriately respond to signals from wild type oocytes to undergo the process of cumulus expansion (see below), suggesting that the cumulus cells in double mutant $Gdf9^{+/-}$ $Bmp15^{-/-}$ follicles are developmentally compromised (Su et al. 2004). The nature of these molecular defects is currently unknown but may be related to changes in cumulus cell metabolism (Su et al. 2008). Mouse and human BMP15 are mitogens for granulosa cells (Otsuka et al. 2000; McNatty et al. 2005), and transgenic overexpression of mouse BMP15 in oocytes causes normal but accelerated follicle development and subsequently, an early onset of acyclicity (McMahon et al. 2008). Even though GDF9 and BMP15 are highly conserved, there appears to be species-specific differences regarding their function within the ovary (Juengel and McNatty 2005). Homozygous sheep mutations in BMP15 have an ovarian phenotype that appears similar to the mouse *Gdf9* knockout. The BMP15 mutations when carried as only a single copy in sheep result in an increased ovulation rate, while no phenotype has been associated with *Bmp15* or *Gdf9* heterozygous mutations in mice. In humans, mutations in BMP15 and GDF9 have been infrequently found to be associated with premature ovarian failure (Di Pasquale et al. 2004, 2006;Simpson 2008), though heterozygous mutations in BMP15 have not been reported for twinning in humans (Zhao et al. 2008). Because of their restricted expression pattern and ability to modulate fertility, BMP15 and GDF9 might be good candidates for contraceptive development. Initial experiments demonstrate that sheep immunized against BMP15 or GDF9 have abnormal folliculogenesis and ovulation rates (McNatty et al. 2007; Juengel et al. 2002). Targeting antibodies to N-terminal peptides appear to be the most efficient means to neutralize their bioactivity (McNatty et al. 2007).

5 Novel Regulatory Mechanisms That Control Follicle Growth and Differentiation

Although many early stages of follicle growth can occur independently of pituitary gonadotropins, ovarian follicles, and more specifically granulosa cells, rely on FSH for follicular antrum formation and for continued growth and differentiation during the antral follicle stages. Moreover, recent studies provide new insights into the multiple signaling pathways that are stimulated in granulosa cells by FSH. This glycoprotein hormone is known to activate adenylyl cyclase, leading to the production of cAMP and the activation of protein kinase A (PKA). There is no doubt that activation of this classical pathway is essential for many aspects of granulosa cell differentiation. However, FSH can also activate the PI3K pathway (likely via a SRC tyrosine kinase) leading to the phosphorylation and activation of AKT, which phosphorylates and thereby inactivates FOXO1 (Gonzalez-Robayna et al. 2000). As mentioned above, FOXO1 has the potential to regulate cholesterol metabolism in granulosa cells, thereby preventing premature increases in precursors for steroidogenesis (Liu et al. 2008a). FOXO1 can also reduce the expression of genes regulating granulosa cell proliferation and differentiation (Park et al. 2005; Liu et al. 2008a). As mentioned, because of the embryonic lethality of the *Foxo1* null mutation, the effects of disrupting *Foxo1* in granulosa cells have not yet been analyzed in vivo. However, the disruption of *Pten* in granulosa cells leads to increased activation of the PI3K pathway, and therefore increased phosphorylation and degradation of FOXO1, resulting in enhanced proliferation, ovulation, and the formation of corpora lutea that persist for unusually prolonged periods of time (Fan et al. 2008a). Surprisingly, although FOXO1 is expressed at elevated levels in granulosa cells, PTEN protein levels are remarkably low. Therefore, factors other than, or in addition to, PTEN may serve to control the PI3K pathway in granulosa cells. These results indicate that the functions of PI3K pathway components in granulosa cells are complex and likely to be stage- and context-specific (Fan et al. 2008a). Thus, disruption of *Pten* in the somatic cells of the mouse ovary causes distinctly different effects from the disruption of this gene in oocytes, as described above (Castrillon et al. 2003; Liu et al. 2007). Furthermore, although natural mutations or disruption of *Pten* in other tissues leads to tumor formation, the disruption of *Pten* alone in granulosa cells did not lead to granulose cell tumors (Fan et al. 2008a), perhaps because other factors impact the PI3K pathway in these cells.

FSH and LH have recently been shown to activate RAS via a SRC tyrosine kinase-mediated process (Wayne et al. 2007). Activated RAS then leads to the phosphorylation and activation of downstream kinases, MEK1 and MAPK3/1 (also known as ERK1/2) (Wayne et al. 2007). Strikingly, KRAS is expressed at high levels in granulosa cells of small and antral follicles but the role of KRAS in granulosa cells remains to be determined (Fan et al. 2008b). Expression in granulosa cells of a constitutively active form of KRAS, $KRAS^{G12D}$, which is frequently associated with various cancers including ovarian cancer and cell transformation,

does not stimulate proliferation or tumor formation in these cells (Fan et al. 2008b). Rather, the $KRAS^{G12D}$ expressing granulosa cells cease dividing, do not exhibit apoptosis, and fail to differentiate, i.e., they become senescent. As a consequence, the abnormal follicle-like structures persist and accumulate in the ovaries of the $KRAS^{G12D}$ mutant mice. Even when *Pten* is disrupted in the $Kras^{G12D}$ mutant strain, GCTs do not form (Fan et al. 2009). These results indicate that granulosa cells are extremely resistant to the oncogenic insults of mutant *Kras* and the loss of *Pten*. By contrast, if the *Kras* and *Pten* mutations are made in ovarian surface epithelial cells, aggressive tumors appear within 6 weeks of age (Fan et al. 2009).

6 The TGFβ Family in Regulation of Granulosa Cell Growth and Differentiation

The TGFβ family of growth factors has wide-ranging roles in female reproduction. Various family members are expressed from the major ovarian cell types (i.e., oocytes, granulosa cells, thecal cells), though many of the effects appear to center on control of granulosa cell growth and differentiation that then impact folliculogenesis and oocyte development. Many recent studies have analyzed the role of this family by cre/loxP-mediated conditional deletion in granulosa cells. Two Cre recombinase lines are particularly used for granulosa cell deletion: *Amhr2cre*, a knockin of Cre recombinase into the anti-Mullerian hormone receptor type II locus (Jamin et al. 2002), and *Cyp19-Cre*, a transgenic line that contains a portion of the aromatase gene that limits Cre expression to granulosa cells and luteal cells (Fan et al. 2008a). While both Cre lines are expressed in granulosa cells, subtle differences may exist in their expression pattern, with *Cyp19-Cre* being expressed in slightly later stage follicles than *Amhr2-Cre* (Fan et al. 2008a).

Follistatin, a BMP and activin antagonist, was the first gene to be conditionally deleted from granulosa cells (Jorgez et al. 2004). Ovaries from follistatin knockout mice have almost complete loss of germ cells prior to birth (Yao et al. 2004). *Fst* conditional knockout female mice (cKOs) demonstrate premature ovarian failure, with few remaining follicles found by 8 months of age (Jorgez et al. 2004). Fertility defects are accompanied by changes in the levels of serum hormones, including increases in follicle stimulating hormone (FSH), luteinizing hormone (LH), and decreases in serum testosterone. Loss of follistatin within the ovary likely results in increased activin activity and possibly, BMP activity. The loss of intraovarian activins results in a different phenotype. Activin is a homo or heterodimer of two related β subunits: βA and βB. Mice null for βA die shortly after birth (Matzuk et al. 1995), but mice deficient for βB have normal size litters but defects in nursing (Vassalli et al. 1994). Ovaries from βB deficient females overproduce the βA subunit (Vassalli et al. 1994), suggesting that any intraovarian reproductive phenotype that is caused by loss of βB may be masked by a compensatory gain in activin A. Thus, the stepwise removal of the activin subunits by conditional deletion in granulosa cells eventually culminates in female sterility when no activin subunits

are expressed (Pangas et al. 2007). While there are multiple defects in folliculogenesis in the activin deficient ovary (Pangas et al. 2007), one of the most obvious defects is the progressive and abnormal accumulation of corpora lutea that is accompanied by increases in serum FSH and progesterone. Other defects include preantral follicles undergoing early luteinization and an increased number of antral follicles. There are likely additional defects in granulosa cells during ovulation because the increase in antral follicle numbers is not reflected in the number of ovulated oocytes, which is significantly decreased. Even though mutations in the activin signal transduction pathway have been implicated in cancer development, and activins have been shown to be critical for growth inhibition in some cell types (i.e., breast and prostate cancer cells) (Cocolakis et al. 2001; Zhang et al. 1997), no tumors develop in the activin-deficient mouse model. Thus activin, like TGFβ, may have variable oncogenic or tumor suppressor properties that are cell-type or context-specific. For example, in granulosa cells, activin appears to play a predominant role as a growth promoter, and its role in the promotion of GCTs has been established in the inhibin alpha knockout mouse. Deletion of inhibin α results in sex-cord stromal tumors in male and female mice and premature death due to development of a cancer cachexia like syndrome (Matzuk et al. 1992, 1994). Genetic removal of the activin type II receptor, deletion of the activin downstream transcription factor *Smad3*, or injection of a chimeric activin binding receptor-murine Fc protein, slows, though does not prevent, tumor growth in inhibin α-deficient mice (Matzuk et al. 1992, 1994; Coerver et al. 1996; Li et al. 2007a, b; Looyenga and Hammer 2007), demonstrating that activin signaling plays a growth promoting role.

The role of the TGFβ family in ovarian follicles has also been investigated by deletion of the SMAD transcription factors, which are part of the TGFβ family canonical signaling pathway. SMAD2 and SMAD3 signal for activin, GDF9, and TGFβ, while SMAD1, SMAD5, and SMAD8 signal for the BMPs and AMH. An additional SMAD, SMAD4, is shared by all members of the TGFβ family. Conditional mutations for these SMADs have been generated in granulosa cells (Li et al. 2008; Pangas et al. 2006b, 2008). Conditional deletion of *Smad4* results in age-dependent infertility, with defects in steroidogenesis, ovulation, cumulus cell function, and eventually premature ovarian failure (Pangas et al. 2006b). Unlike the activin-deficient mouse model, *Smad4* cKO ovaries show an increase in preantral follicle death, a decrease in the number of antral follicles, and no accumulation of CLs. Similar to the activin-deficient ovary, small follicles appeared to luteinize prematurely, and even though *SMAD4* is a known tumor suppressor gene, no tumors developed in *Smad4* cKO mice. Cumulus cells in the *Smad4* cKO are defective and undergo a disorganized or limited cumulus cell expansion. The defects in preantral follicle growth and cumulus cells may be attributable to the inability of GDF9 to fully function through the SMAD pathway when *Smad4* is deleted.

A similar phenotype to *Smad4* cKO female mice is seen in granulosa cell conditional knockouts of the activin/TGFβ signaling SMADs (AR-SMADs), *Smad2* and *Smad3* (Li et al. 2008). SMAD2 and SMAD3 have both unique and redundant roles in various tissues, but appear to have redundant functions in

granulosa cells because single conditional knockouts of *Smad2* or *Smad3* in granulosa cells have no discernable reproductive phenotype (Li et al. 2008). However, double *Smad2 Smad3* cKO mice using *Amhr2cre* have reduced litter sizes and become infertile after 5 months of age with disrupted follicle development (i.e., fewer antral follicles), luteinized follicles, and reduced ovulation, with severe defects in cumulus cell function. The phenotypes of conditional knockouts for the BMP SMADs (BR-SMAD) have phenotypes that differ dramatically from the other SMAD conditional knockout models. Single conditional mutants *Smad1* or *Smad5*, or *Smad8* KO mice, are viable and fertile. The combinations of double conditional Smad1 Smad8, or Smad5 Smad8, are also fertile. However, reproductive phenotypes are seen in double conditional Smad1 and Smad5, or triple conditional Smad1 Smad5 Smad8 mice (Pangas et al. 2008). Both *Smad1 Smad5* dKO or *Smad1 Smad5 Smad8* tKO have initial fertility defects with decreased litters per month and increasing infertility with age. These mice also develop GCTs with full penetrance by 3 months of age. In addition, the majority of Smad1, Smad5 dKO and Smad1, Smad5, Smad8 tKO mice show peritoneal implants and lymphatic metastases over time. The Smad1, Smad5 dKO and Smad1, Smad5, Smad8 tKO models were the first in vivo demonstration that the BMP SMADs may have a critical tumor suppressor function.

The phenotypes of the various SMAD and activin/inhibin knockouts in the ovary suggest a potential interaction between the various BMP and TGFβ/activin pathways in controlling granulosa cell growth and differentiation. Of the knockouts generated in the TGFβ family, only two TGFβ family mouse models develop GCTs: the inhibin α KO and the BR-SMAD cKOs. Part, though not all, of the phenotype of the inhibin α KO has been attributed to activin's tumor promoting activity in part via SMAD3 (Li et al. 2008) (see above). In the BR-SMAD cKO mouse models, an examination of the phosphorylation status of the AR-SMADs demonstrated that SMAD2 and SMAD3 are nuclear and phosphorylated, indicating pathway activation; thus, it has been suggested that part of the phenotype of the BR-SMAD cKO may be due to dysregulated AR-SMAD (i.e., *Smad2* and *Smad3*) pathway regulation (Pangas et al. 2008). Thus, one of the roles of the BR-SMADs may be to antagonize or cross-regulate the AR-SMADs to control cell proliferation. The role of additional signaling pathways in tumorigenesis in the BR-SMAD cKO models is still under investigation.

Disrupting the function of TGFβ family ligands and their signaling pathways not only influences somatic cell function, but eventually results in improper oocyte development. As originally proposed by John Eppig (1991), oocytes secrete factors now known to include GDF9 and BMP15, which based on mouse knockout studies are known to control specific somatic cell functions, including optimal expansion of the cumulus oocyte complex prior to ovulation (Elvin et al. 1999a; Pangas and Matzuk 2005; Vanderhyden et al. 2003). Most recently, Eppig et al. (2008, 2007) have published novel results indicating that oocyte-derived factors regulate cumulus cell production of key metabolic substrates presumed essential for oocyte quality and viability (Eppig et al. 2005; Sugiura et al. 2005a, b). Specifically, oocytes do not make their own cholesterol, fatty acids, or glucose. Rather the

oocytes, most probably by release of BMP15 and GDF9, control cumulus cell expression of specific genes within the cholesterol biosynthetic pathway and the glycolytic pathway. As noted above, FOXO1 also appears to regulate genes in the cholesterol biosynthetic pathway, and thus there may be a functional link between FOXO1 and the oocyte derived growth factors BMP15/GDF9, though this remains to be determined.

7 New Mediators of Ovulation and Luteinization

Based on recent studies, we know that the LH surge can stimulate PKA, AKT, and RAS signaling cascades, and that each of these appears critical for ovulation (Fan et al. 2008b). Most importantly, the seminal studies of Marco Conti and colleagues have shown that LH rapidly induces in granulosa cells the expression of the EGF-like factors amphiregulin (AREG), betacellulin (BTC), and epiregulin (EREG) (Conti et al. 2005). These factors bind the EGF receptors present on granulosa cells and induce the expression of downstream target genes, *Has2*, *Ptgs2*, and *Tnfaip6*, which in cultured cells are targets of ERK1/2 (Shimada et al. 2006). Disruption of the EGF ligand-receptor signaling pathway in mice compromises ovulation, indicating that activation of this pathway is essential for LH-induced ovulation to occur (Hsieh et al. 2007). Moreover, mice in which ERK1 and ERK2 have been disrupted in granulosa cells exhibit normal follicle growth but fail to ovulate or luteinize (unpublished observations). Thus, the critical importance of LH induction of the EGF-like factors and activation of the EGF receptor pathway is mediated, in large part by the activation of ERK1/2 in granulosa cells as well as in cumulus cells (Shimada et al. 2006). Specifically, the prostaglandins (PGE2) and the EGF-like factors produced by granulosa cells then activate specific PGE and EGF receptors present in cumulus cells leading to the expression of specific genes involved in expansion of the cumulus oocyte complex and oocyte maturation (Shimada et al. 2006). The genes involved in expansion include factors essential for making and stabilizing the hyaluronan matrix (*Has2*, *Ptgs2*, *Tnfaip6*, *Vcan*, and *Ptx3*) but also additional genes frequently associated with innate immune responses, including components of the Toll-like receptor pathway, *Cd34*, *Cd52*, *Alcam*, many potent cytokines, such as IL6, as well as transcription factors *Runx1* and *Runx2* (Shimada et al. 2006; Liu et al. 2008b, 2009; Richards et al. 2008).

The impact of cytokines on ovarian function represents a relatively new area of investigation. Recently, IL6 alone has been shown to stimulate expansion of the cumulus oocyte complexes and induce the expression of specific genes involved in this process (Liu et al. 2009). These observations indicate that in clinical situations where levels of IL6 are elevated, such as chronic infections, endometriosis, and possibly PCOS, this and other potent cytokines may disrupt the normal functionality of granulosa and cumulus cells. IL6 acts via specific receptors (IL6ST, also known as gp130) present on cumulus cells as well as the oocyte. Moreover, IL6 can increase the expression of *Stat3* and *Il6st* in cumulus cells and the oocyte present in

preovulatory follicles and enhance reproductive outcomes, suggesting that this pathway significantly influences oocyte quality (Liu et al. 2009). Of note, mice null for *gp130*/*Il6st* exhibit defects in zygotic cell division, suggesting that IL6 and related cytokines regulate oocyte function (Molyneaux et al. 2003). Because the induction of IL6 is regulated not only by AREG (Liu et al. 2009) but also by the progesterone receptor (PGR) (Liu et al. 2009; Kim et al. 2008) and possibly CEBPβ (unpublished observations) that are essential for ovulation, it is tempting to speculate that IL6 may mediate some key process downstream of PGR and CEBPβ in granulosa cells. Moreover, the expression of SNAP25, an important component controlling neuronal-like secretion of cytokines from granulosa cells, is also regulated by PGR (Shimada et al. 2007). Thus, the importance of locally produced and secreted ovarian cell derived cytokines during ovulation needs to be analyzed further and may be relevant for several ovulation-related processes including rupture, COC transport, and fertilization (Richards et al. 2008). In this regard, it is important to note that cytokines have recently been shown to influence the fertilization process by enhancing sperm motility and capacitation (Shimada et al. 2008).

Because mice null for the nuclear receptor interacting protein *Nrip1* (also known as RIP140) also exhibit impaired ovulation and reduced expression of *Areg*, *Ereg*, and many other ovulation related genes, it is possible that NRIP1 regulates transcription of the *Areg* gene, a critical early event in the ovulation process (Tullet et al. 2005; Nautiyal et al. 2010). Because NRIP1 also impacts metabolic pathways and inflammatory events in other tissues (Nichol et al. 2006), NRIP1 may also regulate additional events critical for ovulation.

Targeted disruption of the transcription factor Steroidogenic Factor 1 (SF1; now known as nuclear receptor subfamily 5, group a, member 1, NR5a1) in mice provided the first major evidence that this nuclear receptor was essential for pituitary, gonad, and adrenal formation (Luo et al. 1994). Conditional deletion of this gene in granulosa cells has documented further that there is a critical role for SF1 in early follicle formation and development (Pelusi et al. 2008). More recently, a conditional knockout of *Lhr1* (*Nr5a2*; an orphan receptor highly similar to *Nr5a1*) in murine granulosa cells has been reported (Duggavathi et al. 2008). Although *Nr5a2* is also expressed in granulosa cells of small and growing follicles, evidence from the conditional disruption of *Nr5a2* in granulosa cells indicates that it plays a more critical role in events associated with ovulation and luteinization than in the early stages of follicle growth. Impressively, the *Lrh1* null mice exhibit impaired ovulation *and* luteinization suggesting that this nuclear factor plays a critical role in both of these processes. Thus, in this in vivo context, these two nuclear receptors appear to exhibit distinct, rather than overlapping, functions. One explanation for this may be related to the impact of specific signaling cascades and phosphorylation of either SF1 or LRH1 that alters their ability to bind key regulatory elements in target genes via a switch-type mechanism (Weck and Mayo 2006). Additionally, LRH1 and SF1 exhibit similar functions when overexpressed in cultured rat granulosa cells with the notable exception that SF1, but not LRH1, can override the inhibitor effects of DAX on FSH stimulation of estradiol and progesterone

biosynthesis (Saxena et al. 2007). However, an essential role for DAX in the ovary is doubtful based on evidence that targeted disruption of DAX does not alter normal follicle development, but does prevent normal testis development (Yu et al. 1998). Thus, DAX is not a potent determinant of ovarian development. The differences in the functions of SF1 and LRH1 also appear to be related, in part, to genes that are selectively regulated by SF1 (*Amh*, *Inha*) (Pelusi et al. 2008) compared with LRH1 (*Cyp19*, *Cyp11a1*, and *Ptgs2*) (Duggavathi et al. 2008). Recently, mice null for the estrogen-specific sulfotransferase (*Sult1e1*) have been generated and exhibit impaired ovulation and cumulus expansion (Gershon et al. 2007), suggesting that LRH1 or other transcription factors potently induce expression of this gene in response to the LH surge. Serum and presumably intraovarian levels of estradiol are elevated in these mice disrupting normal feedback mechanisms. In addition, sulfated estradiol is an inactive form unable to bind estradiol receptors thereby potentially altering ovarian cellular functions. However, the mechanisms by which Sult1e1 disruption impairs ovulation have not been defined. Based on the genes regulated by ERK1/2, LRH1, and NRIP1, it is tempting to speculate that NRIP1 may be an important coregulator and/or activator of LRH1 in the ovary and that ERK1/2 may be required for specific phosphorylation events. Because LRH1 is also important for liver metabolism (Lee and Moore 2008) and embryonic stem cells (Mullen et al. 2007), it will be interesting to determine what specific genes this factor controls in ovarian cells that are distinct from those regulated in liver or embryonic stem cells. One other mutant mouse model in which both ovulation *and* luteinization are impaired is the *Cebpb* null mouse (Sterneck et al. 1997). Thus, it will be important to determine how ERK1/2, CEBPβ, LRH1, and NRIP1coordinately regulate a select number of genes.

8 New Regulators of Oocyte Maturation and Meiosis

The meiotic arrest of oocytes is controlled by critical levels of cAMP within the oocyte. For many years somatic cells were thought to be the source of cAMP that was delivered to the oocyte via gap junction because the disruption of gap junctions elicited spontaneous resumption of meiosis (Norris et al. 2008; Gittens and Kidder 2005). However, recent molecular studies have identified and highlighted a critical role for specific G-protein coupled receptors, especially GPR3 and possibly GPR12 in controlling intraoocyte production of cAMP and thereby suppressing meiotic maturation in oocytes of antral follicles (Mehlmann et al. 2004; Hinckley et al. 2005). Specifically, disruption of *Gpr3* in mice led to premature resumption of meiosis and ovarian "aging" (Mehlmann et al. 2004). Human oocytes also express functional *Grp3* but not *Gpr12* (DiLuigi et al. 2008). Collectively, these studies provide the first evidence that oocytes themselves express a receptor that allows these cells to generate their own cAMP. These observations indicated that intraoocyte cAMP levels were unlikely to be controlled exclusively by transport from somatic cells to the oocyte as previously thought. Furthermore, phosphodiesterase

Pde3a and adenylyl cyclase 3 (*Adcy3*) are selectively expressed in murine and human oocytes (Vaccari et al. 2008). Disruption of *Pde3a*, which increases cAMP, or *Adcy3*, which reduces cAMP, either prevents or stimulates, respectively, precocious oocyte maturation (Vaccari et al. 2008). Moreover, the phenotype of mice null for both *Gpr3* and *Pde3a* indicates that *Gpr3* is the major source of cAMP that disrupts meiosis in the *Pde3a* null mice (Vaccari et al. 2008) because meiosis is restored in the double mutant mice. These results provide additional evidence for intra-oocyte control of cAMP production and degradation. Although the specific ligands for these orphan G-protein coupled receptors remain to be convincingly characterized, sphingosine 1-phosphate is a likely candidate (Uhlenbrock et al. 2002).

Thus, factors that regulate ligand production or that modify receptor activity provide potential targets for designing molecules that specifically target oocytes and thereby regulate fertility by blocking meiosis or by eliciting premature resumption of meiosis. For example, a molecule that would selectively bind to ZP1, 2, or 3 and deliver a potent signal to the oocyte might be engineered and delivered via novel nanoparticles. In addition, specific PKA anchor proteins (AKAPs) are present in oocytes and appear to be key regulators of the cAMP/PKA pathway that controls meiosis in the mammalian oocyte (Burton and McKnight 2007). Therefore, targeting these molecules might also provide novel ways to control fertility.

Perhaps one of the most dramatic and provocative approaches developed to study the dynamics of follicle and oocyte growth comes from studies of Woodruff and Shea (Pangas et al. 2003; West-Farrell et al. 2008; Xu et al. 2006). These investigators and their colleagues have shown in mice that maintaining a 3-D follicle structure within an inert but supportive extracellular alginate matrix permits in vitro follicle growth and oocyte maturation in response to hormones. Moreover, these in vitro matured mouse follicles could ultimately be stimulated to release oocytes capable of being fertilized in vitro and subsequently give birth to viable, healthy pups. These approaches open new and exciting possibilities for preserving ovarian tissues in woman undergoing radiation treatment and who subsequently wish to conceive. Although tedious and currently untested in nonrodent species, these approaches may provide a way to mature follicles and thereby reduce the genetic changes that are observed in oocytes matured in vitro compared with in vivo. These studies may also provide a method to study differences that may exist between individual follicles or oocytes, and potentially be able to provide a "biomarker" (either somatic or germ cell) that may be used to determine the capacity of individual eggs when fertilized to develop into viable offspring.

9 Summary

New insights into regulators of early oocyte and follicle formation (*Nr5a1*, *Nobox*, *Sohlh1*, *Lx8*, *Foxo3*, *Foxl2*, *Wnt4*, *Ctnnb1*), follicular growth (*Gdf9*, *Bmp15*, *Foxo1*, *Smads*, *Inha*, *Inhba*, *Inhbb*) as well as ovulation and luteinization (*Cebpb*, *Nr5a2*,

Areg, *Ereg*, *Btc*, *Nrip1*, *Kras*, *Mapk1/3*, *Il6*) indicate that multiple factors and signal transduction pathways act in a cell specific and context specific manner. To regulate fertility, cumulus oocyte complexes remain an attractive target if one could prevent expansion, alter oocyte/cumulus cell interactions or oocyte maturation by disrupting the actions of specific cytokines or other factors without altering the functions of other major organs. These might provide new avenues for contraceptive research as well as improving fertility in women with endometriosis and PCOS.

Information being derived from new approaches such as the exponential increase in knowledge of microRNAs should provide additional insights into factors and combined sets of factors that regulate genes in a cell and context specific manner (Otsuka et al. 2008; Nagaraja et al. 2008; Hong et al. 2008; Fiedler et al. 2008; Mishima et al. 2008). Because microRNAs control the levels of more than one mRNA, local disruption of these molecules may also provide tools for regulating fertility, cancer, and development. Because the delivery of small molecules by a variety of nanotechnology approaches is being aggressively pursued by many, these approaches may enhance the specificity and cell specific delivery of key regulatory molecules that can impact fertility at critical sites and times.

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Estrogen Signaling in the Regulation of Female Reproductive Functions

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Contents

Abstract Estrogens influence fertility and infertility in animals. This chapter reviews the use of estrogen as a contraceptive through the regulation of its production and action. It is concluded that the use of specific agonists and antagonists of estrogen action that avoid the global and unwanted side effects of estrogen offers new potential methods of contraception.

Keywords Aromatase · Contraception · Estrogen receptors · Estrogens

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1 Introduction

Estrogens have an important role to influence fertility and infertility in mammals. They are members of the steroid hormone family produced principally by the gonads and placenta, but in numerous other tissues also such as breast, bone, skin, vasculature, adipose mesenchymal cells, and numerous sites in the brain. They were shown to have negative and positive feedback effects on the hypothalamic–pituitary axis (Diczfalusy and Fraser 1998). It was established also that estrogens acted on target organs such as the uterus, hypothalamus, pituitary, bone, mammary tissue, and liver, as well as having local actions within the gonads (Hisaw 1947; Hall et al. 2001). These properties of estrogen were exploited by Pincus et al. (1958) in the development of the contraceptive pill for women. This extensive list of target tissues is important when considering targeting estrogens as contraceptives as will be discussed below. Estrogens were shown to act on target cells via nuclear transcription factors, or estrogen receptors (ER), of which two have been identified, ERα and ERβ (Jensen and DeSombre 1973; Kuiper et al. 1996). More recently, there is evidence of a membrane form of ER that might transmit the estrogen signal (Levin 2009).

However, our knowledge of the regulation of the biological actions of estrogens is incomplete. Extrinsic estrogens were shown to have actions other than infertility as observed with the contraceptive pill. Compounds with estrogenic activity developed for the agricultural and plastics industries were shown to cause infertility and tumors in mammals (Sharara et al. 1998). Children born of pregnant women treated with diethylstilbestrol had malformation of the reproductive tracts and development of vaginal cancer (Swan 2000). The known mechanisms of estrogen action could not explain the benefits of phytoestrogens used for hormone replacement therapy (Adlercreutz 1995).

The aim of this chapter is to review the use of estrogen as a contraceptive through the regulation of its production and action. After reviewing the mechanisms for estrogen production and signaling, we shall examine the actions of estrogen that allow us to evaluate their novel contraceptive potential.

2 Production of Estrogens

Estrogens are produced from androgens by the enzyme known as aromatase, which is a member of the cytochrome P450 superfamily and its gene designation is CYP19A1. The gene encoding the aromatase enzyme is some 120 kb long of which 91 kb comprise an extensive 5′ untranslated region. This contains a number of 5′ untranslated first exons which are spliced into the coding region in a tissue-specific fashion. The splicing in each case occurs at a common 3′ junction upstream of the start of translation (Simpson et al. 2002). Thus, the coding region is always the same regardless of the tissue site of expression. This occurs because of the

presence upstream of each of these 5′ untranslated exons of a tissue-specific promoter. These promoters have different *trans*- and *cis*-acting elements regulating their activity. Hence, the expression of aromatase in each of the tissue-specific sites of expression is different. The distribution of aromatase activity in the body includes the gonads, hypothalamus, adipose tissue, bone, and placenta, as well as in some tumors and endometriotic tissues (Simpson et al. 2002). Thus, expression in ovarian granulosa cells and Sertoli cells of the testes is driven by a proximal promoter PII which contains a couple of CREs and hence binds CREB and is regulated by factors which stimulate adenylyl cyclase, leading to increased cyclic AMP and PKA activation. In granulosa cells and Sertoli cells, FSH appears to be the primary trophic hormone responsible for this activation. In the case of adipose tissue, the primary promoter being utilized is the distal promoter I.4, which is regulated by Class I cytokines such as interleukin 6 and TNFα in the presence of glucocorticoids. This response is mediated by a JAK1/STAT3 regulatory pathway. In the case of the placenta, the most distal promoter, promoter I.1, is employed which is some 91 kb upstream from the start of translation. Aromatase expression in adipose tissue is normally quite low; however, in breast adipose tissue in the presence of a tumor, aromatase expression increases three- or fourfold. This increase is due to the use of promoter PII, which is driven by inflammatory factors such as prostaglandin E_2 produced by the tumorous epithelium, which also activates adenylyl cyclase and hence cyclic AMP. Other transcription factors and response elements are also employed by each of these promoters, for example in the case of promoter PII, a monomeric orphan member of the nuclear receptor family is also absolutely required for expression and binds to a nuclear receptor half site downstream of the CREs. In the case of the ovary, this nuclear receptor appears to be SF1, whereas in the tumorous mesenchymal cells of the breast it is LRH1, a closely related member of the nuclear receptor superfamily (Clyne et al. 2002).

3 Cellular Mechanisms of Action

ER is a protein that functions as a major component in the mechanisms of estrogen action, where it binds estrogens to initiate the tissue responses. There are two separate ER proteins, ERα and ERβ, which have distinct tissue expression patterns (Mueller and Korach 2001) in both humans and rodents. ERα and ERβ are encoded from separate genes and chromosomal locations, *ESR1* and *ESR2*, and likely arose due to gene duplication. Development of the homologous recombination technology has allowed scientists to develop unique experimental animal models (Hewett et al. 2005). Gene-targeted knock-out mouse models lacking these receptors exhibit distinct phenotypes (Couse and Korach 1999).

Entry of the hormone into the target cell is thought to be by diffusion where it becomes bound by the ER, which is located primarily in the nucleus, but can also be associated with other cellular organelles such as the plasma membrane. The nuclear ER–estrogen complex can regulate genes, positively or negatively, by

binding directly to specific unique DNA sequences, referred to as estrogen response elements (ERE) contained in the promoter region of regulated genes. Once the hormone receptor complex is formed there is believed to be recruitment of co-regulatory proteins (coactivators or corepressors) which associate directly with the receptor protein at the promoter, in addition to the general transcription complex, producing increased or decreased mRNA levels and associated protein production, and resulting physiological responses (Couse et al. 2006).

An alternatively described mode of action involves an indirect mechanism where the ER does not bind directly to the DNA but interacts with existing transcription factors (e.g., f*os*/*jun*), which is referred to as the tethered mechanism of nuclear receptor gene transcription. To elicit the many actions of the hormone this "genomic" mechanism typically occurs over the course of hours in most tissues and has been shown to involve unique gene groups at different times (Hewitt et al. 2003).

Another mode of action of estrogen is thought to involve a nongenomic mechanism. Such a mechanism has been shown to occur very rapidly within minutes of hormone exposure. Components of this cellular mechanism are shown to be the ER protein itself located in or adjacent to the plasma membrane involving interactions with adaptor proteins such as striatin, caveolin-1 or Shc, or through other, recently described nonER plasma membrane-associated estrogen-binding proteins, such as GPR30 (Otto et al. 2009; Levin 2009), resulting in cellular responses such as activation of MAP kinases which can then act to prime the genomic actions.

Besides the two previously mentioned cellular mechanisms, a third ER activity can also occur which involves the ligand independent activation of the receptor protein. Such an action has been shown experimentally in cells and experimental animal models, involving the activation of kinase cascades (e.g., MAPK or IP3K) by growth factors or other membrane signaling agents (Curtis et al. 1996). The extent to which any of these specific mechanisms are involved in mediating the physiological actions of estrogen is still requiring considerable study to develop effective biomedical understanding and therapeutic application.

4 Estrogens and Contraception

Estrogens, estradiol-17β in particular, are essential for fertility in mammals. They are known to act at key points in the reproductive process in females, such as:

- Development of the ovulatory follicle(s)
- Triggering the midcycle preovulatory surge of gonadotropins
- Altering the consistency of cervical mucus to facilitate sperm transport
- Preparing the endometrial lining of the uterus for implantation

Alterations to the production and or actions of estrogen can disrupt these processes leading to infertility.

4.1 Regulation of Estrogen Production

Targeting the intrinsic production of estrogen as a means of contraception is complex and can lead to untoward side effects. This is exemplified by the use of aromatase inhibitors in breast cancer therapy. In recent years, inhibitors such as Arimidex, Letrozole, and Exemestane have supplanted Tamoxifen in first and second line therapy as well as adjuvant and neoadjuvant therapy for breast cancer. However, use of these compounds results in significant contraindications such as bone loss, joint arthralgia, and possibly cognitive defects. This is because these compounds inhibit the catalytic activity of the aromatase enzyme and thus inhibit its activity globally not only in the breast but in other sites where estrogens have important roles such as the reproductive tissues, bone, brain, and cardiovascular system. Thus, there is no tissue specificity in their action. The only way to achieve this would be to take advantage of the specificity presented by the use of tissue-specific promoters to design inhibitors which block expression of aromatase specifically in a given tissue. This is theoretically feasible. For example, in the postmenopausal woman LRH1 uniquely regulates aromatase expression in the breast but not, as far as we understand, in other tissue sites of expression. Thus LRH1 is a potential target for breast-specific ablation of aromatase expression (Simpson et al. 2002).

In the case of premenopausal women, the use of aromatase inhibitors appears to be less effective since they do not reduce circulating estrogen levels to the extent that they do in the postmenopausal situation and are therefore less likely to be useful as contraceptives. Furthermore, their use could lead to collateral problems of estrogen deficiency.

4.2 Regulation of Estrogen Action

Estrogens can be produced and act locally or they can be secreted and act distally. In both cases, they act on the target cells via specific receptors. The actions of estrogen will be governed by the rate at which they are secreted and metabolized, the presence if any of binding proteins, and the concentrations of receptors. Alterations in metabolism of estrogen or the properties of their binding proteins have not offered any potential as a contraceptive. However, there are agonists and antagonists of the receptors that may offer a new approach.

Contraception has been a successful medical treatment for a number of years. Early studies of Pincus, Greep, Hertz, Greenblatt, and others showed the use of estrogen or estrogen receptor agonists could effectively inhibit gonadotropin secretion and subsequent stimulation of the ovary and ovulation (Pincus et al. 1958). From those early years, using estradiol itself, estrogen derivatives or synthetic estrogens included high doses resulting in concerns over the potential side effects of increasing cancer susceptibility in endocrine responsive tissues such as the

endometrium and breast. Years of investigation have now resulted in a therapeutic approach involving much lower dosing schemes and development of neuroendocrine selective agonists.

Advancement from both clinical cases (e.g., aromatase or ER mutant patients) and experimental animal models, e.g., aromatase knockout (ArKO) (Fisher et al. 1998, Britt and Findlay 2002, 2003) and ER knockout (ERKO) (Couse and Korach 1999) mice, has provided new insights into both the mode of action and the specific estrogen receptor (i.e., ERα or ERβ) signaling molecule involved. Such knowledge allows us to know which type of selective estrogen receptor modulator (SERM) or tissue selective estrogen complex (TSEC) will be effective in contraceptive development and regulation of the hypothalamic pituitary gonadal axis. It appears that the primary mediator of negative feedback is ERα, although studies have implemented ERβ as possibly involved in the ovulatory (proestrus) LH surge required for ovulation. Additionally, ERβ has been shown to be required in the ovary for effective follicle ovulation. Therefore, blocking the LH surge with an ERβ selective antagonist maybe a highly effective approach at both the neuroendocrine and target tissue (e.g., ovarian) level. Since ERβ has minimal, if any mitogenic activity in the uterus or breast, the most common side effect should not be a concern. Another option would be a tissue selective delivery of estrogen or a derivative to neuroendocrine sites to act as an agonist and contraceptive. Such an approach does not concern itself with the specificity of the compound and receptor involved, but rather to the tissue selective delivery and action. Finally, use of an ERα selective agonist to induce negative feedback has the age-old problem of also stimulating ERα activities potentially associated with carcinogenesis. So, the side effects may outweigh the usefulness of that earlier approach, based on our current knowledge of LH regulation. It is obvious that understanding the precise mechanism for ER regulation of LH expression will also provide alternative modes of contraceptive development such as through other signaling pathways or the target tissue selective action at the ovary.

5 Conclusions

While the contraceptive pill containing very low doses of estrogen remains a very effective method, the use of receptor-specific agonists and antagonists that avoid the global and unwanted side effects of estrogen offers new potential methods of contraception. Receptor agonists and antagonists rather than aromatase inhibitors would appear to be a preferable option to explore at this stage.

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Progesterone Receptors and Ovulation

Orla M. Conneely

Contents

Abstract The steroid hormone, progesterone, plays a critical role in the regulation of female ovulation. The physiological effects of progesterone are mediated by two nuclear receptor transcription factors, PR-A and PR-B, which are produced from a single gene and upon binding progesterone regulate the expression of specific gene networks in reproductive tissues. Both null mutation of the PR gene to delete both receptor proteins and selective disruption of the PR-A isoform lead to a failure of ovulation due to disabled follicular rupture in response to gonadotrophin stimulation. Recent studies have revealed that the LH stimulus that triggers ovulation is transduced by PRs residing in mural granulosa cells that induce expression of paracrine signals that interact with cumulus cells to control cumulus matrix function and expansion to facilitate follicular rupture.

Keywords Ovulation · Progesterone

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1 Introduction

The primary function of the ovary is to orchestrate female reproductive capacity by supporting the development and release of a fertilizable oocyte (Richards 1994; Richards et al. 2002; Russell and Robker 2007) and regulating production of hormones essential for maintenance of estrous cyclicity in the absence of fertilization and of pregnancy after successful oocyte fertilization. Oocyte maturation occurs within the ovarian follicle whose early establishment begins in the embryo. At birth, a finite pool of primordial follicles is established consisting of an oocyte surrounded by a single layer of somatic cells, the squamous granulosa cell layer (Eppig 2001). As primordial follicles begin to grow, the granulosa cells transition into a cuboidal cell layer marking the emergence of a primary follicle which further develops through proliferation and deposition of multiple layers of granulosa cells into a secondary or preantral follicle that becomes surrounded by an additional thecal cell layer. At puberty, reproductive competence is established and maintained by a highly coordinated cyclic hormonal control of the hypothalamic–pituitary–ovarian axis (McGee and Hsueh 2000; Richards 1994). The release of pituitary-derived follicle stimulating hormone (FSH) during each reproductive cycle stimulates recruitment and maturation of a subset of preantral follicles. These follicles undergo expansion of cumulus granulosa cells surrounding the oocyte and form a fluid-filled antrum that marks the mature preovulatory follicle (Fig. 1). At this stage, granulosa cells within the follicle are segregated into two functionally distinct sublineages, mural granulosa cells (MGCs) that line the follicular cell wall in contact with the basal lamina and cumulus cells that envelop the oocyte and support its development. The FSH stimulus is followed mid-cycle by a transient surge of pituitary-derived luteinizing hormone (LH) that serves as the primary trigger for follicular rupture and release of a fertilizable oocyte as well as differentiation of remaining granulosa cells into a luteal phenotype to form a functional corpus luteum.

Substantial evidence has accumulated in recent years to indicate that the initial LH signal is transduced by LH receptors residing predominantly in the mural granulosa and thecal cell layers of the preovulatory follicle (Eppig 1979a, b; Peng et al. 1991; Wang and Greenwald 1993). The LH-activated signaling pathways in these follicular cell layers then lead to production of secreted factors that can act in

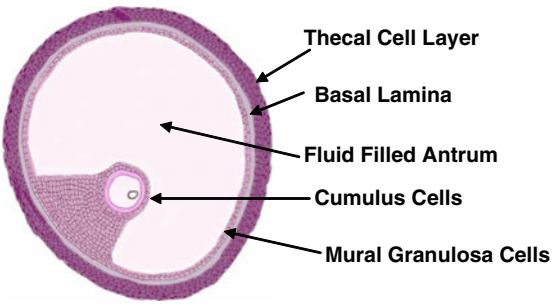

Fig. 1 Mature preovulatory follicle

a paracrine manner to transmit the ovulatory signal to cumulus cells leading to cumulus expansion and ultimate oocyte extrusion (Richards 2007; Russell and Robker 2007).

The advent of high-density gene array profiling technologies coupled with mouse models of genetic ablation of specific LH-regulated genes have uncovered a complex myriad of signaling pathways that mediate all aspects of the ovulatory response to LH. This chapter will focus on the role of progesterone (P) and its LH-regulated ovarian receptors (PRs) in mediating the LH response leading to ovulation.

2 Progesterone Receptors Control LH-Induced Follicular Rupture but Not Luteinization

Is it well established that P is a critical regulator of ovulation and that the physiological responses to P are mediated primarily at the level of transcription by two PR protein isoforms arising from a single gene (Conneely et al. 2003). Although classically viewed as an endocrine hormone, evidence that ovary-derived P may participate in *autocrine* regulation of ovarian function first emerged with the demonstration that LH can stimulate transient expression of PR mRNA and protein in granulosa cells isolated from preovulatory follicles (Natraj and Richards 1993; Park-Sarge and Mayo 1994) and that the antiprogestin, RU486, can inhibit ovulation (Loutradis et al. 1991). Definitive proof that intraovarian PRs are essential mediators of ovulation was provided by analysis of the ovarian phenotype of mice carrying a null mutation of the progesterone receptor gene (PRKO). Despite exposure to superovulatory levels of gonadotrophins, PRKO mice fail to ovulate. Analysis of the histology of these mice has revealed normal development of intraovarian follicles to the tertiary follicular stage (Lydon et al. 1995). The follicles contain a mature oocyte that is fully functional when isolated and fertilized in vitro. However, follicular rupture is effectively eliminated. Despite the ovulatory block, the preovulatory granulosa cells within these follicles can still differentiate into a luteal phenotype and express the luteal marker, P450 side chain cleavage enzyme (Robker et al. 2000). Thus, PRs are required specifically for LH-dependent follicular rupture leading to ovulation but not for differentiation of granulosa cells to form a corpus luteum (luteinization).

Both PR isoforms of PRs (PR-A and PR-B) are induced in preovulatory follicles in response to LH stimulation (Natraj and Richards 1993). Examination of the selective contributions of the individual PR isoforms in mediating the ovulatory function of P using mice in which expression of either the PR-A (PRAKO) or PR-B (PRBKO) isoform was selectively ablated has revealed that the PR-A isoform is both necessary and sufficient to regulate the ovulatory functions of P while the PR-B isoform is dispensible for ovulation (Mulac-Jericevic et al. 2003, 2000). Stimulation of immature PRAKO mice with superovulatory doses of gonadotrophins indicated that superovulation is severely impaired in these mice relative to their wild-type counterparts, but unlike PRKO mice is not completely absent. In contrast, superovulation was unaffected in PRBKO mice. Histological analysis of

the ovaries of PRAKO animals showed numerous mature anovulatory follicles that contained an intact oocyte and were arrested at a similar stage to that previously observed in PRKO mice. The requirement for PR-A in ovulation is likely conserved between mouse and human species given the relatively higher levels of PR-A vs. PR-B expression observed in the human ovary.

3 Molecular Signaling Pathways That Mediate PR-Dependent Follicular Rupture

Induction of ovarian PR expression occurs as early as 4 h and is maximal within 8 h after receipt of an LH stimulus. The spatial expression pattern of PRs is highly restricted to MGCs, while cumulus and thecal somatic cells as well as the oocyte are devoid of PRs (Ismail et al. 2002). These findings indicate that the effects of P on the cumulus oocyte complex are indirect and secondary to a PR-dependent transcriptional program activated in MGCs that is capable of transmitting paracrine signals to regulate cumulus cell function.

Because the PRKO mouse has a specific defect in follicular rupture while LH-induced luteinization of granulosa cells is maintained, high-density differential array analysis using this model provides an excellent approach to delineate the signaling pathways regulated by PRs that are specific to follicular rupture. Recent employment of these approaches has begun to provide important new insights into the molecular mechanisms of P-induced follicular rupture.

Although a comprehensive list of PR-regulated gene signatures in the ovary is currently unpublished, we and others have used Affymetrix U74A microarrays to compare gene array expression profiles in wild type vs. PRKO ovaries. In our study, total RNA was prepared from mice that were stimulated with PMSG followed 48 h later by HCG for 12 h. Under these conditions, the LH-dependent expression of PRs and their previously identified downstream target, ADAMTS-1 is optimal (Espey et al. 2000). Of 12,500 genes interrogated in this analysis, 462 PR-dependent genes were identified of which 294 were selectively upregulated and 168 downregulated more than 1.5-fold in wild type vs. PRKO mice. Thus, PRs mediate the expression of a complex array of gene signatures in response to LH. The following summary will focus on progesterone-regulated signaling pathways activated in MGCs that are critical for follicular rupture and involved in paracrine communication with cumulus cells to regulate their function during ovulation.

4 Regulation of Cumulus Matrix Components by PRs

In response to the LH surge, cumulus cells adjacent to the oocyte recede away from the latter and deposit a hyaluronin (HA)-rich protective extracellular matrix (ECM) as cumulus expansion occurs. ECM deposition occurs in response to the induction of

hyaluronin synthetases (HAS) and is stabilized by hyaluronin binding proteoglycans including versican, TNF-stimulated gene-6 (TSG-6), and pentraxin-3 (PTX-3) (Richards 2007; Russell and Robker 2007). The HA-binding protein, versican, serves as a substrate for the ECM protease, ADAMTS-1, whose mRNA expression is coinduced in MGCs along with versican and is under transcriptional control by PRs (Robker et al. 2000). While the latent form of this enzyme is produced in MGCs, it is processed to a mature secreted form that accumulates along with versican in the cumulus cell matrix. Loss of PR-dependent expression of ADAMTS-1 in ADAMTS-1 null mice results in severe impairment of ovulation underscoring the critical role of this enzyme in mediating the ovulatory functions of progesterone. Additional PR-induced proteases that may contribute similar functions in remodeling of the cumulus ECM include Cathepsin-L (Robker et al. 2000) and ADAM8 (Sriraman et al. 2008). Finally, consistent with the essential role of the PR-A isoform of PRs in regulating the ovulatory function of progesterone, PR-dependent expression of ADAMTS-1 is almost entirely dependent on the PR-A isoform of PRs.

5 Paracrine Growth Factor Signaling by PRs to Cumulus Cells

The epidermal growth factor-like (EGF-L) ligands, amphiregulin, epiregulin, and betacellulin are rapidly induced in MGCs by LH and play a critical paracrine function in regulation of cumulus cell expansion (Conti et al. 2006). Upon proteolytic processing of membrane-bound precursor forms of these growth factors, the active secreted products interact with their cognate receptors on cumulus cells leading to resumption of oocyte meiosis (Hsieh et al. 2009) and formation of the ECM during cumulus expansion in part through upregulation of components of the ECM including HAS-2, TSG-6, PTX-3, and ADAM8 (Conti et al. 2006; Shimada et al. 2006; Sriraman et al. 2008). Recent studies have shown that the LH-induced expression of both amphiregulin and epiregulin is under transcriptional control of PRs and is reduced in PRKO mice (Shimada et al. 2006). Although cumulus expansion in PRKO mice appears morphologically normal, these findings underscore an important role for PRs in paracrine growth factor-mediated regulation of components of the cumulus ECM that are essential for cumulus expansion and function during ovulation.

6 Transcriptional Programs Downstream of PRs

Recent studies have uncovered a hierarchy of transcription factor-mediated control of ovulation in response to progesterone in LH stimulated granulosa cells, whereby P activation of PRs regulates expression of a subset of transcription factors that in turn activate gene signaling pathways that ultimately control the function of cumulus cells. Among these are the nuclear transcription factors, RUNX1, PPARγ,

and hypoxia inducible factors (HIFs), all of which are induced by LH in a PR-dependent manner in MGCs (Jo and Curry 2006; Kim et al. 2009, 2008).

RUNX1 (AML1) is a nuclear transcription factor required for hematopoietic cell differentiation that is upregulated by PRs in granulosa cells (Jo and Curry 2006) and plays an important role in the ovary in transcriptional regulation of expression of prostaglandin-endoperoxidase synthase 2 gene (Ptgs2, cox-2) (Liu et al. 2009a). PTGS2 is a critical regulatory enzyme required for production of prostaglandins and activation of the prostaglandin inflammatory axis that is essential for cumulus expansion and ovulation. The activation of RUNX1 by PRs therefore links progesterone to regulation of prostaglandin-mediated control of cumulus cell function.

The nuclear receptor PPARγ is a critical mediator of LH-induced ovulation and targeted deletion of this transcription factor in granulosa cells leads to failure of follicular rupture (Kim et al. 2008). At a molecular level, the effects of PPARγ are mediated through activation of expression of downstream target genes that include interleukin-6 (IL-6), endothelin-2 (ET-2), and cyclic GMP-dependent protein kinase II (cGKII), all three of which have been shown to be dependent on PRs for their activation (Liu et al. 2009b; Palanisamy et al. 2006; Sriraman et al. 2006). ET-2 is essential for follicular rupture and functions in part through activation of cGKII, while IL-6 promotes cumulus expansion through activation of downstream signaling pathways that induce expression of key components of the ECM including HAS2, TSG-6, PTX-3, and PTGS2 (Liu et al. 2009b).

The hypoxia inducible transcription factors, HIFs, are members of the bHLH-PAS family of transcription factors that function as sensors of cellular oxygen levels. Recent studies have identified three HIFs, HIF-1a, HIF-2a, and HIF-1b, as PR-regulated genes in granulosa cells whose expression is reduced in PRKO mice (Kim et al. 2009). Pharmacological blockade of HIF transcriptional activity is sufficient to inhibit follicular rupture and interestingly inhibits expression of the PR-dependent genes ET-2 and ADAMTS-1. Thus, PRs regulate distinct transcriptional programs in MGCs that are capable of secondary transcriptional activation of partially overlapping signaling pathways that in turn converge on cumulus cells to regulate structural maintenance and remodeling of the ECM and facilitate follicular rupture.

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Contraception Targets in Mammalian Ovarian Development

Eileen A. McLaughlin and Alexander P. Sobinoff

Contents

Abstract In the human ovary, early in pre-natal life, oocytes are surrounded by pre-granulosa follicular cells to form primordial follicles. These primordial oocytes remain dormant, often for decades, until recruited into the growing pool throughout a woman's adult reproductive years. Activation of follicle growth and subsequent development of growing oocytes in pre-antral follicles are major biological checkpoints that determine an individual females reproductive potential. In the past decade, great strides have been made in the elucidation of the molecular and cellular mechanisms underpinning maintenance of the quiescent primordial follicle

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pool and initiation and development of follicle growth. Gaining an in-depth knowledge of the intracellular signalling systems that control oocyte preservation and follicle activation has significant implications for improving female reproductive productivity and alleviating infertility. It also has application in domestic animal husbandry, feral animal population control and contraception in women.

Keywords Fertility control · Granulosa cells · Oocyte · Primary follicle · Primordial follicle

1 Introduction

In the mammalian ovary, early in pre-natal life, oocytes are surrounded by pre-granulosa follicular cells to form primordial follicles. These primordial oocytes remain dormant, often for decades, until recruited into the growing pool throughout a woman's adult reproductive years (Choi and Rajkovic 2006; Reynaud and Driancourt 2000). Once in the primordial follicle, there are only two fates awaiting the oocyte; either the germ cell will be directed to grow and eventually be ovulated or more likely, the oocyte will become atretic. In most mammals, greater than 99% of the oocytes are lost – with only a tiny proportion selected for ovulation. The growing follicles doomed to atresia are easily recognisable within the ovary as they display several markers of apoptotic cell death (Krysko et al. 2008). In contrast, the mechanisms that underpin the maintenance, selection and maturation of the very small population of good-quality and presumably functional oocytes in primordial follicles are only just becoming clear.

Bi-directional signalling between the oocyte and the surrounding somatic cells is considered fundamental in the delicate balance of positive and negative forces controlling the maintenance and activation of the primordial follicle pool (Hutt et al. 2006a; McLaughlin and McIver 2009; Skinner 2005). Both the maintenance of healthy follicles and the highly regulated and selective release of primordial oocytes into the growing pool (Jin et al. 2005) involves cross-talk between an every growing list of cytokines and growth factors (Dissen et al. 2009; Picton et al. 2008; Trombly et al. 2009). Excitingly in the past few years, great strides have been made in characterising the intracellular signalling pathways activated during pre-antral follicle development (John et al. 2007; Reddy et al. 2008) providing fundamental knowledge and insight into the molecular systems responsible for ensuring production of functional oocytes for fertilisation (McLaughlin and McIver 2009).

Throughout the globe, many couples are unable to control their fertility, with an estimated 200 million plus women in the developing world either not using any form of contraception or relying on traditional methods only (Rowlands 2009). In contrast, in the Western world, both access and use of contraception are high; however, the vast majority of women still rely on the oral contraceptive pill or on barrier methods, principally the condom (Rowlands 2009). Ostensibly, there have been many advances in the "pill" over the past 50 years including improved

formulations and the introduction of a range of local vaginal and intrauterine, injectable or sub/transdermal hormonal-based contraceptives. Barrier methods with complementary spermicide/microbicide activity are also under development – particularly as adjuncts for the control of sexually transmitted diseases, such as HIV/AIDS (Rowlands 2009). Since no novel methods of contraception have been introduced since the 1960s, recent insights into the basic cellular mechanisms underpinning follicle maintenance and oocyte development will inform biotechnology strategies for the manipulation of reproduction in humans.

2 Ovarian Folliculogenesis and Exhaustion of the Primordial Follicle Pool

In mammals, primordial germ cells (PGCs) migrate early in embryonic development to colonise the naive gonad where they differentiate to become oogonia. Folliculogenesis begins with recruitment of somatic pre-granulosa cells to the oocyte to form the primordial follicle (Fig. 1) (Dickinson et al. 2010). In rodents and lagomorphs, synchronous primordial follicle formation occurs during the first

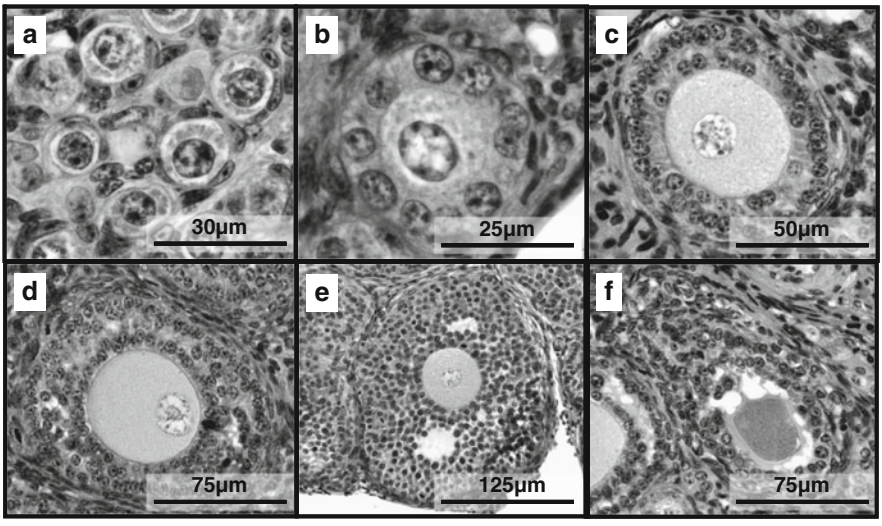
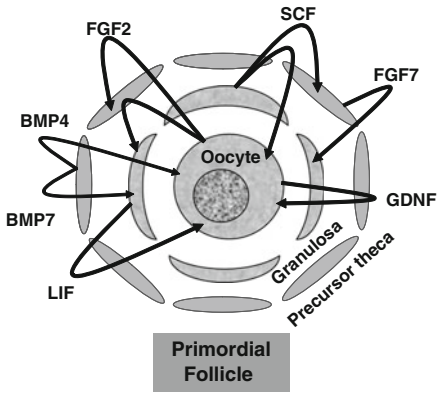


Fig. 1 Photomicrographs depicting the architecture and classification of ovarian follicles during folliculogenesis (**a**) primordial follicles; quiescent oocytes surrounded by a single layer of squamous granulosa cells, (**b**) primary follicle; primary oocytes characterised by a single layer of cuboidal granulosa cells, (**c**) secondary follicle; enlarged oocytes surrounded by a second layer of granulosa cells and marked by the acquisition of a thecal cell layer surrounding the follicle, (**d**) pre-antral follicle; large oocytes surrounded by multiple layers of granulosa cells, (**e**) antral follicle; mature follicle characterised by the presence of a fluid filled cavity within the granulosa cell layer known as an antrum, (**f**) atretic follicle; degenerated follicles found at all stages of follicular development characterised by "detached" granulosa cells and apoptotic oocytes

few days and weeks of early post natal life (Hutt and Albertini 2006; Pedersen and Peters 1968). In contrast, in livestock and primates including the human, follicle formation and activation occur asynchronously during foetal life (Lintern-Moore et al. 1974; Wandji et al. 1996), resulting in ovaries at birth containing both growing pre-antral and early antral follicles in the neonatal ovary (Picton et al. 1998).

Endocrine mechanisms are thought to trigger primordial follicle formation and these are mediated by the maternal hormonal milieu. High levels of maternal oestrogen in the foetal ovary act to maintain intact germline nests and the subsequent decrease of oestrogen and progesterone allows follicle formation (Chen et al. 2007, 2009; Nilsson et al. 2006; Nilsson and Skinner 2009; Pepling et al. 2009). Similarly, a progesterone and oestrogen endocrine-based mechanism of primordial follicle activation has been postulated as both hormones act in in vitro culture experiments to decrease primordial follicle recruitment during the first wave of folliculogenesis in rodents (Kezele and Skinner 2003). Multiple perinatal mechanisms establish the size of the primordial follicle reserve with follicle loss resulting from apoptotic germ cell loss, substantial autophagy and ovarian morphogenesis comprising active extrusion of non-apoptotic germ cells, all resulting in substantial depletion of the follicle population (Rodrigues et al. 2009). As progesterone and oestrogen levels have been noted to drop in bovine and primate foetal ovaries during mid to late gestation coincident with follicle assembly and growth initiation, this further implicates a steroid-based negative regulatory mechanism (Kezele and Skinner 2003; Nilsson and Skinner 2009; Yang and Fortune 2008). Interestingly, earlier studies indicate that there are increased populations of pre-antral and antral follicles in juvenile oestrogen-deficient aromatase knockout (ArKO) mice, also supporting the notion that primordial follicle activation is dependent on a decline in oestrogen levels (Britt et al. 2004). Furthermore, diethylstilbestrol inhibits follicle formation and development in neonatal mouse ovaries in vitro, by acting through oestrogen receptor alpha (EPα) (Kim et al. 2009a, b). Latterly, the androgen, testosterone, has been demonstrated to increase follicle activation in vitro (Yang et al. 2010) and extrapolating from this observation is the hypothesis that excess intra-ovarian androgen is linked to polycystic ovary syndrome (PCOS) (Yang et al. 2010).

In the primordial follicle, and throughout most of its subsequent growth and development, the oocyte is arrested in prophase I of the first meiotic division and only re-enters meiosis or germinal vesicle breakdown upon ovulation (Fig. 1) (Pedersen 1969, 1970). The resting primordial follicles in the ovarian pool are sequentially stimulated to activate and grow, at which point a majority become atretic (Rodrigues et al. 2008; Tingen et al. 2009), with a small minority developing through pre-antral and antral stages into mature Graffian follicles (McLaughlin and McIver 2009; Skinner 2005) (see Fig. 1). Following activation, the rapidly enlarging oocyte synthesises an acellular extracellular matrix, the zona pellucida, and this process is supported by proliferating granulosa cells (Fig. 1). Finally, during antral follicle development, the oocytes become meiotically competent (Zheng and Dean 2007). A surge of luteinising hormone initiated just prior to ovulation resulting in nuclear maturation, completion of the first meiotic division and first polar body extrusion, then re-arrest in meiosis II at metaphase II (Hutt and Albertini 2007) (Fig. 1).



Factor	Effect	Reference
SCF	Stimulates follicular recruitment and promotes precursor theca function	Jin et al., 2005; Hutt et al., 2006
LIF	Stimulates follicular recruitment	Nilsson et al., 2002
FGF2	Promotes SCF expression and cell growth	Nilsson et al., 2001; Nilsson and skinner, 2004
FGF7	Promotes SCF expression	Kezele et al., 2005
GDNF	Autocrine signalling promotes follicular recruitment	Dole et al., 2008
BMP4, BMP7	Stimulates follicular recruitment and survival	Nilsson and Skinner, 2003; Lee et al., 2001; Lee et al 2004

Fig. 2 Ovarian cytokine and growth factor signalling factors involved in primordial follicle recruitment. Abbreviations: *SCF* stem cell factor; leukaemia inhibitory factor; *FGF* fibroblast growth factor; *GDNF* glial-derived neurotrophic factor; *BMP* bone morphogenic factor

In female mammals, the primary determinant of successful reproductive performance is the initial size and then the controlled release of primordial follicles from the resting pool (Maheshwari and Fowler 2008). Studies of total non-growing ovarian follicle populations have determined that the rate of decline in quiescent functional follicles in the adult ovary increases with age (Hansen et al. 2008).

So how does the ovary regulate primordial follicle population dynamics? Recently, quantification of the relative spatial positions and inter-follicular distances between the quiescent primordial follicles and growing pre-antral follicles in neonatal mouse ovaries indicated that follicles were significantly less likely to have started growing if they had one or more primordial follicles closely adjacent (Da Silva-Buttkus et al. 2009). This observation is consistent with the notion that primordial follicles may, as has been previously hypothesised (McLaughlin and McIver 2009), produce diffusible factor(s) that inhibit neighbouring primordial follicles from activating and initiating development (Adhikari and Liu 2009). Thus, the use of positive and negative paracrine signalling mechanisms (Fig. 2) may allow the ovary to both maintain an ovarian pool of follicles throughout the reproductive lifespan, while providing a highly selected supply of functional oocytes for ovulation (Adhikari and Liu 2009; Edson et al. 2009; McLaughlin and McIver 2009).

3 Early Folliculogenesis: Roles of Cytokines, Chemokines, Hormones and Growth Factors

Folliculogenesis in the mammalian ovary has been well characterised into major morphologically distinct entities based on the size of the oocyte and

number of granulosa cells (see Fig. 1) surrounding the oocyte (Pedersen and Peters 1968). More generally these stages are classified as either pre-antral and gonadotrophin independent, comprising the primordial, primary and secondary follicles or the antral, gonadotrophin-dependent phase consisting of the antral, pre-ovulatory, ovulatory follicles and the corpus luteum (see Fig. 1) (Pedersen and Peters 1968; 1971). Studies of naturally occurring mutant mice, particularly mice with Kit mutations (Geissler et al. 1981), were able to determine some of the signalling cascades inherent to normal folliculogenesis (Hutt et al. 2006b). Subsequently, large scale genomic and more recently proteomic approaches have identified a number of paracrine and autocrine signalling pathways associated with primordial follicle and pre-antral follicle development (Arraztoa et al. 2005; Holt et al. 2006; Kezele et al. 2005b; Serafica et al. 2005; Wang et al. 2009).

Key findings from these studies have included the identification of ligands and their receptors localised in primordial follicles, which are implicated as key regulators of the primordial to primary and secondary follicle transition as well as atresia and maintenance of quiescence (see Figs. 2 and 4).

Intracellular signalling pathways initiated, via a wide range growth factors and pleiotrophic cytokines, are known to activate mammalian primordial follicles in mammalian ovarian explant culture systems. These include basic fibroblast growth factor (FGF2) (Garor et al. 2009; Nilsson et al. 2001), vascular endothelial growth factor A (VEGFA) (Artac et al. 2009; McFee et al. 2009), platelet-derived growth factor (PDGF) (Nilsson et al. 2006), kit ligand/stem cell factor (KIT-L/SCF) (Hutt et al. 2006b), leukaemia inhibitory factor (LIF) (Nilsson et al. 2002), keratinocyte growth factor (KGF) (Kezele et al. 2005a), bone morphogenic proteins (BMP 4 and 7) (Craig et al. 2007; Lee et al. 2001, 2004; Nilsson and Skinner 2003), glial-derived neurotrophic factor (GDNF) (Dole et al. 2008) and the neurotrophins (NGF, NT4, BDNF, NT3) (Dissen et al. 2002; Dole et al. 2008; Nilsson et al. 2009; Paredes et al. 2004; Romero et al. 2002; Spears et al. 2003) (see Fig. 2).

Repressors of follicle activation include anti-Müllerian hormone (AMH) which, when added to ovarian explant cultures, inhibits the primordial to primary follicle transition in rodents (Durlinger et al. 2002a). However, no mouse model exists that supports this claim as over-expression of AMH in vivo results in germ cell loss and ovarian degeneration (Behringer 1995; Lyet et al. 1996) probably via activation of an AMHRII-mediated pathway (Mishina et al. 1999). Interestingly, prolonged exposure to AMH initiates follicle development in human ovarian explant cultures (Schmidt et al. 2005). Recently, the chemoattractive chemokine SDF-1 (also known as CXCL-12) was identified as second inhibitor of primordial follicle activation in vitro (Holt et al. 2006). Manipulating the primordial follicle pool remains an attractive method of controlling female fertility though as yet no unique and most importantly reversible contraceptive agent targeting primordial follicle activation has been identified.

4 Multiple Activator and Repressor Pathways Converge to Regulate Activation of the Primordial Follicle

Numerous studies point to multiple stimulatory and negative pathways converging to regulate the activation of the primordial follicle. In in vitro culture systems, large numbers of primordial follicles spontaneously enter the growing pool – and treatment with exogenous cytokines, chemokines, hormones and growth factors increases (or decreases) the proportion of activated follicles. Notably treatment with antagonists or function blocking antibodies will also suppress but not abolish primordial follicle activation indicating multiple possible redundant endogenous mechanisms (Holt et al. 2006; Hutt et al. 2006b; Kezele et al. 2002; Nilsson et al. 2007).

Studies of null or mutated mice indicate that many of the ligands or their receptors implicated in follicle activation are not essential (Dono et al. 1998; Stewart et al. 1992). A recent example is the knock-in mutation (Kit^{Y19F}) mice, which despite a complete abrogation of the PI3K pathway have normal folliculogenesis, ovarian morphology and are fertile (John et al. 2009).

In conclusion, multiple cytokine and growth factor-activated pathways must undertake cross talk to produce an intracellular balance of positive and negative signals, thus ensuring the long-term stability of quiescent primordial follicles, with the release of selected oocytes from repression into the growing population from this precious and finite resource. Targeting the intracellular pathways activated by these pleiotrophic cytokines/growth factors is an attractive prospect as a mechanism to influence follicle growth. Great strides have been made in the past 5 years in the characterisation of these pathways and the development of primordial follicle-specific contraceptive pharmacological agents is now a very real possibility.

5 Intracellular Signalling in Oocytes and Pregranulosa Cells in Primordial Follicles

A member of the forkhead transcription family *FoxO3a* is a central player in the pathway(s) implicated in primordial follicle activation (Fig. 3). FoxO3a is a well-characterised regulator of embryogenesis, tumorigenesis and the maintenance of differentiated cell states through direction of key cellular processes such as stress responses, cell cycle arrest and programmed cell death (Hosaka et al. 2004; Kaufmann and Knochel 1996).

FoxO3a null mice suffer from a lack of primordial follicles in early neonatal life coupled with increased numbers of growing follicles and a subsequent increase in oocyte degeneration of newly growing follicles (Castrillon et al. 2003; Hosaka et al. 2004). Localised initially to granulosa cells (Richards et al. 2002), FoxO3a was thought to mediate its suppressive effects on primordial oocyte recruitment by increasing the expression in the oocyte of a cell cycle inhibitor, p27kip1 (or Cdkn1b), whilst concurrently decreasing cyclin D1 and D2 expression, thereby arresting the cell cycle (Brenkman and Burgering 2003) (Fig. 3). However,

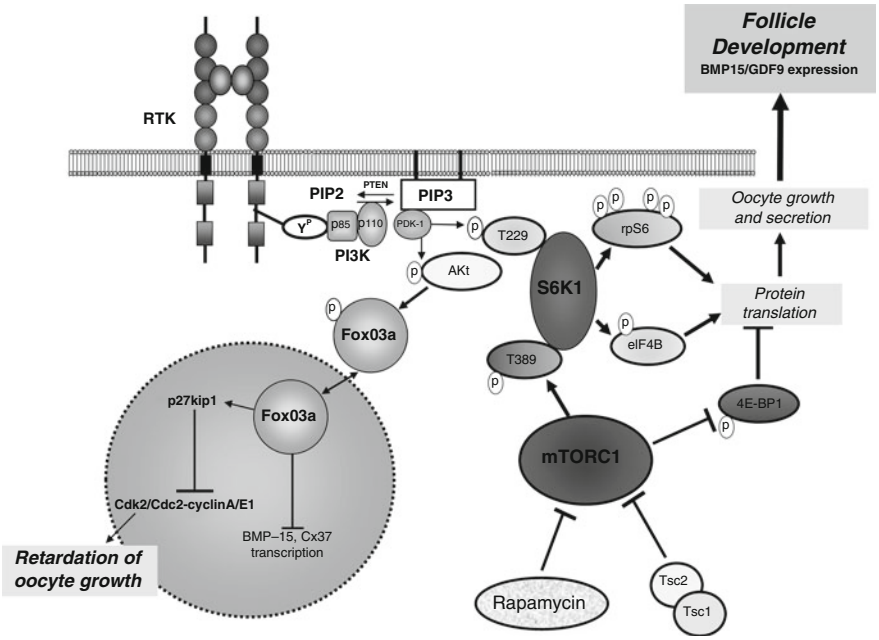


Fig. 3 The oocyte PTEN-PI3K – FoxO3a and mTORC pathways govern follicle activation through control of initiation of oocyte growth and maintenance of oocyte quiescence. Abbreviations: *PIP2* Phosphatidyl inositol bisphosphate; *PI3K* Phosphoinositide 3-kinase; *PIP3* Phosphatidylinositol (3,4,5)-trisphosphate; *PDK1* 3-Phosphoinositide-dependent protein kinase, S6K1, p70 S6 kinase; *PTEN* phosphatase and tensin homolog; *Akt* Protein Kinase B; *rpS6* Ribosomal protein S6; *FoxO3a* Forkhead box O3A; *GSK3* Glycogen synthase kinase 3; *p27kip1* Cyclin-dependent kinase inhibitor 1B (CDKN1); *Cdk2* Cyclin dependent kinase 2; *BMP15* Bone Morphogenic Protein 15; *Cx37* Connexin 37; *GDF9* Growth Differentiation factor 9; *mTORC1* mammalian Target of Rapamycin Complex 1; *eIF4B* eukaryotic initiation factor 4B; *TSC1/2* tuberous sclerosis complex 1/2; *4E-BP1* Eukaryotic translation initiation factor 4E binding protein 1

characterisation of the p27kip1 deficient mouse revealed accelerated postnatal follicle assembly and a vast increase in the founding primordial follicle population, which then underwent premature activation (Rajareddy et al. 2007). Consequently, it was established that p27kip1 controls oocyte development by suppressing the functions of Cdk2/Cdc2-Cyclin A/E1 in the diplotene arrested oocytes (Rajareddy et al. 2007), while simultaneously activating a caspase-mediated apoptotic cascade, thus also inducing follicle atresia (Rajareddy et al. 2007).

6 Signal Transduction: The Phosphatidylinositol 3-Kinase (PI3K) and the mTOR Pathways

Akt (Protein Kinase B) signalling was predicted to be activated via extracellular receptor tyrosine kinase (RTK) signalling pathways that regulate the phosphorylation control of FoxO3a inactivation via nuclear exclusion (Junger et al. 2003).

The use of multiple germ cell-specific knockout mice indicates that extracellular signals transduced through the PI3K (phosphatidylinositol 3-kinase) pathway (Reddy et al. 2005) are fundamentally important for the regulation of early follicular development (Liu et al. 2006) (Fig. 3). The majority of the constituents of one PI3K pathway, including GSK-3α and GSK-3β, Akt, Foxo3a, (Liu et al. 2007b), FoxO3a and p27kip1 (Rajareddy et al. 2007) have been demonstrated to be present in growing mouse oocytes. It was proposed that stimulation of the PIK3 pathway through an RTK (possibly c-kit though not exclusively) (see John et al. 2009) results in the phosphorylation and functional suppression of FoxO3a and thus the release of quiescent oocytes into a state of active growth and development (Liu et al. 2007a). This hypothesis was supported by evidence that FoxO3a is capable of both suppressing BMP15, connexin 37 and connexin 43 production in mouse oocytes (Fig. 3), important factors in oocyte-granulosa and inter-granulosa cell communications, and also to up-regulate expression of p27 in the oocyte nucleus, ultimately resulting in the suppression of oocyte growth and follicular activation (Liu et al. 2006).

Phenotypic analysis of a null mouse with an oocyte-specific conditional knock-out of Pten (phosphatase and tensin homolog deleted on chromosome 10), a major negative regulator of PI3K (phosphatidylinositol 3-kinase) revealed the entire primordial follicle pool becomes activated and all primordial follicles become depleted in early adulthood, causing premature ovarian failure (Fig. 3). The authors concluded that the oocyte Pten-PI3K pathway governs follicle activation through control of the initiation of oocyte growth (Reddy et al. 2008) (see Fig. 3). This was subsequently confirmed with the development of an oocyte-specific inducible [Vasa-Cre(ERT2)] conditional knock-out mouse (John et al. 2008). Using this model, targeted ablation of Pten was shown to activate the PI3K/Akt pathway leading to hyperphosphorylation of Foxo3 and primordial follicle activation. Moreover, Foxo 3 was shown to control primordial follicle activation via a nucleocytoplasmic shuttling mechanism; the phosphorylation of this transcription factor catalysing its movement from the nucleus to the cytoplasm.

More recent studies of Akt1 null females indicate that they have both reduced fertility and abnormal oestrous cyclicity (Brown et al. 2009). In early postnatal life, Akt1 null ovaries display abnormal folliculogenesis and this is followed in early adulthood by a significant decrease in the primordial follicle population (Brown et al. 2009), reinforcing the notion that the PI3K/Akt pathway is critical for primordial follicle development.

Subsequent to their studies of the Pten null mouse, another member of the PI3K pathway, 3-phosphoinositide-dependent protein kinase-1 (Pdpk1), was implicated in follicle activation (Fig. 3). In stage-specific Pdpk1 null mice, the majority of primordial follicles were depleted prematurely, causing ovarian failure by early adulthood (Jagarlamudi et al. 2009; Reddy et al. 2009). This outcome was linked to the suppression of Pdpk1– p70 S6 kinase 1 (S6K1)-ribosomal protein S6 (rpS6) signalling (Jagarlamudi et al. 2009; Reddy et al. 2009), thus continuing to implicate the PI3K/Pten/Pdpk1 signalling pathway as central to the molecular oocyte network that controls the primordial follicle population. Thus, reproductive ageing in

females appears fundamentally linked to the dysregulation of this signalling pathway in oocytes resulting in subfertility and premature ovarian failure.

A second pathway appears to function synergistically with the PI3K–Pten network described above. Oocyte-specific deletion of a negative regulator of Target of Rapamycin Complex 1 (mtorc1), tumour suppressor tuberous sclerosis complex 1 (Tsc1), results in the premature activation of the entire pool of primordial follicles and follicular depletion in early adulthood, causing premature ovarian failure (Adhikari et al. 2010). Not surprisingly, oocyte-specific tumour suppressor tuberous sclerosis complex 2 (Tsc2), which also negatively regulates (mtorc1), also functions to maintain the primordial follicle population in quiescence (Adhikari et al. 2009). As described for Tsc1 null mice, the absence of the Tsc2 gene in oocytes results in a phenotype in which the primordial follicles are prematurely activated and depletion of follicles in early adulthood, causing premature ovarian failure (Fig. 3). These findings' results suggest that the Tsc1–Tsc2 complex is required to establish the quiescent state of primordial follicles via suppression of mtorc1 activity and that activation of the primordial follicle is dependent on mtorc1 activity in oocytes (Adhikari et al. 2009).

Supporting somatic cell lineages also play a major role in controlling and nurturing primordial follicle activation and development. A key example is the forkhead transcription factor Foxl2, as mutations in the FOXL2 gene are associated with ovarian failure in both humans and mice (Duffin et al. 2009; Uda et al. 2004). Foxl2-deficient mice display major defects in primordial follicle activation with consequent follicle loss. In addition, roles in gonadal development and sex determination have also been suggested (Uda et al. 2004). Features of Foxl2 null animals point towards a new mechanism of premature ovarian failure, with all major somatic cell lineages failing to develop around growing oocytes from the time of primordial follicle formation (Uda et al. 2004).

7 Promoting and Regulating Early Follicle Growth and Development

Many members of the TGFβ superfamily act as paracrine growth factors and are expressed by both ovarian somatic cells and oocytes in a developmental-stage specific manner. A well characterised marker of ovarian follicular reserve is AMH – originally identified in Sertoli cells of the foetal testis and known to promote the regression of the Müllerian ducts during differentiation of the male reproductive tract (Munsterberg and Lovell-Badge 1991). AMH is also expressed in ovarian granulosa cells (Durlinger et al. 1999) and as outlined above both acts as an inhibitor of the initiation of primordial follicle growth and decreases the sensitivity of follicles to the FSH-dependent selection for dominance in both mice (Visser and Themmen 2005) and humans (Dumesic et al. 2009).

During folliculogenesis, AMH expression is initiated in the granulosa cells of primary follicles, peaks in granulosa cells of pre-antral and small antral follicles and

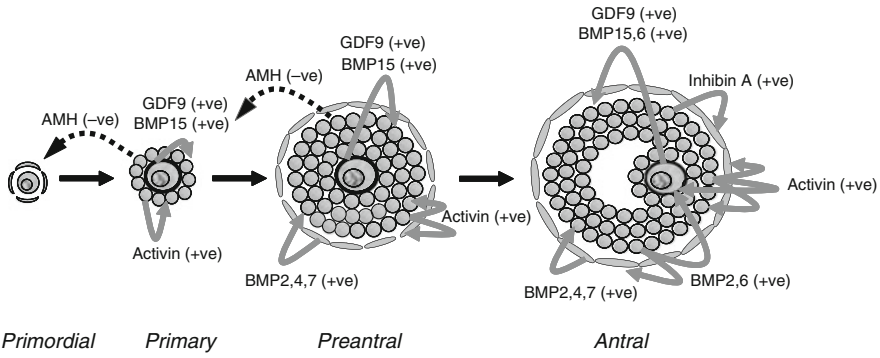


Fig. 4 Potential signalling interactions (+ve, stimulatory; −ve inhibitory) of TGF-β superfamily members involved in the primary to early antral follicle stages. Abbreviations: *AMH* anti Müllerian hormone; *GDF* growth differentiation factor; *BMP* bone morphogenic protein

gradually diminishes in pre-ovulatory follicles (Dumesic et al. 2009; Durlinger et al. 2002b; Visser and Themmen 2005). This continued expression of AMH until the antral stage indicates a continual function in folliculogenesis beyond inhibiting the initiation of primordial follicle growth (Fig. 4). AMH inhibits FSH-stimulated follicle growth in both the mouse and human, suggesting that AMH is one of the factors determining the sensitivity of ovarian follicles for FSH (Durlinger et al. 2001) and is therefore a dominant regulator of early follicle growth (Dumesic et al. 2009).

In mammals, FSH also acts as the predominant survival factor for selected antral follicles, preventing the spontaneous onset of follicular apoptosis. Within this subpopulation, the follicles with the highest FSH sensitivity become dominant and continue to develop into Graffian follicles. On the other hand, AMH inhibition of FSH follicle sensitivity may play a role in antral follicle selection (McGee and Hsueh 2000; Durlinger et al. 2001). On binding to AMH receptors located on the granulosa cells of small pre-antral follicles (Fig. 4), AMH signalling activates SMAD transcriptional regulators which reduce LH receptor expression, leading to a decrease in aromatase (Diclemente et al. 1994) and therefore oestradiol levels (Andersen and Byskov 2006) and reduced FSH sensitivity (Kevenaar et al. 2007a, b).

Recent studies in normo-ovulatory women have indicated that serum AMH levels decrease with age in pre-menopausal women (de Vet et al. 2002), and that there is a direct correlation between serum AMH levels and antral follicle number (van Rooij et al. 2002), thus reflecting the size of the primordial follicle pool (Fig. 4). Therefore, AMH levels can be used to indicate ovarian follicle reserve (Visser et al. 2006) and to determine treatment strategies for women undergoing assisted conception (Macklon et al. 2006; Nelson et al. 2007, 2009).

Given that AMH plays important roles in both primordial follicle activation and antral follicle selection, it is a tempting target for a contraceptive agent. One possible avenue involves the use of recombinant AMH, or AMH agonists and/or antagonists for the long-term control of female fertility. In the case of contraception, the use of recombinant AMH and/or agonists that mimic the endogenous

growth factor could be used to halt primordial follicle recruitment, augmenting or providing an alternative form of hormonal contraceptive with the added benefit of possibly preserving the primordial follicle pool and therefore prolonging the reproductive life cycle. Indeed, advancements in recombinant technology have allowed the production of bio-activated AMH, which could possibly be used in such a role (Weenen et al. 2004).

GDF9, an oocyte-specific member (McGrath et al. 1995) of the growth and differentiation subfamily of TGFβ growth factors and it's receptor, bone morphogenic receptor type II, are expressed in rodent and human primary follicle stage oocytes and granulosa cells (Aaltonen et al. 1999; Hayashi et al. 1999), suggesting a role in paracrine signalling within the follicular microenvironment (Vitt et al. 2002). GDF9 has been shown to be essential for the development of primary follicles (Juengel et al. 2004), plays a crucial role in somatic ovarian cell development (Hreinsson et al. 2002) and is a key regulator of normal cumulus cell function (Gilchrist et al. 2008; Su et al. 2004) (Fig. 4).

GDF9 null mice have abnormal primary follicles which fail to develop beyond the primary stage, are unable to form a theca, and have impaired meiotic competence (Dong et al. 1996; Yan et al. 2001). In addition, in vitro cultures of ovarian tissue supplemented by GDF9 in both rats and humans enhance the progression of early to late stage primary follicles (Hreinsson et al. 2002; Nilsson and Skinner 2002). Interestingly, the abnormal follicles seen in GDF9 null mice consist of enlarged oocytes surrounded by a single layer of cuboidal granulosa cells. While these granulosa cells are typical of primary follicles, ultrastructural analysis of oocytes obtained from GDF9 null mice revealed that these oocytes had progressed to advanced stages of differentiation and were capable of resuming meiosis after in vitro maturation (Carabatsos et al. 1998). GDF9 signalling is also required for pre-antral follicle growth and ovulation (Elvin et al. 1999b) (Fig. 4), and GDF9 expression has been detected in the oocytes of both murine and human antral follicles (Elvin et al. 1999b; Gilchrist et al. 2004; Hreinsson et al. 2002). Culture of rat granulosa cells isolated from antral follicles has shown that GDF9 stimulates proliferation, but also suppresses FSH-induced granulosa cell differentiation, as indicated by lower progesterone and oestradiol levels with attenuated LH receptor formation (Vitt et al. 2000; Yamamoto et al. 2002). These results suggest that GDF9 may regulate antral follicle development by ensuring continued granulosa cell proliferation and preventing premature luteinisation (Fig. 4).

In addition to its proposed role in ensuring antral follicle development, GDF9 has also been implicated in ensuring pre-antral follicular survival by suppressing granulosa cell apoptosis and inducing thecal cell androgen production. In a recent study by Orisaka et al. down regulation of GDF9 expression in cultured rat granulosa cells led to a subsequent increase in caspase-3 activation and granulosa apoptosis (Orisaka et al. 2006). GDF9 was also capable of preventing ceramide induced apoptosis in granulosa cells cultured from pre-antral follicles, but had no affect on pre-ovulatory follicles (Orisaka et al. 2006). Studies involving double mutant GDF9 and inhibin-α knockouts have also shown GDF9 is required to induce theca cell differentiation, as indicated by the formation of theca-like cells in

developing follicles lacking thecal cell-specific markers Cyp17a1 and LH receptor (Wu et al. 2004). This observation is supported by a number of in vitro studies, which have demonstrated that GDF9 increases androgen production, *Cyp17a1* and *c-kit* expression in rat pre-antral follicles (Elvin et al. 1999b; Orisaka et al. 2009; Solovyeva et al. 2000).

Further studies have shown GDF9 signalling is also essential for cumulus development and metabolism (Elvin et al. 1999a; Su et al. 2008, 2009). During ovulation, GDF9 induces cumulus cell expansion, a process associated with the intricate association of cumulus cells with the oocyte throughout the ovulatory process and subsequent fertilisation. This process is essential, as it protects the oocytes during follicular extrusion,and assists fertilisation. Treatment of isolated granulosa cells in vitro with recombinant GDF9 has been shown to influence the expression of a suite of genes involved in cumulus cell expansion (*HAS2*, *COX-2*, *StAR*, *uPA* and *LHR*) and induce the same process in oocytectomised cumulus cell-oocyte complexes in vitro (Elvin et al. 1999b). These results are also supported by RNAi studies which show that selective knockdown of GDF9 in mature mouse oocytes reduces cumulus expansion in vitro (Gui and Joyce 2005).

GDF9 is also a viable target for a contraceptive agent due to its essential requirement for oocyte maturation. Immunocontraceptive studies in sheep have found that antisera generated against peptides corresponding to the first 1–15 amino acid residues on the N-terminus of GDF9 cause anovulation in ewes following primary and single booster vaccinations (McNatty et al. 2007). This raises the possibility of inhibitory/antisense compounds which target the N-terminus of GDF9 being used as potential human contraceptives in the near future.

Bone Morphogenic Proteins (BMPs) are the largest group of multifunctional growth factor cytokines belonging to the TGFβ superfamily. BMPs are expressed in numerous cell types and tissues and are involved in a wide variety of biological processes including mesoderm patterning, neurogenesis, bone formation and angiogenesis (David et al. 2009; Furtado et al. 2008; Morikawa et al. 2009; Xiao et al. 2007). First reported in mammalian ovary development in 1999, a whole host of BMPs have subsequently been identified in the oocyte, granulosa and thecal cells of the ovarian follicle (Knight and Glister 2006; Shimasaki et al. 2003).

BMP15 expression remains constant throughout folliculogenesis, being detected initially in oocytes and granulosa cells in the primordial follicle stage in the human ovary (Aaltonen et al. 1999; Margulis et al. 2008; Teixeira Filho et al. 2002), in primary follicle stage oocytes in the mouse (Dube et al. 1998) and in primordial follicle stage oocytes in the sheep (McNatty et al. 2001) suggesting a species-specific role for BMP15 (Fig. 4).

Knockout studies conducted in the mouse have shown females lacking a functional BMP15 gene are sub-fertile, due to impaired ovulation and fertilisation (Yan et al. 2001). Sheep with homologous point mutations corresponding to the chromosomal location of BMP15 are infertile, with follicular development beyond the primary stage being impaired (Galloway et al. 2000; Hanrahan et al. 2004). In humans, studies have led to the discovery of various missense mutations and polymorphisms in the BMP15 gene, which have all been associated with primary

and secondary amenorrhea (Di Pasquale et al. 2004, 2006). Interestingly, these mutations were described as having a similar phenotype to sheep with BMP15 homologous point mutations, with impaired follicular development beyond the primary stage (Di Pasquale et al. 2004; Galloway et al. 2000). When combined, these studies suggest that BMP15 may play a "curtailing" role in the transition of primary to secondary follicles during human folliculogenesis (Fig. 4).

In terms of BMP15's mechanism of action, follow-up in vitro experiments on isolated human granulosa cells have shown that treatment with recombinant BMP-15 in culture stimulates granulosa cell growth, while treatment with recombinant "mutant" BMP15 had no effect (Di Pasquale et al. 2004). This advocates that BMP-15 may exert its effect on follicular development by stimulating granulosa cell proliferation at the primary stage. Interestingly, co-culture with both recombinant wild type and mutant BMP15 had no effect on granulosa growth, suggesting an antagonistic effect (Di Pasquale et al. 2004) (Fig. 4).

As well as its role in the transition of primary to secondary follicles, BMP15 has also been implicated in the suppression of FSH-induced progesterone synthesis in rat and ruminants, and the stimulation of cumulus cell expansion and metabolism in the mouse (McNatty et al. 2005; Yoshino et al. 2006; Sugiura et al. 2007). Although BMP15 has been found to play varying roles beyond primary follicle growth, given the species-specific nature of BMP15's function, and the lack of analogous studies, it is unknown whether human BMP15 mimics any of these reported functions.

A recent study into the expression of BMP15 in human oocyte and cumulus granulosa cells has mapped BMP15 expression in pre-ovulatory stage oocytes and pre-ovulatory/ovulatory stage cumulus cells (Chen et al. 2009). BMP15 expression was found to increase significantly during late stage pre-ovulatory oocytes, suggesting a role for BMP15 in the final stages of oogenesis (Fig. 4). Additionally, the level of BMP15 significantly decreased in cumulus cells surrounding ovulatory oocytes compared with those surrounding pre-ovulatory oocytes. This decreased level of BMP15 in cumulus cells after oocyte maturation, coupled with the fact that BMP15 suppresses progesterone synthesis in mammalian models and is involved in human granulosa cell growth, suggests that this protein has the ability to act as an inhibitor of the premature luteinisation of cumulus cells (Chen et al. 2009; Di Pasquale et al. 2004; Gilchrist et al. 2008; McNatty et al. 2005; Otsuka et al. 2001).

BMP15 is an autosomal homologue of GDF9, both of which are expressed from a very early stage of follicular growth and play key roles in promoting follicular growth beyond the primary stage (Di Pasquale et al. 2004; Dong et al. 1996; Galloway et al. 2000) (Fig. 4). BMP15 and GDF9 have also been shown to act synergistically in mice during development of the oocyte-cumulus cell complex (Yan et al. 2001). In terms of fertility regulation, BMP15 immunisation studies in sheep have found that both active and passive immunisations are able to influence the biological activity of BMP15 in ewes (Juengel et al. 2002). Furthermore, recent immunocontraceptive studies in sheep have found that antisera generated against peptides corresponding to the first 1–15 amino acid residues on the N-terminus of BMP15 cause anovulation in ewes following primary and single booster vaccinations (McNatty et al. 2007). This raises the possibility of inhibitory/antisense

compounds which target the N-terminus of BMP15 being used as potential contraceptives in the near future. Another novel concept for a BMP15-based contraceptive agent involves the use of recombinant BMP15 mutants as potential antagonists. As described above, in vitro experiments using a mutant recombinant protein ($BMP15^{Y235C}$) showed that the mutant BMP15 was able to antagonise the stimulatory effects wild-type BMP15 on granulosa cell growth (Di Pasquale et al. 2004). Additionally, in the study through which this mutation was identified, the women who are heterozygous carriers of the Y235C mutation are infertile, with an impaired follicular phenotype. Therefore, the Y235C mutation may antagonise wild-type BMP15 in vivo (Di Pasquale et al. 2004) and underpin development of a BMP15 antagonist, or recombinant $BMP15^{Y235C}$, as a possible contraceptive.

In a recent paper by McMahon et al., recombinant human BMP15 and GDF9 were shown to undergo phosphorylation, and that this phosphorylation was required for normal bioactivity (McMahon et al. 2008). This study is novel, in that it is the first to report any member of the TGFβ superfamily as phosphoproteins. More interesting though was the fact that dephosphorylated BMP15 and GDF were capable of antagonising their wild-type counterparts by competitively binding to BMP receptors and failing to induce the BMP/Smad pathway (McMahon et al. 2008). These results raise the interesting possibility of using phosphorylation as a method of BMP15 and GDF9 regulation in the context of fertility control. The use of modified recombinant BMP15 and GDF9 incapable of undergoing phosphorylation could also be theoretically used as potential antagonists, and therefore as possible contraceptives.

8 Conclusions

Recent improvements in our understanding of the intracellular signalling systems, such as the PI3K and Tsc1 pathways, that control maintenance of the primordial follicle population and transduce the as yet elusive extracellular signals necessary for primordial follicle activation, have significant implications for the design of new contraceptive agents for women. As primordial follicle activation requires close communication between oocyte and somatic cells and many cytokine and chemokine factors have a clearly demonstrated role in releasing oocytes into the growing pool, then if the trigger is oocyte generated, an early response must include suppression of FOXO3A and mTORC activity and ultimately regulation of the "folliculogenesis clock" (Matzuk et al. 2002). Once activated to grow, the oocyte orchestrates and coordinates the development of mammalian ovarian follicles, the rate of follicle development being controlled by the oocyte (Eppig et al. 2002). Importantly, we are also beginning to elucidate those pathways activated by members of the TGFβ superfamily that regulate and support oocyte development. Pharmacological inhibition of these signalling pathways may hold the key development of non-steroidal ovarian contraceptives for the twenty-first century.

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Proteomics of Embryonic Implantation

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Contents

Abstract Implantation is a complex process involving an intricate cascade of molecular interactions between the implanting blastocyst and the receptive endometrium. The molecular basis of endometrial receptivity and the mechanisms by which the blastocyst first adheres to the luminal epithelium and then penetrates into the stroma are only just beginning to be resolved. Advances in "omics" technologies, particularly proteomics and metabolomics, are set to have a major impact on the development of this field. In the wake of this information, novel targets for contraceptive intervention may become apparent.

Keywords Embryo implantation · Proteomics · Secretomics

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1 Introduction

Implantation of the developing blastocyst is an absolute requirement for reproduction. From the embryo's viewpoint, its goal is to invade the maternal tissue and gain access to nutrients that are essential for survival and development. Implantation is a complex process in which a semiallogeneic embryo needs to be accepted by the maternal endometrium. For this to occur, a bi-directional communication between the blastocyst and the endometrium is required. This dialog enables a synchronous development of the viable embryo and the development of endometrial receptivity followed by embryo apposition, adhesion, and invasion into the stroma (Dominguez et al. 2002).

Endometrial receptivity is a self-limited period in which the endometrium acquires a functional and transient ovarian steroid-dependent status that allows a blastocyst to be received and which further supports implantation through the mediation by immune cells, cytokines, growth factors, chemokines, and adhesion molecules (Kämmerer et al. 2004; Giudice 1999a; Dimitriadis et al. 2005). This specific period, known as "the window implantation," opens 4–5 days after endogenous or exogenous progesterone stimulation and closes 9–10 days afterwards (Finn and Martin 1974; Martín et al. 2002).

Implantation itself is governed by a collection of endocrine and autocrine signals of embryonic and maternal origins as well as by the corresponding embryo–endometrial dialog. Understanding the activity and function of the molecules involved in this dialog will enable us to use them as predictors of either endometrial receptivity or embryo quality.

Recently, major advances in the genomics of the endometrium (Horcajadas et al. 2007) and oocytes (Bermúdez et al. 2004) have been achieved with the microarray and bioinformatics technologies available, to provide a vast amount of information regarding gene expression in these tissues and cells. However, gene expression is only one aspect of the complex regulatory network that allows cells to respond to intracellular and extracellular signals. Unlike the genome, the proteome itself is dynamic, complex, and variable. Furthermore, it depends upon the developmental stage of the cells, reflecting the impact of both internal and external environmental stimuli. Proteomics is often considered the next step in the study of biological systems (Fig. 1) and is more complicated than genomics, mostly because the proteome differs from cell to cell, while an organism's genome is constant, albeit with exceptions. To date, lack of sensitivity has been a stumbling block for the global introduction of proteomics into the field of human reproduction. However, new developments in mass spectrometry using protein profiling and peptide sequencing have been implemented to elucidate the underlying biological processes. In this chapter, we will review the state-of-the-art of proteomics during embryonic implantation (Fig. 2).

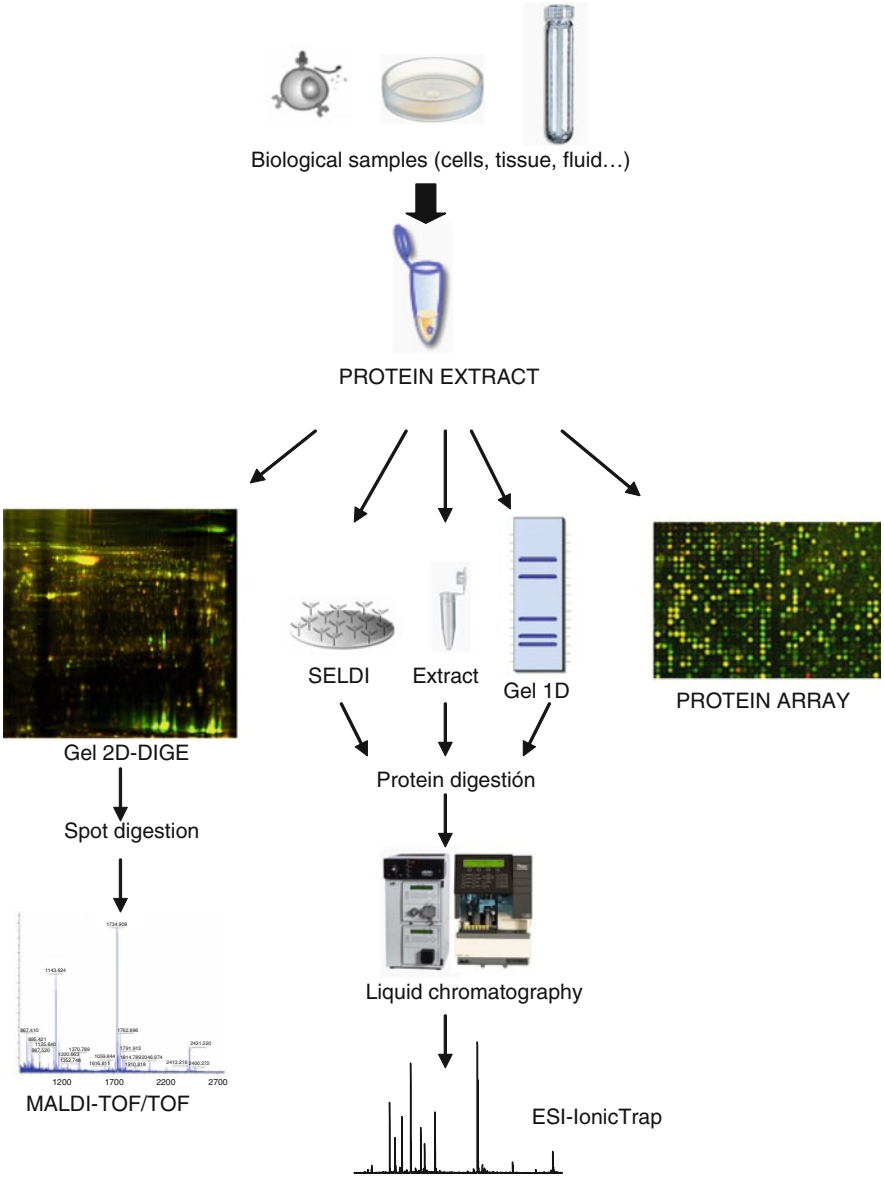

Fig. 1 Strategies of proteomic analysis. Proteins are extracted from biological samples, fractionated, separated, and analyzed by differential techniques. In gel-based methods (*left*), different protein samples are labeled with different fluorescent dyes and are then mixed together. Next, proteins are separated into two-dimensional difference gel electrophoresis (2D-DIGE) according to their isoelectric point and molecular mass. Gels are scanned by lasers and those spots corresponding to proteins with a differential pattern of expression are identified. Finally, these proteins are identified by mass spectrometry (MALDI-TOF/TOF). In the chromatographic separation methods (*center*), protein extracts, protein fractions (SELDI), or one-dimensional gel bands

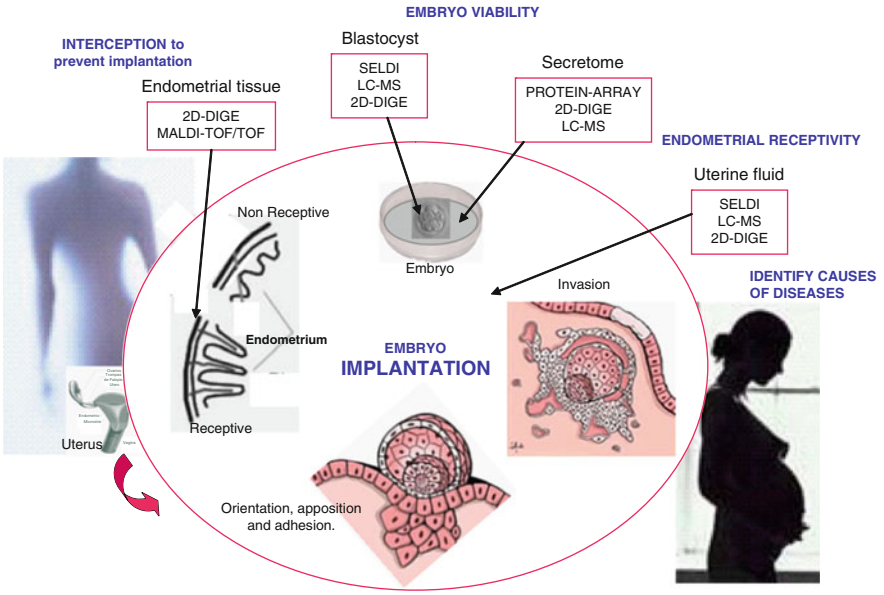

Fig. 2 Application of proteomic technologies to the study of embryo implantation. This figure shows the proteomics approaches that can be used to study the embryo implantation process. The identification of differentially expressed proteins will allow us to understand this complex biological process and to use them as key interceptive markers, to prevent embryo implantation, as markers of endometrial receptivity, embryo viability or as causes of disease

2 Proteomic/Secretomics of the Human Embryo

A crucial aspect in implantation is the concept of "embryo viability," that is, acquisition of the ability to recognize, adhere, and invade the endometrial tissue. The selection of appropriate embryos for their transfer to the uterus is a critical issue in the field of reproductive medicine. Morphological evaluation remains the primary method of embryo assessment during IVF cycles, but its limited predictive power and inherent inter- and intraobserver variability limits its value (Guerif et al. 2007). Consequently, there is a need to objectively identify those embryos with the highest implantation potential based on specific genomic, proteomic, and/or metabolomic profiles.

Katz-Jaffe et al. (2006a) developed a method to analyze the proteome of individual human blastocysts and to identify differentially expressed proteins prior to

Fig. 1 (Continued) (SDS-PAGE) are digested enzymatically, and the peptide mixture is separated by liquid chromatography (HPLC). Usually, peptides are analyzed and typically identified by an electrospray ionization mass spectrometer coupled with a linear ion trap. Other methods are based on proteins arrays (*right*). These arrays are membranes that contain a certain number of pre-absorbed antibodies that correspond to different proteins

implantation. Cryopreserved individual embryos for research were obtained from couples undergoing infertility treatment and were donated with consent and analyzed by time-of-flight mass spectrometry. Differential protein expression profiles were observed between early and expanded blastocysts, and also by developing blastocysts as opposed to degenerate embryos. Significantly, several up-regulated and down-regulated proteins were detected in degenerating embryos. A search in the protein databases highlighted several candidates, including an inhibitor of Tcf-4 (transcription factor mediating Wnt signaling) and an apoptotic protease-activating factor. Degenerating embryos displayed a significant up-regulation of several potential biomarkers which may be involved in apoptotic and growth-inhibiting pathways. Therefore, these data linked the proteomic profiles to embryo morphology.

Given the technical and ethical difficulties implied in handling human embryos, research is progressing through the application of noninvasive proteomics to study the molecules both produced by the embryo and secreted into the surrounding medium to identify novel biomarkers of embryo development and viability. In this perspective, little is known about the peptide/protein production and consumption of human embryos.

Several groups have focused on the identification of the specific molecules secreted by the embryo into conditioned media, which are considered critical for embryo viability, such as IL-1α and IL-6 (Baranao et al. 1997), IL-1α (Sheth et al. 1991), or soluble human leukocyte antigen G (HLA-G) (Desai et al. 2006; Fuzzi et al. 2002). These later studies revealed higher pregnancy rates when soluble HLA-G was detected in the conditioned media of day 3 embryos. However, the results were not consistent as pregnancies were obtained from HLA-G negative embryos. Since individual mammalian embryos have very different developmental potentials, even within the same cohort, it will be necessary to evaluate several parameters for the definitive indication of developmental competence and embryonic viability.

The proteomics platform has been successfully employed to analyze the secretome of mammalian embryos throughout preimplantation development, and a database of secretome profiles representing preimplantation development has been created (Katz-Jaffe et al. 2006b). This work revealed that human embryos produce distinctive protein profiles every 24 h of their development ($P < 0.05$) with proteins that are differentially expressed, while others remain constant across the different embryonic stages. The correlation of day 5 secretome data with ongoing blastocyst development revealed an 8.5 kDa protein biomarker that was significantly up-regulated ($P < 0.05$). The best candidate for this biomarker was ubiquitin, which has been implicated in the implantation process in some mammalian species. Ongoing research focuses on the identification of other proteins and also on the correlation of these unique protein profiles with both viability and ongoing successful pregnancy.

Recently, our group reported the partial embryonic secretome (proteins secreted/consumed) by the human blastocyst related to their implantation ability (Domínguez et al. 2008). The aim of this work was to identify changes in the protein profile of the culture media from human blastocyst cultured for 24 h, which either implanted

or did not implant, using protein-array technology. Furthermore, a statistical approach was performed to compare each of these media with a medium that did not contain blastocysts (control medium). When the protein profile of the blastocyst culture medium was compared with the controls, soluble TNF receptor 1 and IL-10 increased significantly, whereas MSP-a, SCF, CXCL13, TRAILR3, and MIP-1b decreased significantly. Specifically, CXCL13 and GM-CSF also decreased significantly in the implanted blastocyst media compared with the media from nonimplanted counterparts with a similar morphology.

We have also investigated the secretome profile of implanted blastocysts which developed after performing an embryo biopsy for preimplantation genetic diagnosis and were subsequently grown in a sequential system or cocultured with endometrial epithelial cells (EEC) (Dominguez et al. 2008a). The results after having applied protein-array technology showed a different protein pattern in these two culture systems. Interestingly, IL-6 was the most abundantly secreted protein in the EEC coculture, which enables us to conclude that the IL-6 present in the media is consumed/metabolized by the blastocyst and could be necessary for the developmental process. Furthermore, IL-6 could be considered a potential predictor of blastocyst selection, as an alternative to the usual morphological criteria.

Definitive identification of the key development proteins will provide insights into the cellular and biochemical processes occurring during human embryonic development. In addition, these data could contribute to the development of a noninvasive viability assay to be used in both clinical IVF and animal biotechnology. The identified differences in the protein profile of the culture media in the presence of implanted vs. nonimplanted blastocysts could be used as a potential marker of embryo viability and, therefore, a useful tool other than morphology to select the more appropriate blastocysts to be transferred.

3 Proteomics of the Human Endometrium

The dynamics of the endometrial transition from the nonreceptive stage to the receptive stage at the proteomic level deserves further attention to understand endometrial receptivity and to identify potential molecules for interception. DeSouza et al. (2005) employed a quantitative approach to assess the proteomic repertoire using isotope-coded affinity tags (ICAT), affinity purification and liquid chromatography coupled online to Mass Spectrometry (LC-MS) between proliferative and secretory endometria. Only five proteins showed a consistent differential expression, of which the glutamate NMDA receptor subunit zeta 1 precursor and FRAT1 were the most interesting proteins. The utility of these proteins as indicators of receptivity endometrial is open to further research.

Our group compared the proteomes of prereceptive (day LH+2) vs. receptive (LH+7) endometrial biopsies obtained from the same fertile woman ($n = 6$) in the same menstrual cycle. Biopsies were analyzed using two-dimensional fluorescence

difference gel electrophoresis (2D-DIGE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Seventy-eight differentially expressed proteins were found in the receptive vs. prereceptive endometrium, with 44 and 34 up- and down-regulated spots, respectively. It is interesting to emphasize two of the most consistently and differentially expressed proteins, Annexin A2 and Stathmin 1, which may prove important in predicting the receptivity status and could, therefore, be possible targets for interception (Dominguez et al. 2008b).

Proteomic techniques have been also used to search for proteins which are differentially expressed in endometriosis. This gynecological condition occurs when endometrial tissue becomes implanted in ectopic sites outwith the uterus, usually within the peritoneal cavity (Kitawaki et al. 2002; Donnez et al. 2002). Tabibzadeh et al. compared the 2D-PAGE of peritoneal fluid (PF) of women with and without endometriosis. However, the gels exhibited a limited number of protein spots, and the identity of the majority of the protein spots with an abnormal expression in endometriosis was neither determined by immunoblotting nor mass spectrometry (Tabibzadeh et al. 2003). Instead, they showed marked differences in the amount and type of the PF proteins present in six women with mild endometriosis, in six women with severe endometriosis, in six women with infertility and no endometriosis, and in six fertile controls using 2-DE. The proteins observed in women with infertility and without endometriosis did not differ from those of healthy controls, and mild endometriosis was associated only with a mild reduction of proteins in the 35–40 kDa and pI 5.7–6.0 ranges when compared with controls. However, a more marked decrease in the same protein spots was observed in women with severe endometriosis, which also presented a two- to fourfold increase in the amount of other numerous proteins seen in severe endometriosis when compared with controls.

Fowler et al. (2007) investigated the effects of endometriosis on the proteome of the human eutopic endometrium by using 2D-PAGE and mass spectrometry. Several deregulated proteins were identified including (1) molecular chaperones such as heat shock protein 90 and annexin A2, (2) proteins involved in the cellular redox state, such as peroxiredoxin 2, (3) molecules involved in protein and DNA formation/breakdown, including ribonucleoside-diphosphate reductase, prohibitin and prolyl 4-hydroxylase, and (4) secreted proteins, such as apolipoprotein A1.

In a similar work, Zhang et al. (2006) designed a study to search for endometriosis-specific proteins using 2-DE, and mass spectrometry. The 2-DE protein patterns of the average gels of the sera samples from women with or without endometriosis were compared, and different protein spots were detected with a discrepancy that was at least threefold. After the comparative proteomic study, the authors found 13 protein spots from serum that correlated with 11 known proteins which were expressed differently between women with and without endometriosis. While some of the differentially expressed proteins originated from the cytoskeleton, others were regulatory proteins of the cell cycle, associated with signal transduction, or with immunological function. Such proteins include the G antigen family

B1 protein, actin-related protein 6, actin like-7-anhydrase I, Dentin matrix acidic phosphoprotein I, CD166 antigen, and cyclin A1, among others.

4 Proteomics of Human Endometrial Fluid

The viscous fluid secreted by the endometrial glands provides nutrients for blastocyst formation and constitutes a microenvironment where the embryo–endometrial dialog occurs prior to implantation. It is also an important compartment for the assessment of endometrial maturation (Beier-Hellwig et al. 1989; Giudice 1999b; Lindhard et al. 2002; Herrler et al. 2003; Beier 1974; Maathuis and Aitken 1978; Beier and Beier-Hellwig 1998). Furthermore, uterine secretions are less complex in terms of their protein repertoire and may serve as a pool of biomarkers for functional endometrial operation.

Endometrial secretion has been shown to contain (1) proteins originating from the transudation of serum, (2) leakage products of apoptotic epithelial cells, and (3) proteins secreted from the glandular epithelium. This secretion undergoes significant changes in protein content during the transition from the proliferative phase to the secretory phase (Maathuis and Aitken 1978). Endometrial secretion composition varies during the menstrual cycle as a result of the changes in the ovarian steroid serum concentration (Beier and Beier-Hellwig 1998). Estradiol (E_2) regulates transudation by blood vessel dilatation and permeability, and progesterone (P) controls the secretory activity of the endometrial glands. Furthermore, endometrial secretion contains cytokines such as leukemia inhibitory factor (LIF) (Laird et al. 1997), glycodelin (PP14) (Li et al. 1993a), macrophage colony-stimulating factor (M-CSF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) (Classen-Linke et al. 2000), insulin-like growth factor binding protein 1 (IGFBP-1), interleukins (Simon et al. 1996; Makkar et al. 2006), as well as steroid and nonsteroid hormones (estrogen, progesterone, prolactin, human chorionic gonadotrophin, and precursors) (Stone et al. 1986; Licht et al. 1998).

In the past, the protein patterns of uterine secretions throughout the menstrual cycle have been analyzed by electrophoresis. These analyses revealed three different protein patterns that are typical of the equivalent phases of the menstrual cycle: intermediate phase, proliferative phase, and secretory phase. The results showed characteristic "families" of proteins bands, corresponding to 63 proteins, and some of them were identified by their molecular mass (Beier-Hellwig et al. 1989).

In another work (Van der Gaast et al. 2003), endometrial fluid obtained transcervically by aspiration immediately prior to embryo transfer was analyzed and the protein profile in each sample was determined. Although uterine fluid aspiration is a safe method, sometimes the material obtained is not enough for analysis or it may be diluted as a result of uterine washing, making the results difficult to consider. These studies also demonstrated that endometrial secretion can be obtained for analysis immediately prior to embryo transfer in IVF cycles without disrupting implantation (Li et al. 1993b; Olivennes et al. 2003).

More recently, Van der Gaast et al. (2008) investigated the effect of ovarian stimulation in IVF on endometrial secretion and markers of receptivity in the midluteal phase. The endometrial fluids obtained during this period in the stimulated cycle were compared with the spontaneous cycle. Protein composition was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and gels were stained with Coomassie brilliant blue. The protein pattern was obtained by measuring the relative density of each band by means of scanning laser densitometer and the GelScan XL software package. In this pilot study, ovarian stimulation did not alter the investigated markers of endometrial maturation in the midluteal phase.

Classically, two-dimensional electrophoresis, based on a combination of isoelectric focusing and SDS-PAGE, was the only method available to analyze the protein complement in a sample with high resolution. The introduction of protein chips and mass spectrometry has facilitated protein identification. Presently, our group is working on the proteomic analysis of endometrial fluid from natural cycles using liquid chromatography online with electrospray ionization-ion trap mass spectrometry (ESI-LC-MS) techniques. This approach circumvents one of the major challenges of endometrial research, that of investigating endometrial performance during the window of implantation without disrupting endometrial function and the subsequent process of implantation.

The current technical limitations of applying proteomics to the study of protein patterns in endometrial fluid is that the majority of identified proteins correspond to serum proteins, thus masking the identification of proteins present at low

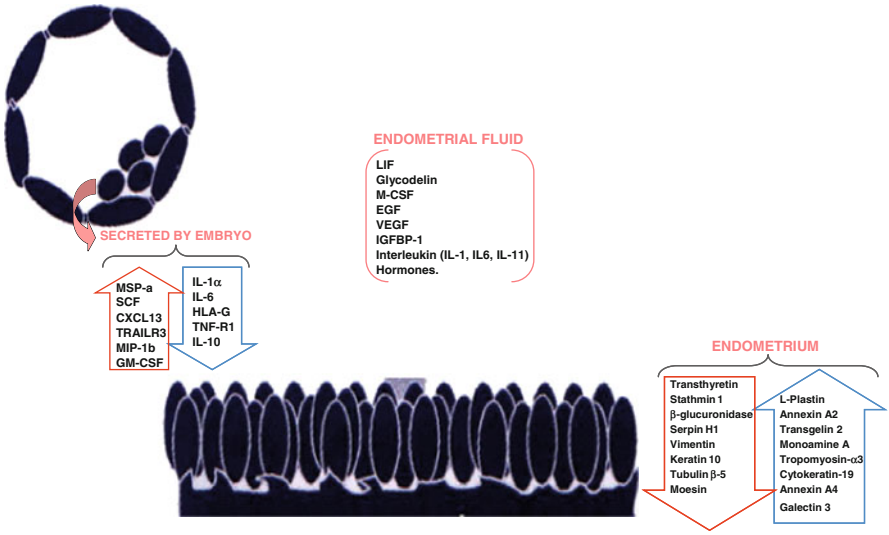

Fig. 3 Proteins implicated in the implantation process. Proteins found up- and down-regulated by different authors in the following compartments:endometrial tissue, endometrial fluid, and embryo secretion, at the time of implantation

concentrations which may be of great interest, such as biomarkers for endometrial receptivity, embryo development, diseases, and/or contraceptive intervention.

5 Conclusions

The significant histological, biological, and physiological events that occur during implantation are ultimately the result of regulated changes in gene transcription, together with posttranscriptional, posttranslational, and epigenetic modifications that ultimately control expression the embryonic and endometrial proteomes (Fig. 3). In recent years, "omics" techniques have advanced to the point that rapid identification of genes or proteins of interest can be readily secured. However, technical limitations still exist which complicate the unification of the results obtained.

Proteomics together with genomics and metabolomics are complementary approaches that provide diverse but comparable perspectives, which will improve our understanding of the complexity of the implantation process, including the identification of key biomarkers. The next step will be to integrate this information into a system biology approach to develop models for functions of interest, such as embryo viability, endometrial receptivity, and the embryo–endometrial dialog.

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Evaluation of Plasma Membrane Calcium/Calmodulin-Dependent ATPase Isoform 4 as a Potential Target for Fertility Control

Elizabeth J. Cartwright and Ludwig Neyses

Contents

Abstract The array of contraceptives currently available is clearly inadequate and does not meet consumer demands since it is estimated that up to a quarter of all pregnancies worldwide are unintended. There is, therefore, an overwhelming global need to develop new effective, safe, ideally non-hormonal contraceptives for both male and female use.

The contraceptive field, unlike other areas such as cancer, has a dearth of new targets. We have addressed this issue and propose that isoform 4 of the plasma membrane calcium ATPase is a potentially exciting novel target for fertility control.

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The plasma membrane calcium ATPase is a ubiquitously expressed calcium pump whose primary function in the majority of cells is to extrude calcium to the extracellular milieu. Two isoforms of this gene family, PMCA1 and PMCA4, are expressed in spermatozoa, with PMCA4 being the predominant isoform. Although this gene is ubiquitously expressed, its function is highly tissue-specific.

Genetic deletion of PMCA4, in PMCA4 knockout mice, led to 100% infertility specifically in the male mutant mice due to a selective defect in sperm motility. It is important to note that the gene deletion did not affect normal mating characteristics in these mice. This phenotype was mimicked in wild-type sperm treated with the non-specific PMCA inhibitor 5-(and 6-) carboxyeosin diacetate succinimidyl ester; a proof-of-principle that inhibition of PMCA4 has potential importance in the control of fertility.

This review outlines the potential for PMCA4 to be a novel target for fertility control by acting to inhibit sperm motility. It will outline the characteristics that make this target drugable and will describe methodologies to identify and validate novel inhibitors of this target.

Keywords Drug target · Non-hormonal contraceptive · Plasma membrane calcium ATPase · Sperm immotility

1 Introduction

1.1 The Need for New Safe, Effective, Non-hormonal Contraception

With the growing global population there is an ever increasing need for safe, effective and accessible contraception for both males and females. Although there are a variety of barrier methods, hormonal steroids, and sterilisation methods in wide use, the contraceptives currently available clearly do not meet global demands since it is estimated that more than a quarter of pregnancies worldwide are unintended (Global Health Council 2002). It is also clear that a variety of contraceptive methods are required as people have changing contraceptive needs throughout their reproductive years and, on a global scale, different societies have very different requirements with respect to contraception. So, although the contraceptive market is truly global it is vital that the method of contraception responds to both individual and societal needs, e.g. in the Western world males are looking for an easy to use contraceptive method, in developing countries females take control of contraception, and in many countries a barrier contraceptive is still a requirement to reduce the spread of infectious disease.

The use of steroids as contraceptives has been widely accepted for several decades; however, although steroidal methods are available for both men and

women it is women that have particularly embraced their use. Both oral and non-oral forms of steroidal contraceptives are available which may combine oestrogen and progestin or progestin alone to prevent pregnancy by interfering with ovulation and the menstrual cycle. These methods can be highly effective when used/taken correctly; for example, the traditional oral contraceptive taken for 21 days is 99% effective at preventing pregnancies. However, problems may arise with compliance, which is why many of the recent efforts in the contraceptive field have focused on developing more "user-friendly" methods of delivering steroidal contraceptives. Steroid-hormone contraceptives are also associated with a number of side effects, many of which prohibit their use in certain groups e.g. women with hypertension, or make their use unacceptable to individuals; their effectiveness is also reduced if, for example, the woman has sickness or diarrhoea.

Steroidal contraceptives designed to suppress gonadotrophins have been the focus of numerous research projects to develop a widely acceptable method of male contraception (Handelsman et al. 1996; Anawalt et al. 1999; Wu et al. 1999; Kamischke et al. 2001; Gu et al. 2003; Hay et al. 2005). These approaches have had varying levels of success as measured by the ability of the treatment to suppress spermatogenesis. There are many aspects to consider when developing a male contraceptive; these include its efficacy, hormonal profiles during treatment and after termination, time taken to restore fertility following termination of treatment and drug safety. It is also essential to consider and assess the attitudes and expectations of the male user, and importantly his female partner, to male contraception in general as well as to the specific regimen under development. A recent study of 9,342 men from nine countries, across four continents, ranging in educational background, income levels and religious beliefs, were surveyed about their attitudes to male fertility control (Heinemann et al. 2005). This study concluded that there is definitely a market for male contraceptive methods, but that attitudes are clearly not homogeneous; a fact that will need to be taken into account when developing a product/products for market.

Thus, there is clearly an ongoing need for the development of new contraceptives and Nass and Strauss (2004) have summarised the strategies that need to be followed to achieve this objective. These include three important processes: (1) the identification and validation of novel targets, (2) enhanced contraceptive drug discovery, development and clinical testing, and (3) to facilitate and coordinate contraceptive research and development in the future (Nass and Strauss 2004). Unfortunately, as summarised by Aitken et al. (2008), there has been a downturn in the amount of research and development carried out in this area by the major pharmaceutical companies; in this analysis, it was calculated that to develop a new contraceptive from validated target to phase III clinical trial would take 15–20 years and cost in the region of $100 million (Aitken et al. 2008). This trend must be reversed if we are meet global contraceptive needs in the twenty-first century.

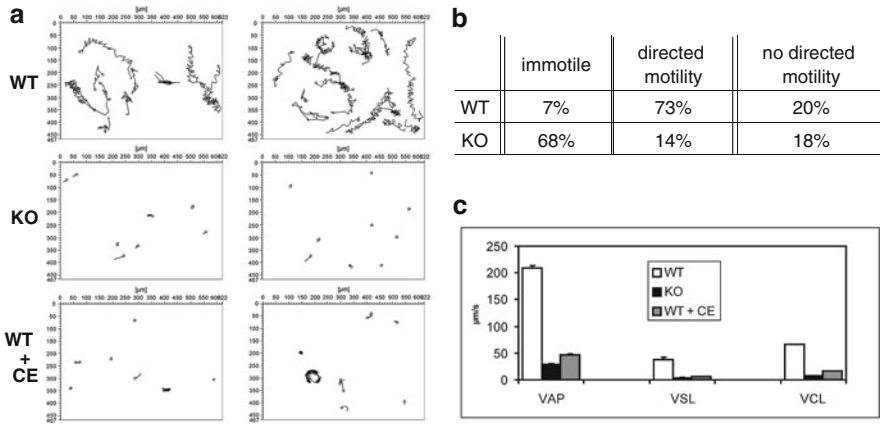
Our own research has addressed one of the most important steps in the development of a new contraceptive; we have identified a potential new target for fertility control: isoform 4 of the plasma membrane calcium ATPase.

2 The Role of PMCA4 in Sperm Motility

We have generated a strain of mice which carries a genetic deletion of the calcium pump PMCA4 (plasma membrane calcium/calmodulin-dependent ATPase, isoform 4) (Schuh et al. 2004). The male mice are 100% infertile due to a selective defect in sperm motility, but otherwise are completely normal under physiological conditions, as are the females. Our findings were later confirmed by Okunade and colleagues who generated a similar PMCA4 knockout mouse model (Okunade et al. 2004).

Mouse spermatozoa express two isoforms of PMCA, PMCA1 and PMCA4. PMCA4, which is by far the dominant isoform, is localised to the principal piece of the sperm tail (Okunade et al. 2004). It is interesting to note that CatSper1–4, which are essential channels controlling Ca^{2+} influx, are required for sperm motility and like PMCA4 are localised to this region of the sperm tail (Ren et al. 2001; Quill et al. 2003; Jin et al. 2007).

By using the now well established technique of gene targeting by homologous recombination in embryonic stem cells, we generated a mouse model in which PMCA4 was deleted in all cells (PMCA4 null mutant; PMCA4 knockout) (Schuh et al. 2004). This has enabled us to evaluate the function of the protein in vivo and to determine the isoform-specific functions of this gene. Breeding mice heterozygous for the deletion of PMCA4 ($PMCA4^{+/-}$) yielded homozygous knockout ($PMCA4^{-/-}$, PMCA4 KO), heterozygotes and wild-type (WT) mice in the expected 1:2:1 Mendelian ratio. However, when homozygous knockout males and females were crossed no pups were born. By analysing the mating behaviour of the PMCA4 KO mice it was clear that both males and females showed normal mating characteristics leading to the development of a vaginal plug, a sign in mice of successful mating. By breeding male or female PMCA4 KO mice with wild-type females and males, respectively, only the female knockout/male wild-type pairings produced litters. It was then evident that the male PMCA4 KO mice had a fertility problem, and that the female null mutant mice had normal fertility. Morphological analysis of the sperm and testis from PMCA4 KO mice appeared normal; by staining sections of the testis with haematoxylin/eosin no histological differences between PMCA4 KO and WT mice were apparent and, in addition, analysis of sperm isolated from PMCA4 KO mice showed no obvious cytological differences when compared with sperm from wild-type littermates. These results led us to investigate the motility of the spermatozoa from the PMCA4 KO mice. Using CASA analysis to study the main motility parameters in sperm we demonstrated a clear functional difference between spermatozoa from PMCA4 KO mice and their wild-type littermates. PMCA4 null mutant sperm showed severely reduced average path velocity (VAP), a virtual lack of progressive velocity (VSL) and impaired track speed (VCL), as shown in Fig. 1. Okunade and colleagues were also able to demonstrate that PMCA4 null mutant sperm are unable to achieve hyperactivated motility (Okunade et al. 2004). We concluded from these data that deletion of PMCA4 leads to a highly specific form of male infertility, characterised by normal mating



	immotile	directed motility	no directed motility
WT	7%	73%	20%
KO	68%	14%	18%

Fig. 1 PMCA4 deficient sperm display highly reduced motility. (**a**) Examples of recordings of sperm motility paths from wild type (WT) and PMCA4 knockout mice (KO). Sperm from WT mice treated with the PMCA inhibitor 5-(-6)-carboxyeosin diacetate succinimidyl ester (CE) mimic the effect of PMCA4 gene deletion on sperm motility. (**b**) PMCA4 deficient sperm display highly reduced motility and low directed motility compared with WT sperm. (**c**) Main motility parameters showed that average path velocity (VAP), progressive velocity (VSL) and track speed (VCL) were severely impaired in PMCA4 deficient and CE-treated sperm. Adapted and reproduced from Schuh et al. 2004 with permission from the American Society for Biochemistry and Molecular Biology (ASBMB)

behaviour but severely impaired sperm motility. We therefore hypothesised that we would be able to mimic the effect of the genetic deletion of PMCA4 by treating wild-type sperm with an inhibitor of PMCA; it must be noted that the inhibitor used in these experiments is not specific solely to isoform 4 of PMCA. In the event, spermatozoa treated with the cell-permeable inhibitor 5-(and 6-) carboxyeosin diacetate succinimidyl ester (CE) displayed severely reduced sperm motility, see Fig. 1.

It is likely that the motility phenotype is linked to the elevated concentration of intracellular Ca^{2+} that was observed in the mutant sperm (Schuh et al. 2004). Ca^{2+} is known to be an important signalling molecule involved in the regulation of sperm motility (Darszon et al. 2005) and a study has clearly shown that PMCA has an essential role in the extrusion of Ca^{2+} and regulation of basal calcium levels in spermatozoa (Wennemuth et al. 2003).

3 The Plasma Membrane Calcium/Calmodulin-Dependent Calcium ATPases

The plasma membrane calcium pump is a 134 kDa protein which belongs to a family of P-type ATPases, comprising a group of ATP-fuelled ion pumps that form a phosphorylated (aspartyl phosphate) intermediate as part of their reaction cycle

(Olesen et al. 2007). All isoforms of PMCA act to extrude Ca^{2+} from the cytosol to the extracellular compartment, in exchange for protons, thereby regulating global intracellular Ca^{2+} levels, local Ca^{2+} levels and/or Ca^{2+} signalling, depending on the cell type and isoform (Carafoli and Stauffer 1994; Wennemuth et al. 2003; Okunade et al. 2004; Schuh et al. 2004; Brini et al. 2007; Oceandy et al. 2007; Strehler et al. 2007; Baggaley et al. 2008; Cartwright et al. 2009).

3.1 Tissue Distribution of PMCA Isoforms and In vivo Specificity of Function

Mammals possess four isoforms of PMCA (PMCA1–4 – gene names *Atp2b1–4*) encoded by four independent genes at distinct chromosomal locations. In humans, these genes are located on chromosomes 12q21-q23, 3p25-p26, Xq28 and 1q25-q32, respectively (Olson et al. 1991; Wang et al. 1994). The four isoforms clearly have distinct functions and expression patterns. PMCA1 has long been viewed as the housekeeping isoform as it is the isoform which is expressed earliest during development (Zacharias and Kappen 1999) and has been identified in the vast majority of tissues/cell types investigated (Strehler and Zacharias 2001). Recent data from genetically modified mice has upheld the theory that PMCA1 is a housekeeping gene, at least during embryonic development, since mice carrying a null mutation in PMCA1 die during a very early stage of embryonic development (Okunade et al. 2004; our own unpublished data). The expression of PMCA isoforms 2 and 3 is much more restricted. Both isoforms are expressed in regions of the brain (Stauffer et al. 1993) with PMCA2 being particularly highly expressed in the Purkinje cells of the cerebellum and PMCA3 in the choroid plexus (Stahl et al. 1992); additionally, PMCA2 is highly expressed in lactating mammary tissue and steriocilia of hair cells associated with the cochlea (Reinhardt et al. 2000; Dumont et al. 2001). PMCA4 has also been found to be expressed in all tissues/cell types investigated; it is the dominant isoform in erythrocytes and spermatozoa and is also expressed in heart, kidney, skeletal muscle, brain, pancreas, small intestine as well as the lens and corneal epithelium of the eye (Brandt et al. 1992; Stauffer et al. 1995; Okunade et al. 2004; Marian et al. 2005; Talarico et al. 2005). Given recent knockout data (Okunade et al. 2004; Schuh et al. 2004) which shows that deletion of PMCA4, unlike PMCA1, does not lead to embryonic lethality, the conventional view that both isoforms 1 and 4 are "housekeeping" enzymes (Stauffer et al. 1995; Garcia and Strehler 1999) is now being replaced by the view that PMCA4 has highly specialised functions, whereas PMCA1 may fulfil the "housekeeping" role.

The study of genetically modified mice has greatly informed our knowledge of the specialised and highly specific functional roles of the PMCA isoforms in vivo. Gene knockout mice, mice which overexpress isoforms of PMCA and those carrying spontaneous mutations have all been studied (Prasad et al. 2007; Cartwright et al. 2009). As has been described above, loss of function of PMCA1

(PMCA1 knockout) leads to early embryonic death. Ablation of PMCA2 function in a gene knockout model and in two spontaneous mutants has been extensively shown to be associated with deafness (Kozel et al. 1998; Street et al. 1998; Takahashi and Kitamura 1999). Loss of PMCA2 function has also been associated with human disease; a mutation in the gene having been associated with hearing loss (Schultz et al. 2005). Little is known about the in vivo function of PMCA3 but it is anticipated that over the coming years a gene knockout model will be generated since there are a number of international consortia whose aim is to generate a gene knockout mouse model of every protein-coding gene in the mouse genome (EUCOMM: European Conditional Mouse Mutagenesis Programme http://www.eucomm.org associated with EUMODIC: European Mouse Disease Clinic http://www.eumodic.org; KOMP: Knockout Mouse Project http://www.nih.gov/science/models/mouse/knockout; NorCOMM: North American Conditional Mouse Mutagenesis Programme http://www.norcomm.org). PMCA4 as described in Sect. 2 has a highly specialised function in regulating sperm motility, it has also been shown that when this gene is overexpressed in the vascular smooth muscle and myocardium it is involved in the regulation of peripheral vascular tone, and cardiac contractility and hypertrophic responses, respectively (Schuh et al. 2003; Oceandy et al. 2007).

4 PMCA4 as a Suitable Drug Target

It is clear that although PMCA4 is ubiquitously expressed its function is highly tissue-specific, with physiological relevance in the sperm. We believe that this identifies PMCA4 as a novel target for developing inhibitors for use as a contraceptive.

4.1 Suitability of the Structure of PMCA4 to Drug Targeting

Hydrophobicity plots and molecular modelling (see Fig. 2) show that PMCA is a ten transmembrane (TM) domain protein with four major intracellular areas: the N-terminal stretch whose function is largely unknown; the loop between TM domains 2 and 3, which is believed to participate in calcium pore formation; the loop between TM domain 4 and 5 containing the ATP binding site as well as the site of high-energy phosphate bond formation; and the C-terminus which contains the calmodulin binding site. Binding of calmodulin is calcium-dependent and leads to disinhibition of the pump. This region also contains the PDZ binding domain, in certain splice variants, which leads to interactions with proteins, many of which are essential to the role of PMCA in Ca^{2+} signalling (Cartwright et al. 2007; Strehler et al. 2007). The regulation of the activity of PMCA and its structural domains has been detailed in a number of reviews to which we refer the reader (Carafoli 1991;

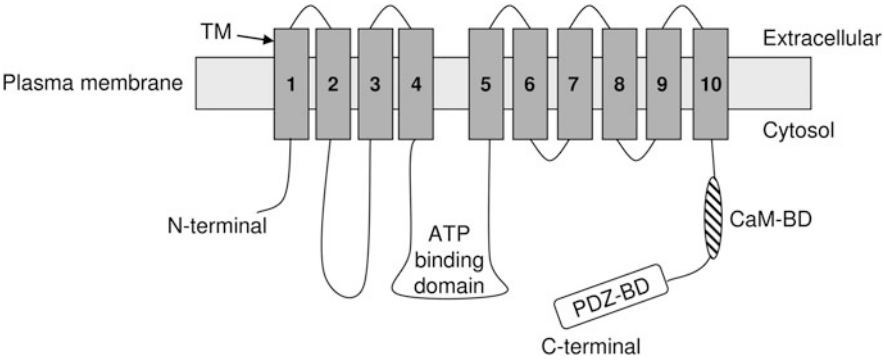


Fig. 2 Illustration of the membrane topology of PMCA4. The ten transmembrane domains (TM) are numbered 1–10. The largest intracellular loop between TM4 and 5 contains the ATP binding domain. When the pump is in the inactive state, the autoinhibitory calmodulin-binding domain (CaM-BD) will interact with regions of the large first and second intracellular loops (between TM2 and 3 and TM4 and 5); binding of Ca^{2+}-calmodulin to the CaM-BD will release this interaction and render the pump active

Strehler and Zacharias 2001; Cartwright et al. 2008; Di Leva et al. 2008). All four of the intracellular domains as well as the extracellular amino acid stretches between TM domain 1 and 2, and between TM domains 3 and 4 are suitable candidates for drug targeting (see Sect. 6). By analogy with most proteins/drug targets, splicing variants are known; one in the intracellular loop between TM domain 2 and 3 and one at the C terminal end. However, the roles of the several known splice variants of the PMCA have not been fully elucidated.

To know the high resolution structure of a transporter and to investigate its interaction with a hit or lead compound using X-ray diffraction analysis would be hugely informative. However, as yet the crystal structure for PMCA has not been identified. The crystal structure of another PII-type ATPase (ten transmembrane domain calcium transport ATPase), the sarcoplasmic reticulum calcium ATPase (SERCA), which has recently been published (Olesen et al. 2007), can be used as a model for PMCA at least until its own crystal structure is identified (Di Leva et al. 2008). Recently, substances have been identified that inhibit SERCA at sub-picomolar levels, which are being developed as anti-prostate cancer agents (Sohoel et al. 2005) providing another strong argument that this class of targets, which includes PMCA4, are potentially ideal targets for drug development.

4.2 Drugability of PMCA4

The type II class of P-type ion-motive ATPases, to which PMCA4 belongs, is a class of drug with a particularly strong track record of therapeutically relevant inhibitors. Notably, pumps with a structure similar to PMCA4 such as the Na^+/K^+

Fig. 3 Alignment of the predicted membrane domains of PMCA4, Na^+/K^+ ATPase and H^+/K^+ ATPase. *Red boxes*: predicted transmembrane domains of PMCA4. *Blue boxes*: predicted transmembrane domains of Na^+/K^+ and H^+/K^+ ATPases. *Yellow boxes*: residues required to bind ouabain. *Green boxes*: residues implicated in omeprazole or lanzoprazole binding. *Asterisks*: amino-acid homology between all three proteins

ATPase and the H^+/K^+ ATPase have well described clinically useful inhibitors, namely, ouabain and omeprazole, respectively. Ouabain (digitalis) has been used within the cardiovascular field for two centuries and omeprazole is a blockbuster antacid.

Although the structural/regulatory/functional similarities between PMCA4 and these other P-type ATPases suggest that PMCA4 itself may be targetable, it is clear that the sequences of these enzymes are sufficiently divergent from PMCA4 to avoid non-specific cross-inhibition. Alignment of the sequences of Na^+/K^+ ATPase and the H^+/K^+ ATPase to that of PMCA4 identifies that the extracellular regions which bind to ouabain and omeprazole align with the putative extracellular domain of PMCA4 between transmembrane domains 3 and 4, suggesting the PMCA4 may be targetable at this region (see Fig. 3). It is interesting to note that the known PMCA inhibitor caloxin 2A1 (Chaudhary et al. 2001) binds in this region; while this compound has no potential for drug development because of its low affinity, this is proof-of-principle that this part of the pump is of strategic importance.

4.3 Target Validation: Modelling Target Action Using Knockout Mice

As described in Sect. 2, PMCA4 was identified as a potential target for fertility treatment based on the data generated by the analysis of PMCA4 knockout mice. Can these mice be considered as an effective genetic model in which to study drug action in vivo? There is clear evidence to suggest that gene knockout models can provide extremely valuable information regarding potential drug action; a retrospective review of gene knockout information encoding targets for 100 best-selling drugs demonstrated that there is an excellent (~85%) correlation between the phenotypes observed in these knockout mice and the efficacy and action of the drug (Zambrowicz and Sands 2003, 2004). Gene knockout models are not only able to provide important information about the target but also any off-target effects and issues that may arise. It must be clear however that gene knockout models are *not*

drugs and the data obtained from their analysis must be considered as part of a package of information. Some of the problems associated with the study of gene knockouts stem from being unable to determine the phenotype due to embryonic lethality, developmental defects or compensatory effect of other genes. These factors however are not an issue in the case of PMCA4 knockout mice.

5 Identification of Hit Compounds

There are currently two groups of compounds known to inhibit members of the PMCA family, caloxins (Szewczyk et al. 2008) and eosin and its derivatives (Gatto and Milanick 1993). These compounds could be used as potential chemistry start points in the identification of specific and efficient inhibitors, which possess characteristics essential to the pharmaceutical industry, as opposed to being suitable only for use in basic research. It is also possible to consider inhibitors of other P-type ATPases such as ouabain and omeprazole as potential start points (as highlighted in Sect. 4.2 and Fig. 3). It would also be prudent to take steps to identify novel inhibitors of PMCA4; an issue which we have begun to address.

To improve the probability of identifying a suitable hit substance active against a target of interest, it is advantageous to develop a high throughput screening approach (HTS) to enable a large number of compounds to be evaluated for their biological activity. In order to assess the ability of compounds to inhibit the activity of PMCA4, we exploited the ATPase activity of PMCA4 to develop a high throughput screen. As mentioned above, the plasma membrane Ca^{2+} pump is a member of the P-type pump family, which is characterised by the formation of a high-energy phosphorylated intermediate during the reaction cycle. It is a widely accepted view that two conformational states of the phosphorylated PMCA exist, E1 and E2 (see Fig. 4) (Di Leva et al. 2008); although it should be noted that there are some criticisms of this view (Scarborough 2003).

PMCA, like other P-type ATPases such as the SERCA, undergoes a reaction cycle in which the enzyme exists in two distinct conformational states known as E1

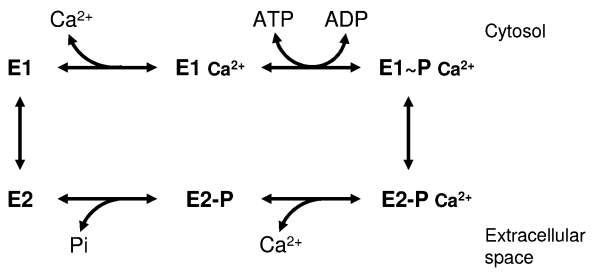


Fig. 4 Illustration of the PMCA reaction cycle. In the E1 conformation, the pump binds calcium with high affinity at the cytoplasmic side. In the E2 conformation, the pump has a lower affinity for calcium which promotes its release into the extracellular space

and E2 (de Meis and Vianna 1979; Krebs et al. 1987; Di Leva et al. 2008). In the E1 state, Ca^{2+} binds with high affinity at the cytoplasmic side of the membrane. The reaction with ATP leads to the phosphorylation of aspartate, forming the aspartyl phosphate intermediate which is characteristic of this family of P-type ATPases, leading to a conformational change in the enzyme from E1∼P to E2-P. In its E2 conformation, the enzyme releases Ca^{2+} to the extracellular space since in this conformation Ca^{2+} is bound at lower affinity. After the Ca^{2+} has been released, the E2-P intermediate is cleaved and the enzyme returns to the E1 conformation for the cycle to begin again.

There are two well established methods for assessing ATPase activity. One is a colorimetric assay which utilises the inorganic phosphate produced by ATP hydrolysis to complex to the triphenylmethane dye – malachite green (see Fig. 5a); this causes a colour change, which can easily be detected via a colorimeter (Osborn et al. 2004). This method is highly amenable to HTS. An alternative method for measuring ATPase activity is based on the coupled enzyme approach. Again, the assay measures the reaction products from ATP hydrolysis and is based on the regeneration of PMCA-dependent released ADP by pyruvate kinase which converts phosphoenolpyruvate to pyruvate. The released pyruvate is converted to lactate by lactate dehydrogenase using NADH (see Fig. 5b). Detection of the rate of decline of NADH over 10 min is used to determine the activity of the pump. This assay has been optimised (Hammes et al. 1998) and adapted for high-throughput screening. This involved reduction of statistical variation by testing multiple cell lines, various transfection methods (we eventually opted for an adenoviral-PMCA4 construct), membrane isolation methods, purity of compounds used in the coupled enzyme

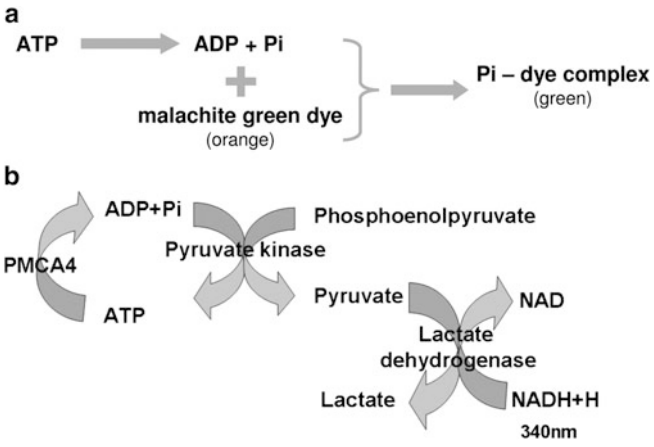


Fig. 5 Assays to detect PMCA ATPase activity. (**a**) Malachite green assay: in which the conversion of ATP to ADP leads to the released inorganic phosphate (Pi) forming a complex with the malachite green dye, leading to a measurable colour change. (**b**) Coupled enzyme assay: in which PMCA hydrolysis is coupled to NADH oxidation with pyruvate kinase, lactate dehydrogenase and phosphoenolpyruvate. PMCA activity is determined by measuring the decreased absorbance of NADH at 340 nm

assay, amount and viscosity (and hence precision) of automated pipetting, temperature, duration, etc. Various SERCA and Na^+/K^+ ATPase inhibitors, calcium/calmodulin activation, carboxyeosin inhibition, as well as the response to different amounts of human PMCA4 expressed in the cells, were used to ascertain that the measured ATPase activity originated from PMCA4.

Since the turn of the century there has been a downward trend in the number of new products reaching the market (Brown and Superti-Furga 2003), so it appears to be prudent that we use a combination of new and established tools/technologies available to enhance the development of small molecules against a known target. One such method is fragment-based drug design in which X-ray crystallography is used to screen fragment libraries for specific binding to a target protein (Hajduk and Greer 2007). This technique has the advantage over HTS in that fewer compounds need to be screened, it detects fragments that bind with low affinity (~100 μM–10 mM) and identifies substantially fewer false positives. A major concern with this technique concerns the ability to express a sufficient quantity and quality of membrane proteins such as PMCA4. To date these types of proteins have proved to be extremely difficult to produce and crystallise.

3-D protein structures and high resolution crystal structures have been used in drug discovery for many years; however, as described above, there is no such high resolution structure available for PMCA4. In its absence, it is possible to use structure activity relationship (SAR) studies to develop the inhibitors identified by HTS. This technology explores structural variants of the hits and attempts to model the relationship between structure and activity. The model can then be used to predict new structures designed specifically to enhance activity.

6 Specificity of Action of Potential PMCA4 Inhibitor

Unlike the majority of other drugs, a contraceptive will be used by healthy people as a preventative method, rather than as a treatment. For this reason, there is little tolerance of side effects and specificity of action is essential. It is therefore vital that the specificity of action of any identified PMCA4 inhibitor is determined. This will include determining that at the dosages used, the resultant inhibitor levels do not act to inhibit other P-type ATPases, it should be specific to isoform 4 of PMCA, and since PMCA4 is a ubiquitously expressed enzyme it is essential that the tissue specificity of the inhibitor is determined.

By analogy with many drug targets, such as kinases, nuclear hormone receptors, etc., the four PMCA isoforms have significant sequence similarities; however, significant divergences exist in strategic locations making it highly likely that drugs can be developed that specifically inhibit isoform 4, but not isoforms 1–3 (see Fig. 6).

Comparison of the amino acid sequences of the four PMCA gene products enabled the degree of homology in both extracellular and intracellular regions of

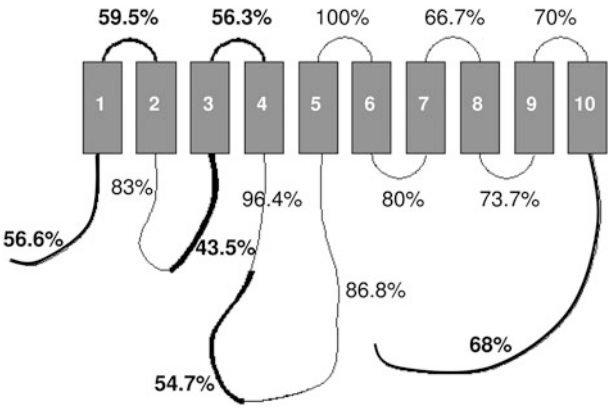


Fig. 6 Homology of the four human PMCA isoforms. Each putative intracellular and extracellular domain of PMCA has been sequence aligned to determine the percentage homology between all isoforms. Regions of low homology are highlighted in *bold*

the pump to be determined. It is clear that there are great sequence variations between isoforms in the extracellular region between TM domains 3 and 4; this region as well as the first extracellular loop are the binding sites of the peptide inhibitors caloxin 2A1 and 1A1, respectively. This alignment also shows that both the N and C termini also differ considerably between isoforms. Clearly, there are several regions of the protein that are sufficiently divergent from other isoforms of PMCA to make the identification of a PMCA4-specific inhibitor entirely likely.

It is a relatively commonly held view that it is necessary to identify genes that are expressed specifically in the target tissue of interest to provide highly specific drugs with a low potential risk of side effects. For example, it has been suggested that identification of genes specifically expressed in the reproductive tissues could lead to the development of contraceptives with highly selective effects (Nass and Strauss 2004). This goal, however, may be unrealistic since the majority of genes and their products are found to be expressed in many tissues, perhaps with distinct temporal expression, or expression associated with the current condition of the tissue. Indeed, it is important to note that many of our most successful drugs do not act in a tissue-specific manner; for example, aspirin, ACE-inhibitors and beta-blockers are targeted against specific proteins but these proteins are expressed in more than one tissue. Conversely, drugs may be targeted against a tissue-specific protein, but have other molecular effects (e.g. HMG inhibitors). Furthermore, it is well known that side effects frequently occur off-target, e.g. HIV protease inhibitors have an exclusively non-human target, but still display severe side effects such as allergies and accelerated atherosclerosis. This is not to say that tissue specificity should not be a goal but a ubiquitous enzyme such as PMCA4, which has tissue-specific function, should certainly not be disregarded based on this property alone.

7 Conclusions

There are many barriers to the development of new contraceptive drugs, an area which currently lags behind demand. Key issues in this context are that safe and effective contraception also needs to be affordable and widely accessible. Moreover, the costs to industry for the development of a drug and its introduction into the market are enormous and the financial rewards may not warrant such an input. There also needs to be an increase in the amount of basic research carried out in this field, leading to the identification and validation of new targets.

We believe we have identified the plasma membrane calcium ATPase isoform 4 (PMCA4) as a credible target for fertility control. It meets the essential criteria for a potentially successful target; gene knockout studies in the mouse have shown it to have a highly tissue-specific role in sperm motility, its sequence is suitably divergent from other family members that it will be possible to develop drugs to inhibit isoform 4 only, and the target (an enzyme) is drugable thus making it a good target for small molecule inhibitors.

The identification and validation of PMCA4 as a target is the first step in a long process in the development of a new contraceptive drug. Identification and validation of a lead compound is currently a work in progress. There must then follow a series of safety and efficacy tests in humans (Phase I, II and III clinical trials) and approval of the drug by the appropriate licensing authorities prior to the introduction of any drug onto the market.

PMCA4 inhibition effectively targets sperm motility and it is anticipated that a pharmacological inhibitor can be developed to act as a contraceptive. Although the sperm themselves would be the logical target for a male contraceptive, this does not preclude the use of such an inhibitor as a female contraceptive. We believe therefore that PMCA4 is an exceptionally strong target with a great potential to meet the needs of both industry and society.

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Part II
Male Reproduction

New Insights into Sperm Physiology and Pathology

R. John Aitken, Mark A. Baker, Geoffry N. De Iuliis, and Brett Nixon

Contents

Abstract Infertility is a relatively common condition affecting approximately one in ten of the population. In half of these cases, a male factor is involved, making defective sperm function the largest single, defined cause of human infertility. Among other factors, recent data suggest that oxidative stress plays a major role in the etiology of this condition. Spermatozoa spontaneously produce a variety of reactive oxygen species (ROS) including the superoxide anion, hydrogen peroxide and nitric oxide. Produced in small amounts, ROS are functionally important in driving the tyrosine phosphorylation cascades associated with sperm capacitation. However, when ROS production exceeds the spermatozoa's limited antioxidant defenses, a state of oxidative stress is induced characterized by peroxidative damage to the sperm plasma membrane and DNA strand breakage in the sperm nucleus. Such oxidative stress not only disrupts the fertilizing potential of human spermatozoa but also the ability of these cells to create a normal healthy embryo. As a result, DNA damage in human spermatozoa is correlated with an increased incidence of miscarriage and various kinds of morbidity in the offspring.

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These insights into the pathophysiology of defective sperm function have clear implications for the diagnosis and treatment of male infertility, particularly with respect to the potential importance of antioxidant therapy. These concepts may also be relevant to the design of novel approaches to male contraception that attempt to replicate the pathological situation.

Keywords Chromatin protamination · Oxidative stress · Reactive oxygen species DNA damage · Spermatozoa

Abbreviations

8OHdG	8-OH, 2′-deoxyguanosine
cAMP	Cyclic adenosine monophosphate
CMA3	Chromomycin
DPI	Diphenylene iodonium
DUOX	Dual oxidase
H_2O_2	Hydrogen peroxide
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOX	NAD(P)H oxidase family
ONOO	Peroxynitrite
PKA	Protein kinase A
ROS	Reactive oxygen species
SOD	Superoxide dismutase

1 Introduction

Male infertility is a relatively common condition affecting around 1 in 20 of the male population (McLachlan and de Kretser 2001). Notwithstanding the high prevalence of this condition, our understanding of the underlying etiology is still rudimentary and therapies are empirical. With the exception of a small percentage of severely infertile males possessing significant deletions of their Y chromosome (Nuti and Krausz 2008), attempts to identify a genetic basis for male infertility have been largely unsuccessful. What we do know is that a majority of such patients possess sufficient numbers of spermatozoa to fertilize the egg in vivo. However, the quality of these gametes is compromised to the point that fertilization and the initiation of normal embryonic development are not possible. The molecular basis of the functional defects present in the spermatozoa of male patients is the subject

of ongoing research. If these mechanistic details could be resolved, the outcomes might have important implications for the diagnosis, treatment and prevention of this distressing condition. Furthermore, a detailed understanding of the mechanisms responsible for defective sperm function might provide valuable information on the selection of suitable targets for male fertility regulation. After all, such patients suffer from no systemic morbidity other than a loss of fertility and, in many ways, might be considered prime examples of the kind of condition that we should like to replicate for contraceptive purposes, i.e., healthy individuals suffering from a selective loss of fertility due to functional defects in their spermatozoa. Elucidating the exact nature of the lesions that are present in the spermatozoa of infertile males is therefore of equal interest to all those wishing to repair, or suppress, the fertilizing capacity of human spermatozoa. In the following chapter, I shall summarize some of the major advances we have made in this area and indicate the possible directions in which this research might progress in the future.

2 Oxidative Stress and Impaired Sperm Function

One of the major causes of defective sperm function to emerge over the past decade has been oxidative stress. Spermatozoa are particularly susceptible to this form of stress because they are richly endowed with substrates for free radical attack in the form of unsaturated fatty acids and DNA. They are also vulnerable because of a highly specialized internal structure characterized by a cytoplasmic space that is extremely limited in terms of both its volume and distribution. As a consequence, these cells are poorly endowed with the cytosolic antioxidant enzymes (catalase, superoxide dismutase (SOD) and glutathione peroxidase) that protect most cells from free radical attack. Furthermore, these cells are active generators of ROS (Tosic and Walton 1946; Aitken and Clarkson 1987). Indeed, excessive production or exposure to ROS has been statistically and causally associated with defective sperm function and DNA damage in a large number of independent studies (Aitken and Clarkson 1987; Aitken and Krausz 2001; Aitken and Baker 2006; Tremellen 2008). In principle, such exposure to ROS could originate from a variety of sources including (1) the presence of activated leukocytes as a consequence of infections in the male reproductive tract (Aitken and Baker 1995), (2) electromagnetic radiation, including heat (Paul et al. 2008) or radio frequency radiation in the mobile phone range (Aitken et al. 2005; Deepinder et al. 2007; De Iuliis et al. 2009), (3) redox cycling metabolites or xenobiotics such as catechol estrogens or quinones (Bennetts et al. 2008), and (4) ROS generated as a consequence of aberrant sperm metabolism (Aitken 2004). Since mitochondria have been shown to be a major source of ROS in human spermatozoa (Koppers et al. 2008), any factor capable of interfering with electron transport in these organelles is a potential inducer of ROS and DNA damage. In this context, a superabundance of free unsaturated fatty acids appears to be particularly significant (Aitken et al. 2006).

3 Impact of Oxidative Stress on Spermatozoa

3.1 Motility Loss

Exactly 30 years ago Thaddeus Mann's group at the University of Cambridge clearly demonstrated the clinical significance of oxidative stress in the etiology of defective sperm function (Jones et al. 1979). These authors observed a correlation between the lipid peroxide content of human spermatozoa and severe motility loss. This relationship between motility loss and oxidative stress is striking and has been repeatedly demonstrated in independent studies (Alvarez et al. 1987; Aitken and Clarkson 1987, 1988; Aitken et al. 1989a, b, 1991; Aitken and Fisher 1994; Tremellen 2008; Agarwal et al. 2008). Thus exposure of human spermatozoa to extracellularly generated ROS induces a loss of motility that is directly correlated with the level of lipid peroxidation experienced by the spermatozoa (Gomez et al. 1998). Similarly, the loss of motility observed when spermatozoa are subjected to an overnight incubation is highly correlated with the lipid peroxidation status of the spermatozoa at the end of the incubation period (Gomez et al. 1998). The ability of antioxidants such as α-tocopherol to rescue sperm motility in vivo and in vitro is yet more evidence that lipid peroxidation is a major cause of motility loss in populations of human spermatozoa (Aitken et al. 1989a; Suleiman et al. 1996; Verma and Kanwar 1999).

The mechanisms by which lipid peroxidation leads to motility loss probably involve changes in the fluidity and integrity of the plasma membrane and a subsequent failure to maintain membrane functions critical to flagellar movement. Disruption of membrane Ca^{2+}/Mg^{2+} ATPase activity as a consequence of decreased membrane fluidity would, for example, lead to a loss of motility secondary to an increase in intracellular calcium (Hong et al. 1984). In addition, ROS-induced lipid peroxidation will disrupt all sperm functions dependent on membrane fluidity including sperm–oocyte fusion and the ability to undergo a physiological acrosome reaction (Aitken et al. 1993a, b).

3.2 DNA Damage

Oxidative stress is also a major cause of DNA damage in mammalian spermatozoa. Using a quantitative PCR approach to calculate lesion frequency, the human mitochondrial genome has been shown to be much more susceptible to DNA damage than the nuclear genome (Sawyer et al. 2003). As a consequence, the integrity of the sperm mitochondrial genome is an excellent marker of oxidative stress, even though this DNA is of no biological significance in its own right because sperm mitochondria do not generally replicate after fertilization. When quantitative PCR was used to compare the lesion frequencies induced in spermatozoa and a variety of other cell types following exposure to H_2O_2, the nuclear

genome of the male gamete was shown to be particularly resistant to oxidative damage (Sawyer et al. 2003). This resistance is thought to mirror the unique manner in which nuclear chromatin is packaged in spermatozoa, as also reflected in the high levels of irradiation required to damage sperm DNA compared with somatic cells (McKelvey-Martin et al. 1997).

During the terminal differentiation of spermatozoa, the chromatin becomes remodeled as nuclear histones are progressively replaced with small positively charged molecules known as protamines. As a consequence of their small size and charge, protamines permit the packaging of sperm chromatin into an extremely small space. In Eutherian mammals, the protamines possess numerous cysteine residues that become oxidized during epididymal transit, establishing a series of inter- and intramolecular disulphide bonds that serve to stabilize the nuclear chromatin structure. Such stabilization renders the DNA more resistant to oxidative stress. The spermatozoa of most marsupial species cannot stabilize in this way because their protamines do not contain cysteines and, as a consequence, their nuclear DNA is significantly more susceptible to oxidative damage that Eutherian spermatozoa (Bennetts and Aitken 2005).

The efficiency of sperm protamination also appears to be a major factor in the etiology of DNA damage in human spermatozoa. Using a fluorescent probe (chromomycin; CMA3) that competes with protamines for binding sites in the minor groove of DNA we, and others, have clearly shown a very tight inverse relationship between the degree of chromatin protamination and DNA fragmentation (Sakkas et al. 1998; Fig. 1). In light of these data, we have advanced a two-step hypothesis to explain the etiology of DNA damage in human spermatozoa.

According to this hypothesis, defective spermiogenesis leads to impaired remodeling of sperm chromatin during the final stages of spermiogenesis. As a consequence of this disrupted spermiogenetic process, spermatozoa are liberated from the germinal epithelium in an imperfect state possessing poorly protaminated chromatin as well as a variety of other defects including the retention of excess residual cytoplasm (Gomez et al. 1996; Aitken et al. 1996; Fischer et al. 2003) and functional impairments that affect the motility of the spermatozoa and their ability to participate in the cascade of cellular interactions (zona recognition, zona-induced acrosome reaction, sperm–oocyte fusion, etc.) that culminate in fertilization of the oocyte (Huszar et al. 2006; Aitken et al. 2009).

The defective chromatin remodeling observed in cases of impaired spermiogenesis constitutes the first step in the etiology of sperm DNA damage by creating a state of vulnerability to attack. The origins of the poor chromatin structure seen in the spermatozoa of male infertility patients are currently unknown. Recent animal data suggest that steroid-induced suppression of FSH or LH/testosterone is correlated with poor protamination of spermatozoa (Aleem et al. 2008). However, endocrine insufficiency is rare in the infertile male population. Another contributory factor may be age since as men age, the quality of spermatogenesis declines and the spermatozoa show clear evidence of poorly protaminated chromatin associated with high levels of DNA fragmentation (Plastira et al. 2007). Impaired chromatin remodeling may also be associated with exposure to cytotoxic chemotherapeutic

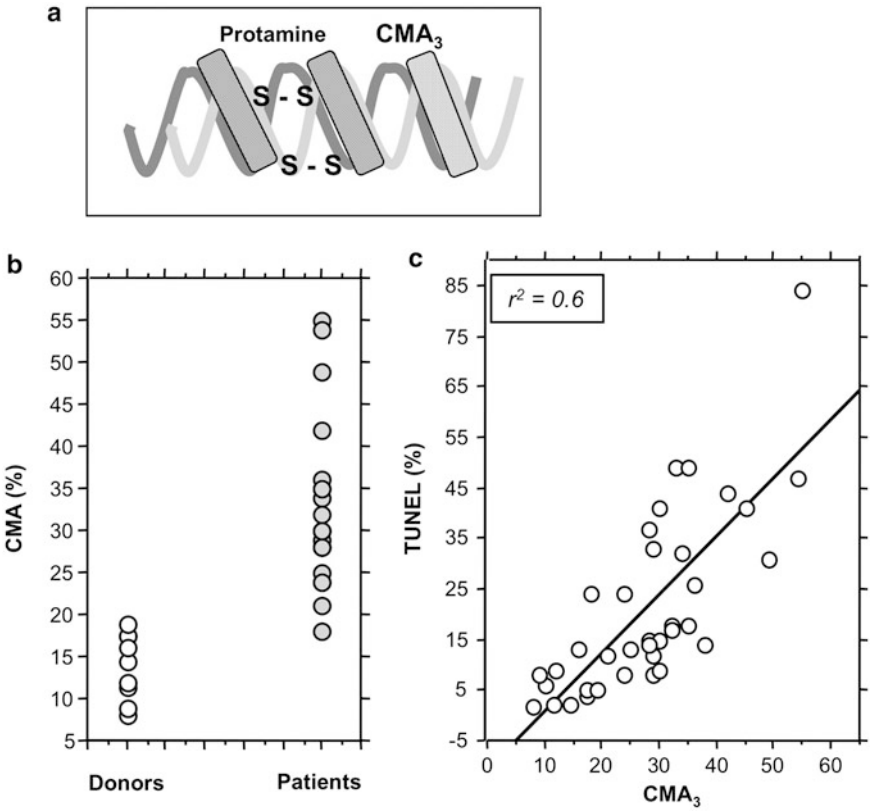

Fig. 1 Chromatin protamination and susceptibility to DNA damage in the male germ line. (**a**) CMA3 (chromomycin) is a fluorescent probe that competes with protamines for binding sites in the minor groove of sperm DNA such that the more impaired the protamination process the more labeling is observed. (**b**) Significant ($P < 0.001$) disruption of the protamination process is evident in the spermatozoa of male infertility patients, who exhibit much higher levels of CMA3 labeling than control donors. (**c**) Disruption of sperm chromatin protamination is highly correlated with the incidence of DNA damage in these cells as measured with a TUNEL assay ($P < 0.001$)

agents such as cyclophosphamide, etoposide, cisplatin, and bleomycin, which can attack testicular germ cells in the late stages of spermatogenesis and impair the carefully orchestrated protamination process (Spermon et al. 2006; Codrington et al. 2007). In a similar vein, organophosphorus pesticides have also been shown to impair chromatin remodeling during spermiogenesis generating a susceptibility to oxidative stress that leads to DNA fragmentation in the germ line (Piña-Guzmán et al. 2006). In this case, the underlying mechanism is thought to involve aberrant phosphorylation of sperm protamines during spermiogenesis. Another possibility is that the alkylation of free thiols on sperm protamines by environmental toxicants impairs the stabilization of sperm chromatin in the epididymis by impeding the creation of disulphide bridges (Sega 1991).

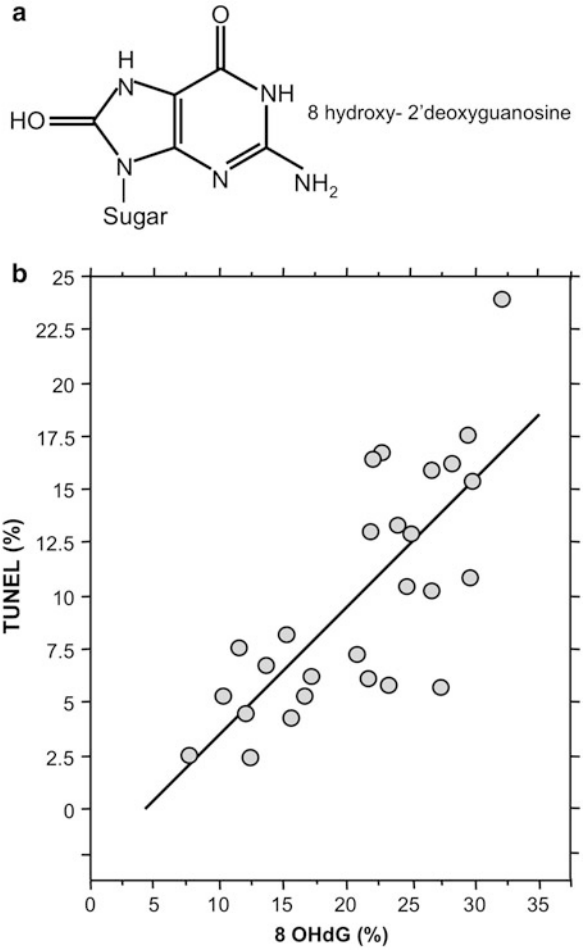

Fig. 2 Most DNA damage in the male germ line is associated with oxidative stress. (**a**) 8OH, 2′-deoxyguanosine (8OHdG) is a marker for oxidative damage to DNA. (**b**) DNA damage in human spermatozoa measured with a TUNEL assay is highly correlated with the formation of oxidative DNA base adducts ($P < 0.001$) (De Iuliis et al. 2009)

The second step in this sequence of events leading to DNA damage in spermatozoa is an attack on the poorly protaminated chromatin to induce DNA fragmentation. According to our two-step hypothesis, this attack is mediated by ROS (Aitken et al. 2009). The significance of oxidative stress in the origins of DNA damage in spermatozoa is indicated by the ability of ROS, such as hydrogen peroxide (H_2O_2), to directly trigger such lesions in human spermatozoa (Aitken et al. 1998a; Li et al. 2006) and by the extremely tight correlated observed between DNA damage in human spermatozoa and the presence of the oxidized DNA base adduct, 8-OH, 2′-deoxyguanosine (8OHdG; Fig. 2). The major DNA adducts found in human

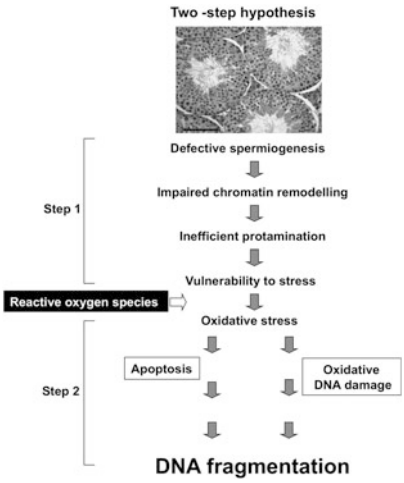

Fig. 3 A two-step hypothesis for the origin of DNA damage in the male germ line (reprinted with permission from the International Journal of Andrology; Aitken et al. 2009)

sperm DNA are 8OHdG and two ethenonucleosides (1,N6-ethenoadenosine and 1, N6-ethenoguanosine). While the former is a direct consequence of oxidative attack on sperm DNA, the latter probably arise from exposure to 4-hydroxy-2-nonenal, a major product of lipid peroxidation (Badouard et al. 2008). These findings, taken in conjunction with our own data revealing a high correlation between DNA damage and 8OHdG expression (Fig. 2), suggest that oxidative stress is the major reason that vulnerable chromatin becomes attacked by free radicals emanating from such sources as infiltrating leukocytes, redox-cycling xenobiotics, aberrant sperm metabolisms, or failed antioxidant defense systems.

Thus, in summary, while spermatozoa are vulnerable to oxidative stress and lipid peroxidation, the unique packaging of these cells during the final stages of spermatogenesis normally renders the DNA resistant to such damage, particularly in Eutherian mammals. When DNA damage does occur, it is thought to be the result of a combination of factors including poor chromatin remodeling during spermiogenesis followed by a free radical attack on the exposed DNA (Fig. 3). The DNA damage that results from these processes is detrimental to reproductive success in terms of successful fertilization, carriage of pregnancy to term and the health and normality of the offspring (Aitken 2004; Aitken and Krausz 2001; Aitken and Marshall Graves 2002; Aitken and Baker 2006).

4 The Physiological Role of ROS

If ROS are so dangerous to spermatozoa, then why have these cells evolved a capacity to generate these highly reactive molecules? The answer appears to lie in a poorly understood physiological process that is characteristic of mammalian spermatozoa – capacitation. Capacitation is a maturational process that spermatozoa

must undergo in the female tract if they are to interact successfully with the oocyte and commence the cascade of intercellular interactions leading to fertilization. As a biological phenomenon, capacitation has been acknowledged since the pioneering work of Austin (1951) and Chang (1951) more than 50 years ago. However, as a biochemical entity, the nature of capacitation has only become apparent in the past decade. The most significant finding over that period has been the discovery that sperm capacitation involves a dramatic increase in the level of tyrosine phosphorylation exhibited by the sperm tail, particularly in the midpiece (Visconti et al. 1995; Lin et al. 2006). This signal transduction pathway is driven by cAMP, mediated by the promiscuous tyrosine kinase, pp60cSrc, and modulated by the redox status of the cells (de Lamirande and Gagnon 1993, 1995; Aitken et al. 1995, 1998b; Leclerc et al. 1997, 1998; Baker et al. 2006). ROS generation is thought to exert a positive influence on tyrosine phosphorylation in spermatozoa through its ability to influence the intracellular levels of cAMP. The case for ROS involvement in cAMP generation and tyrosine phosphorylation has now been made for human (Aitken et al. 1995, 1998b), rat (Lewis and Aitken 2001), mouse (Ecroyd et al. 2003), bovine (Rivlin et al. 2004), and equine (Baumber et al. 2003) spermatozoa, via mechanisms that involve the stimulation of adenylyl cyclase activity (Zhang and Zheng 1996; Aitken et al. 1998b; Lewis and Aitken 2001; Rivlin et al. 2004). It is also possible that ROS, particularly H_2O_2 may enhance tyrosine phosphorylation through the selective suppression of tyrosine phosphatase activity. The latter enzyme contains a key cysteine residue in the catalytic domain that must be in a reduced state for phosphatase activity to be expressed. Direct exposure of tyrosine phosphatase enzymes to H_2O_2 leads to oxidation of this cysteine and a decline in enzyme activity (Hecht and Zick 1992).

The precise nature of the ROS triggering this tyrosine phosphorylation cascade is still uncertain. A pivotal role for H_2O_2 generation has been suggested by experiments demonstrating that direct exposure to this oxidant leads to the stimulation of tyrosine phosphorylation and capacitation in suspensions of human, hamster, or bovine spermatozoa (Bize et al. 1991; Aitken et al. 1998b; Rivlin et al. 2004). Similarly, the artificial creation of oxidizing conditions by exposing spermatozoa to extracellularly generated ROS using the glucose oxidase or xanthine oxidase systems has been shown to stimulate capacitation and tyrosine phosphorylation in several species (man, hamster, bull, and horse) via mechanisms that can be reversed by the addition of catalase (Bize et al. 1991; Aitken et al. 1995; Baumber et al. 2003; Rivlin et al. 2004). The biological importance of H_2O_2 has been further emphasized by the ability of catalase to inhibit the spontaneous induction of tyrosine phosphorylation in capacitating mammalian spermatozoa (Aitken et al. 1995). In addition, catalase has been shown to suppress sperm functions such as hyperactivation, the acrosome reaction, and sperm–oocyte fusion that are all ultimately dependent on the attainment of a capacitated state (Bize et al. 1991; Griveau et al. 1994; Aitken et al. 1995).

Other studies have suggested key roles for superoxide anion and/or nitric oxide in the induction of sperm capacitation. The inhibitory action of SOD constitutes the primary evidence for a role for superoxide anion in the capacitation process

(de Lamirande and Gagnon 1995; de Lamirande et al. 1997; de Lamirande and O'Flaherty 2008; O'Flaherty et al. 2006). How the presence of a membrane impermeant enzyme (SOD) scavenging a membrane impermeant free radical (superoxide) manages to disrupt the capacitation process is still uncertain. It would necessitate superoxide being generated on the external surface of the sperm plasma membrane and then entering the cell through some form of anion channel to exert its intracellular biological effects. Notwithstanding uncertainties about superoxide's mechanism-of-action, evidence for the involvement of this radical in the capacitation process has been presented for buffalo, equine, and bovine spermatozoa (O'Flaherty et al. 2003; Burnaugh et al. 2007; Roy and Atreja 2008) as well as for human sperm cells. The intracellular mechanism by which superoxide anion stimulates capacitation appears to involve similar changes to those precipitated by H_2O_2 including cAMP production and the enhancement of PKA-dependent tyrosine phosphorylation (Leclerc et al. 1997). In reality, the extremely rapid intracellular dismutation of superoxide to H_2O_2 under physiological conditions probably means that both forms of ROS are involved in the regulation of sperm capacitation in vivo.

If superoxide and H_2O_2 are involved in the regulation of sperm capacitation, how are these ROS generated? One possibility that has been raised is the presence of a plasma membrane NAD(P)H oxidase (NOX) capable of generating ROS (Lewis and Aitken 2001; Aitken et al. 1997, 2003, 2007). Although evidence for NADPH oxidases such as DUOX, NOX5, and NOX2 in the male germ line has been obtained, there is no convincing data that they are involved in regulating the capacitation process (Banfi et al. 2001; Shukla et al. 2005; Baker et al. 2007; Sabeur and Ball 2007). Although addition of NADPH to mammalian spermatozoa does stimulate the cAMP-dependent tyrosine phosphorylation events associated with sperm capacitation (Aitken et al. 1995; Lewis and Aitken 2001; Urner and Sakkas 2003), no superoxide signal has been recorded in the presence of this cofactor (de Lamirande et al. 1998). Moreover, the redox signal detected in sperm suspensions following stimulation with NADPH in the presence of the chemiluminescent probe, lucigenin (Vernet et al. 2001; Lewis and Aitken 2001), appears to reflect the secondary generation of ROS following activation of the probe by a one-electron reduction mediated by reductases, such as cytochrome b5 and cytochrome P450 reductase (Aitken et al. 2004a; Baker et al. 2004, 2005): in the absence of lucigenin, no superoxide is generated in the presence of NAD(P)H. How exogenous NADPH generates its biological effects is currently uncertain although one possibility is the spontaneous oxidation of NADPH to hydrogen peroxide in the extracellular space (Ford 2004).

Another form of oxygen free radical that is thought to be involved in the regulation of sperm capacitation is nitric oxide (NO). NO is thought to be generated by nitric oxide synthases present in the sperm head and/or flagellum (Herrero et al. 1996, 2003). However, an alternative mechanism has also been suggested involving a nonenzymatic induction of NO generation as a result of an interaction between mitochondrial H_2O_2 and arginine (Aitken et al. 2004b). Whatever the origins of this radical species, its mechanism-of-action appears to be very similar to superoxide

and hydrogen peroxide in that it stimulates the tyrosine phosphorylation events associated with sperm capacitation. It is also probable that superoxide and NO interact to form the peroxynitrite anion (ONOO) (Aitken et al. 2004b) and that this radical species plays a key role in promoting the phosphorylation events associated with capacitation (Herrero et al. 2001).

Overall, there can be no doubt that spermatozoa generate ROS. In fact, they were the first cells in which the cellular generation of reactive oxygen metabolites was recorded (Tosic and Walton 1946). It is also clear that there is not one site of redox activity in these cells but several, each with a potential to generate ROS under certain conditions (Aitken et al. 2003). Clearly, the use of chemiluminescent reagents such as luminol and, particularly, lucigenin to examine ROS generation by mammalian spermatozoa has been problematical (Aitken et al. 2004a). Chemiluminescence readouts cannot be standardized and may reflect a variety of oxidoreductase activities not directly related to ROS generation. A new generation of fluorescent reagents suitable for the cellular assessment of superoxide and H_2O_2 generation using flow cytometry is now available and should facilitate research in this field (De Iuliis et al. 2006; Kadirvel et al. 2009). One of the results of using such reagents is an awareness of just how important the sperm mitochondria are as a source of ROS (Vernet et al. 2001; Koppers et al. 2008). In many of the papers (including our own) suggesting that NOXs are important sources of ROS during capacitation, one of the primary lines of evidence has been the suppressive effects of diphenylene iodonium (DPI), a flavoprotein inhibitor which is known to suppress NADPH oxidase activity. However, it is not often appreciated that DPI is also a potent inhibitor of mitochondrial ROS generation as a result of its ability to inhibit the respiratory electron transport chain (Li and Trush 1998). Thus at this stage, it is not possible to rule out the mitochondria as a major source of the redox activity that drives the capacitation process. Indeed, human sperm mitochondria have been observed to undergo a morphological transformation during capacitation (Vorup-Jensen et al. 1999). Furthermore in rodents, attainment of the ability to capacitate during epididymal transit is associated with the phosphorylation of several mitochondrial proteins, the development of a mitochondrial membrane potential, and activation of mitochondrial ROS generation (Aitken et al. 2007).

5 Conclusions: Oxidative Stress in Infertility and Prospects for Contraception

In summary, it is clear that a fundamental aspect of sperm physiology is their ability to generate ROS. Physiologically, these metabolites appear to be heavily involved in the regulation of sperm capacitation, particularly the cAMP-induced increase in tyrosine phosphorylation that characterizes the capacitated state. Evidence has been obtained for the involvement of specific ROS such as H_2O_2, superoxide, NO, and ONOO in the regulation of this process. However, the reactivity of these

metabolites is so great that they are all potential activators of the tyrosine phosphorylation pathway associated with sperm capacitation. Indeed, in this respect spermatozoa are not so different from somatic cell types where the redox regulation of tyrosine phosphorylation is a widely recognized phenomenon (Nakashima et al. 2005). Where spermatozoa differ from most other cell types is in their susceptibility to oxidative stress. The unique architecture of these cells means that they are largely devoid of the cytoplasm that would normally accommodate the antioxidant enzymes that protect most cell types from oxidative stress. Moreover, these cells possess multiple targets for free radical attack as well as mitochondria that are prone to electron leakage and the generation of ROS. They may even possess professional ROS generating enzymes belonging the NOX family. All of these factors contribute to the important role that oxidative stress plays in etiology of defective sperm function. Free radical attacks on the unsaturated fatty acids that dominate the metabolomic profile of these cells result in the induction of lipid peroxidation accompanied by a loss of motility, while similar attacks on the nuclear and mitochondrial genomes result in oxidative base adduct formation and DNA strand breaks.

While it is clear from the foregoing that human spermatozoa are particularly vulnerable to oxidative stress, can the involvement of free radicals in the etiology of male infertility be used as the basis for creating a novel approach to male contraception? To be of significance as a viable contraceptive target a given molecule should be specific to the male germ line, functionally important and susceptible to pharmacological intervention. A majority of the systems used by spermatozoa to generate or scavenge free radicals are shared with somatic cells. However, some are specific. For example, spermatozoa possess specific forms of thioredoxin that, among other things, support the protective action of thioredoxin-dependent peroxidases (peroxiredoxins) that abound in these cells (Miranda-Vizuete et al. 2004). These molecules are functionally important in protecting cells against oxidative stress, possess a well-defined biochemical activity and are inhibitable. In principle, targeting such molecules should recreate the infertility phenotype that we see in patients characterized by defective sperm function associated with high levels of oxidative stress. Since oxidative attacks on the male germ line appear to trigger an apoptosis-like response in these cells (Ball 2008), it may only be necessary to create sufficient stress to activate this pathway and an infertility phenotype would be generated. Selective deletion of just one of the thioredoxin-dependent periredoxins (Prx4) has been shown to induce partial testicular atrophy associated with increased germ cell apoptosis (Iuchi et al. 2008). Although these animals were fertile, it should be recognized that there are several periredoxins present in human spermatozoa, creating a high level of redundancy (Baker et al. 2007). If we target the thioredoxins that support these peroxidases a more consistent infertility phenotype might be observed. Contraceptive efficiency would be absolutely critical for this approach because there would be significant risks associated with spermatozoa possessing oxidatively damaged DNA, escaping the suppression of sperm function and fertilizing the oocyte, with implications for the health and well being of the offspring.

Alternative targets for contraception may be identified from the proteomic databases or from studies targeting the proteomic differences between normal spermatozoa and cells possessing specific functional lesions such as an inability to bind to the zona pellucida. The introduction of cutting-edge technologies such as proteomics and metabolomics into the analysis of human spermatozoa will undoubtedly generate more potential targets for contraceptive intervention as our knowledge of the basic biology of these highly specialized cells improves. In this context, it will be up to public sector research institutes and Universities to identify and develop these leads to the point that the pharmaceutical industry is once again motivated to join in the search for a safe effective reversible method for controlling male fertility (Aitken et al. 2008).

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The Epididymis as a Target for Male Contraceptive Development

B.T. Hinton and T.G. Cooper

Contents

Abstract The epididymis is an excellent target for the development of a male contraceptive. This is because the process of sperm maturation occurs in this organ; spermatozoa become motile and are able to recognise and fertilise an egg once they

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have traversed the epididymal duct. However, a number of attempts to interfere in sperm maturation and epididymal function or both have not been successful. The use of transgenic animals has proved useful in identifying a few epididymal targets but has yet to open the doors for drug development. Continuous focus on identifying additional epididymal targets and sperm-specific and epididymal-specific drugs is key to bringing a male contraceptive acting on the epididymis to the public.

Keywords Blood–epididymis barrier · Epididymal gene knockout mice · Epididymal proteins · Epididymal transporters · Epididymis · Sperm maturation · Spermatozoa

1 Introduction

Contraceptives acting by a post-testicular action in the male partner will be designed to take advantage of the physiology of the epididymis. Every spermatozoon entering the ejaculate has passed through this organ that promotes its transit from the testis, fosters its maturation and maintains its quiescence before ejaculation. As these processes take place over a period of about a week, there would appear to be ample time for the fertilising potential of spermatozoa to be compromised. If this could be accomplished, the onset of infertility would be rapid (the time it takes for the spermatozoa, in whatever region they are damaged, to complete epididymal transit and enter the ejaculate) and the infertility would be reversible and almost as fast (the time it takes the unaffected testicular spermatozoa to pass through the no-longer affected organ and replenish the caudal sperm reserves), i.e. about a week in both instances.

Various approaches to such epididymal contraception have been mooted. They are based on (1) promoting peritubular epididymal contractions, which would reduce sperm transit time so that the time for their interaction with epithelial secretions is reduced to a suboptimal level; (2) attacking epididymal epithelial secretion to modify the composition of luminal fluid so that concentrations of sperm maturation-dependent factors are reduced to a suboptimal level; and (3) directly targeting the spermatozoa with inhibitors of sperm function, for example, blocking sperm motility, metabolism, membrane function, vitality; however, none has been successfully implemented (Cooper and Yeung 1999). The challenge for investigators is to uncover potential epididymal targets for contraceptive development.

2 Infertile Males as a Contraceptive Paradigm

The disappointment arising from the difficulties in realising epididymal contraceptive leads has been countered by knowledge that several infertile males demonstrate precisely what is required of the concept of post-testicular contraception, and one

that occurs naturally or can be mimicked in transgenic animals. Such models provide hope that the ultimate goal is not illusory.

Several domestic species occasionally produce individual males that are sterile but are otherwise competent in other male (including copulatory) behaviour. Testicular function is normal, sperm numbers are not diminished but the ejaculates contain morphologically abnormal spermatozoa. The phenotype in these so-called "Dag defect" males is spermatozoa in ejaculates characterised by angulated flagella; they are motile but "swim backwards", that is, the head of the spermatozoon points away from the direction of motion. The origin of the flagella bending is the epididymis, since testicular spermatozoa from these males have straight flagella, but the site in the epididymis where coiling takes place differs among animals (Cooper and Barfield 2006). Male contraceptives could mimic this natural infertility if the causative mechanism were known.

Although tail coiling can be induced in spermatozoa by hypotonic treatments, the osmolality of cauda epididymidal fluid from the Dag defect bulls and boars was not consistently low and the osmolality of fluid from more proximal regions, where the effect may have originated, was not measured. Results of limited analysis of epididymal fluid composition were also inconsistent. The epididymal phenotype was not unusual and one pig examined had the initial segment (Cooper and Yeung 2003), a caput region that may be important in this condition in mice (see below).

3 Transgenic Mice: Epididymal Models of Male Infertility

Several murine models of male infertility display a similar angulated sperm defect to that of the Dag defect of domestic species. Tail angulation is normally present in a minority of cauda epididymidal spermatozoa when exposed to routine media (Eyden and Maisin 1978) but occurs to a far larger extent in certain knockout animals.

3.1 Infertile Male Mice Lacking the Initial Segment and Exhibiting Sperm Flagellar Angulation

3.1.1 c-Ros-Deficient Mice

The best characterised model of post-testicular infertility is the c-*ros* knockout mouse. Loss of this orphan tyrosine kinase receptor leads to male infertility, although the males are still capable of copulating and can be used instead of vasectomised mice to induce pseudopregnancy in female mice (Sonnenberg-Riethmacher et al. 1996). Post-copulatory spermatozoa in the uterus display flagellar angulation that prevents sperm migration beyond the uterotubal junction (Yeung et al. 2000).

Within the epididymis <20% of spermatozoa are angulated but this increases upon release from the cauda epididymidis in routine medium (Yeung et al. 1999). Flagellar angulation is a morphological manifestation of the swollen state (Yeung et al. 2002a) so that removal of the cell membrane with detergent membrane releases the membrane restraint and reduces the percentages of angulated and hairpin bend flagellar forms. Despite the infertility in vivo, c-*ros*-null spermatozoa are capable of fertilising zona-intact eggs in vitro (Sonnenberg-Riethmacher et al. 1996) so the null spermatozoa are capable of undergoing capacitation and the acrosome reaction, confirming that the in vivo infertility stems from a failure of the spermatozoa to reach the eggs as a result of their abnormal morphology.

In man, the c-*ros* gene is widespread along the epididymis, with the exception of the proximal caput epididymidis (Légaré and Sullivan 2004), rather than the high expression in the initial segment and lower in more distal caput segments. However, human male contraception will not involve gene knockouts; rather the mechanisms of infertility induction in these models – the induction of swelling – will be mimicked. The cause of the flagellar swelling in these animal models has been examined by analysing their epididymal pheno- and geno-types. The caput epididymidis of c-*ros*-null mice is smaller than that of the WT (Sonnenberg-Riethmacher et al. 1996) because it fails to develop the initial segment (IS) (Avram and Cooper 2004) with its associated rich vascularity (Wagenfeld et al. 2002). As expected, IS-specific genes are lacking, including CRES and MEP17 (Cooper et al. 2003), but also EAAC1, a sodium-dependent glutamate transporter, is down-regulated in the caput, but not corpus or cauda epididymidis (Wagenfeld et al. 2002). As a consequence, the glutamate content of cauda epididymidal spermatozoa is decreased (Yeung et al. 2004a). The significance of this lowered sperm osmolyte content becomes apparent when the epididymal spermatozoa contact fluids of lower osmolality at the time of ejaculation, and when the osmolytes are needed to remove water that enters osmotically.

At this moment c-*ros* appears to be a promising epididymal target for male contraceptive development. Its receptor and kinase domains are amenable to small molecular weight inhibitors and recently inhibitors of the c-*ros* kinase have been prepared (El-Deeb et al. 2009; Park et al. 2009). The challenge will be to examine the role of this gene in the adult male and determine whether regulating its expression will result in male infertility. If c-*ros* is not a druggable target, then potential downstream genes known to be regulated by c-*ros* are potential targets.

3.1.2 GPX5Tag2 Transgenic Mice

Deliberate targeting of the caput epididymidis with the large T-antigen, to interfere with its function, created two transgenic (TG) lines, one of which, the GPX5Tag2,

was infertile and displayed similar sperm flagellar angulation to that of the c-*ros*-null mouse. Unlike that in the c-*ros*-null-mutant, sperm angulation occurs, as in the Dag defect males, within the epididymis (Sipilä et al. 2002; Yeung et al. 2002b). Cauda epididymidal fluid osmolality is significantly lower in the TG male than that in the wild type, although still higher than that of the female tract (Sipilä et al. 2002). The initial segment is present, despite an apparent hypertrophy, and CRES and MEP17 are down-regulated (Sipilä et al. 2002), as found for the c-*ros*-KO males (Cooper et al. 2003). Unlike the angulated spermatozoa from the c-*ros*-KO males, those from GPX5Tag2 males are unaffected by demembranation and are unable to fertilise eggs in vitro. These results are explicable by the occurrence of hypo-osmotically driven flagellar bending within the epididymis, followed by the normal sulphydryl oxidation that occurs during epididymal transit and stiffens the flagellum into an angulated shape that cannot straighten out when membrane restraints are removed.

This animal model provides clues that changing the epididymal luminal fluid osmotic microenvironment will result in male infertility. The challenge will be to uncover molecules in the epididymis responsible for maintaining osmolarity and discovering approaches that interfere in the function of such molecules. These will include enzymes involved in the synthesis of *myo*-inositol and sorbitol and transporters for ions, glutamate, and L-carnitine.

3.2 Infertile Mice Lacking the Epididymal Initial Segment

Many other models of murine male infertility exist, several of them presenting with angulated spermatozoa or lack of an initial segment but impaired volume regulation may not always be the cause of infertility. (1) The "*viable motheaten*" is an infertile male mouse with a natural mutation of the SH2 domain of the SHP-1 protein tyrosine phosphatase enzyme. This gene co-localises with, and dephosphorylates, c-*ros*; furthermore, the initial segment, as in the c-*ros*-KO, is lacking. The infertility, however, could also stem from testicular defects of spermatogenesis and testosterone secretion (Keilhack et al. 2001). (2) The epididymis of XX*Sry*, sex-reversed, pseudohermaphrodite males has no initial segment (LeBarr and Blecher 1986) and no rich capital vascularity (Le Barr and Blecher 1987) but is infertile because of azoospermia stemming from its abnormal chromosomal complement. The epididymal tubule is shorter in these males as the initial segment never develops (LeBarr et al. 1991), despite normal androgen levels (Le Barr et al. 1986). (3) The G protein-coupled receptor LGR4/GPR48 is expressed in the murine initial segment and the LGR4 knockout mouse is infertile and lacks an initial segment (Mendive et al. 2006; Hoshii et al. 2007). This is a consequence of early developmental changes leading to a hypoplastic organ together with down

regulation of the oestrogen receptor-α, aquaporin-1 and the sodium hydrogen exchanger NHE3 (*Slc9a3*). As a result, there is retention of spermatozoa and fluid within the testis, distension of the rete testis, leading to spermatogenic disruption, and sperm statis in the efferent ducts lumen with an immunological response in the form of granuloma (Mendive et al. 2006). Depending on the genetic background there may be angulation of spermatozoa in the cauda epididymidis (Hoshii et al. 2007).

It seems unlikely that these models will provide specific epididymal targets for contraceptive development. The SHP-1 protein is potentially attractive because it is a druggable target; however, the expression of this protein is ubiquitous and specificity is an issue. However, all models emphasise the importance of the proximal region of the epididymis in male fertility.

3.3 Infertile Mice with Angulated Spermatozoa

3.3.1 Foxi1-Deficient Mice

The forkhead transcription factor *foxi1* regulates gene expression in narrow and clear cells of the epididymis, especially the vacuolar H^+-ATPase proton pump, carbonic anhydrase II and the chloride/bicarbonate transporter. As these proteins modulate the acidity of epididymal, luminal fluid pH is significantly higher in the KO than WT animals (Blomqvist et al. 2006), a feature also found in the c-*ros*-KO males (Yeung et al. 2004b). Sperm tail angulation is also a feature of these animals although the fact that spermatozoa fail to enter the uterus in large numbers suggests there are additional copulatory semen deposition problems. A change in epididymal morphology was also noted with a heavier cauda epididymidis present than that in wild type controls.

Although transcription factors are not normally considered to be druggable, their downstream targets could be possible targets for contraceptive development. Since *foxi1* regulates the expression of three druggable targets, i.e. two enzymes and one transporter, a male contraceptive could be designed to interfere in the function of either one or all three. It is not entirely clear whether all three or a combination of these targets would need to be compromised for male infertility. However, as lowered intraluminal pH is not invariably associated with male infertility (the ammonia transporter Rhcg KO mice have reduced intraluminal pH but are fertile: Biver et al. 2008), the other luminal fluid components could be targeted by contraceptives.

3.3.2 FKBP52-Deficient Mice

FKBP52 is a member of the family of immunophilins and also acts as a chaperone for steroid hormone receptors through Hsp90. Although FKBP52-null mice show partial androgen insensitivity in several reproductive tissues, e.g. external genitalia, the expression of androgen-dependent genes in the epididymis is normal (Cheung-Flynn et al. 2005; Hong et al. 2007). The infertile male phenotype observed in these mice is partially due to the disrupted external genitalia and anterior prostate, leading to poor mating and lack of vaginal plugs, but an epididymal defect has also been suggested (Hong et al. 2007). In the KO male, sperm numbers are decreased and sperm morphology is characterised by flagellar angulation in the cauda (but not caput or corpus), which would render males infertile were mating to be normal. FKBP52 has been shown to bind to spermatozoa, and spermatozoa from the null mice have abnormal morphology and a reduced fertilising ability. Nevertheless, in vitro capacitation and the acrosome reaction occur and fertilisation by these spermatozoa leads to normal embryo development (Hong et al. 2007).

Determining whether this protein is druggable warrants further study, for example, a drug could be designed to enter the epididymal lumen and prevent the interaction between FKBP52 and spermatozoa resulting in male infertility.

3.3.3 Herc4-Deficient Mice

E3 ubiquitin ligase (*Herc4*) is highly expressed in the testis and is involved in the flagging and removal of proteins during the spermatogenic sculpturing of spermatozoa. The knockout mice are subfertile (litter sizes reduced by half) and spermatozoa are less motile and display angulated spermatozoa (Rodriguez and Stewart 2007).

This is not a contraceptive model as infertility is not achieved.

3.3.4 SLO3-Deficient Mice

SLO3 (KSper) has been identified as a pH-dependent potassium channel involved in membrane hyperpolarization during capacitation. The KO males are infertile and their capacitated spermatozoa do not fertilise zona-intact or zona-free eggs *in vitro*. Up to 70% of capacitated spermatozoa exhibit flagellar angulation and display reduced progressive motility and a failure to undergo the acrosome reaction (Santi et al. 2010).

This is a promising lead as a contraceptive because it is a sperm-specific channel involved in a sperm-specific function.

3.4 *Infertile Male Mice with Flagellar Angulation Combined with Testicular Defects*

Other murine models that display angulated sperm defects may not purely reflect epididymal dysfunction; they may also be associated with testicular sperm defects. For example, mice deficient in (1) *ApoER2*, a member of the low density lipoprotein receptor family, which binds epididymal secretions of clusterin (Andersen et al. 2003) and *SePP1* (Olson et al. 2007), and is localised in the initial segment (Andersen et al. 2003). Spermatozoa from the Apolipoprotein E receptor 2 knockout mouse (ApoER-KO) display flagellar angulation that develops within the epididymis, but, unlike those of the GPX5Tag2 males, a large percentage of the spermatozoa can be straightened by detergent. Mitochondrial defects are also observed and PHGPX is reduced in the spermatozoa (Andersen et al. 2003). (2) Secreted hepatic selenoprotein P (*SePP1*) is central to selenium transport and SePP1-KO mice suffer neurological disorders. The null males are infertile with sperm flagellar abnormalities such as hairpin bends, which develop during epididymal transit, but extrusion of axonemes and outer dense fibres and a truncated mitochondrial sheath lacking several mitochondrial gyres is suggestive of testicular damage. Testicular Se is reduced in the *SePP1*-KO males (Renko et al. 2008) and supplementary Se does not reverse the sperm phenotype or infertility of the null males, as the carrier protein is absent (Olson et al. 2005). (3) Acid sphingomyelinase (*ASM*) catabolises sphingomyelin (SPM) to ceramide and phosphorylcholine. Human mutations in this gene (*SMPD1*) lead to lipid storage diseases (e.g. Niemann-Pick disease, NPD) in which SPM and associated lipids (e.g. cholesterol) accumulate in tissues. The *ASM*-KO mouse presents a pathological condition between NPD Types A and B and males suffer reproductive impairment (Butler et al. 2002). Flagellar angulation can be prevented by detergent treatment (indicative of osmotic swelling) and also by treatment with the lacking ASM (Butler et al. 2007), suggesting that membrane changes could also cause angulation.

The flagellar and axonemal defects described in these models point to inadequate spermatogenesis and spermiogenesis, rather than solely the inadequate epididymal function required for post-testicular contraception.

3.5 *Infertile Male Mice Displaying Other Forms of Sperm Tail Angulation*

Several transgenic mouse models are characterised by males producing coiled sperm tails, but they are not the same sort of angulation as that mentioned above. (1) Spermatozoa from *Retinoid X receptor β-deficient* mice display tail angulation, but their infertility stems from oligoasthenozoospermia, as the testis is the main organ affected by lack of this receptor (Kastner et al. 1996). (2) *Spem1-deficient* mice display sperm tail bending that occurs at the sperm neck and reflects more a spermatogenetic failure related to failed cytoplasmic extrusion than an epididymal effect on volume regulation (Zheng et al. 2007). (3) Infertile *Gopc-(Golgi-associated PDZ- and coiled-coil motif-containing protein)-deficient* mice display flagellar coiling within the epididymis, the extent of which is related to migration of the cytoplasmic droplet (Suzuki-Toyota et al. 2004, 2007).

Mimicking the infertility of these males with testicular malfunction would not provide post-testicular contraception.

4 Targeting Other Epididymal Proteins

The importance of the initial segment for fertility may be shown by targeting other initial segment-specific secreted proteins. The genes of some proteins are apparently restricted to the initial segment, whereas others are also expressed in wild type animals in adjacent epithelial structures. In situ hybridization and immunohistochemical techniques have shown expression of genes and proteins limited to the IS, but without complete and serial sectioning of the caput epididymidis, the disposition of the medial and lateral aspects of the caput epididymidis (Blecher and Kirkeby 1978) makes boundaries determined in single sections incomplete. Molecular studies require dissection of tissue that cannot be done accurately when rapid freezing of the tissue is required. Nevertheless, there is some consensus of the regional expression of some proteins. The effect of the knockout of genes expressed in different epithelial structures may affect these epithelia directly but also have down- or up-stream effects on untargeted regions.

4.1 *Infertility in Mice Involving Blockage of the Efferent Ducts*

4.1.1 HE6-Deficient Mice

HE6, derived initially from the human epididymal caput (that largely contains efferent ducts: Yeung et al. 1991), encodes a G protein-coupled protein (Gpr64)

that is specific for the efferent ducts and the initial segment in rodents (Obermann et al. 2003). HE6-KO mice display reduced epididymal weight and sperm numbers, spermatozoa lacking heads and angulated flagella and reduced motility (Davies et al. 2004). This is a consequence of eventual blockage of the efferent ducts leading to dilation of the rete testis and spermatogenic arrest (Davies et al. 2004; Gottwald et al. 2006) and sperm stasis within the epididymis. Interestingly (and worthy of further investigation), and unlike the situation in the LGR4-KO males (Mendive et al. 2006), there is no immunological response. Gottwald et al. (2006) showed that water resorption in the efferent ducts was decreased in HE6-KO mice and spermatozoa accumulated within them so that a sperm-free epididymis resulted; results explained by the inability of the epididymis to cope with increased distal transport or further absorption of larger fluid volumes.

Unlike the LGR4-KO males, the initial segment is still present in these animals (Davies et al. 2004, 2007) and β-galactosidase is still expressed (Davies et al. 2004): gene expression is either decreased (cystatins 8 and 12, lipocalins 8 and 9, a novel β-defensin Defb42 and membrane protein HE9 [mE9], ADAM28, EAAC1) or increased (clusterin/ApoJ and osteopontin/Spp1) (Kirchhoff et al. 2006; Davies et al. 2007).

G protein-coupled proteins are excellent druggable targets, and that early spermatogenic stages persist in the testes of aged males and that there is a lack of immune response to the accumulated spermatozoa, raise the hopes of reversibility. Nevertheless, the longer the period of contraception, the more difficult it will be to clear the tract of the spermatozoa accumulated within the efferent ducts before resumption of fertility.

4.1.2 Pax8-Deficient Mice

Pax8 is expressed in the efferent ducts and the initial segment. Thyroid-deficient Pax8-null mice can survive if given thyroxine, but the males are sterile. The null males are characterised by inconsistent development of parts of the epididymis and efferent ducts, whose presumed occlusion leads to dilatation of the rete testis and eventual spermatogenic shut-down (Wistuba et al. 2007).

The infertility here is related to azoospermia and not a post-testicular action.

4.2 Infertility After Targeting Epididymal Proteins

4.2.1 Immunological Depletion of P34H

Human epididymal protein P34H is secreted in the corpus epididymidis and binds to the spermatozoon over the acrosome and is involved in zona-binding (Boue et al.

1996); sperm levels are related to IVF success (Sullivan et al. 2006) and its loss can lead to human infertility (Boué and Sullivan 1996; Moskovtsev et al. 2007). It is the equivalent of P26h in the hamster in which immunological suppression leads to complete male infertility (Berubé and Sullivan 1994). P34H and related P31h are members of the carbonyl reductase family but whether they have this function in the epididymis remains to be examined.

This enzyme is a druggable target and if its activity were specific to the epididymis, it would be an ideal target for male contraceptive development.

4.2.2 Immunological Depletion of Eppin

Eppin, an epididymal protease inhibitor (Wang et al. 2007), plays a role in post-ejaculatory seminal plug dissolution and release of physically arrested motile spermatozoa. Immunological suppression in monkeys leads to incomplete and irreversible infertility (O'Rand et al. 2006).

The risks of immunological contraception lie in the difficulty in ensuring adequate access of antibodies to spermatozoa within the epididymis (Nieschlag and Henke 2005). However, since Eppin is an enzyme inhibitor it is a druggable target.

4.3 Persistent Fertility After Targeting Epididymal Proteins

For the proteins below, of initial segment origin or not, infertility has not been achieved in knockout models.

4.3.1 SED1-Deficient Mice

SED1 (MFG-E8, lactadherin) is a protein that was identified as being involved in sperm–egg binding. It is secreted by the initial segment and then binds to the surface of the acrosomal region of sperm by intercalation of the discoidin/C domains. SED1 binds to the zona pellucida, but not to the egg plasma membrane (Ensslin and Shur 2003). Male SED1-null mice display a wide range of fertilities, from normal fertility to infertile with controls producing an average of 9 pups per litter compared with nulls that produce an average of 3 pups per litter (Ensslin and Shur 2003; see Shur et al. 2006 for review). Although sperm numbers, motility, morphology and rates of spontaneous and ionophore-induced acrosome reactions are normal, the spermatozoa display low sperm–zona binding.

SED1 could be targeted either in the epididymis or at the site of fertilisation for both male and female contraception.

4.3.2 SPAM1-Deficient Mice

SPAM1 (sperm adhesion protein 1, PH-20), a hyaluronidase, is present in the efferent ducts, initial segment, proximal epididymis, vas deferens and accessory organs (Zhang et al. 2004). It is secreted by the epididymis and is taken up on the sperm head, midpiece and tail during their transit in the epididymis (Martin-DeLeon 2006). SPAM1-KO male mice are fertile (Baba et al. 2002) possibly because of upregulation of other hyaluronidases (Hyalp1: Miller et al. 2007). The null-spermatozoa lack the protein but can take it up upon incubation in epididymal fluid. Upon uptake, these spermatozoa can be capacitated and penetrate oocyte-cumulus complexes as well as wild-type spermatozoa. Even wild type spermatozoa can take up SPAM1 from epididymal fluid, suggesting an undersaturation (Chen et al. 2006) that may have a physiological consequence, since SPAM1 is also secreted by the uterus, binds to spermatozoa and enhances cumulus dispersal (Griffiths et al. 2008a). The uptake of both the epididymal and uterine forms of SPAM1 is mediated by epididymosomes and uterosomes (Griffiths et al. 2008b).

As an enzyme, SPAM1 is druggable, but has yet to be shown to be targetable.

4.3.3 CRISP1-Deficient Mice

CRISP1 (the former protein DE, *c*ysteine-*ri*ch *s*ecretory *p*rotein) is secreted beyond the initial segment binds to spermatozoa and is involved in sperm–egg fusion (Ellerman et al. 2006; Roberts et al. 2006). However, CRISP1 knockout male mice are fertile because the number of spermatozoa, the motility of fresh and capacitated spermatozoa and their morphology are normal (Da Ros et al. 2008). Although the extent of tyrosine phosphorylation is below that of control spermatozoa, their ability to undergo progesterone-induced acrosome reactions is unchanged from that of WT controls. In vitro fertilisation reveals a lowered propensity to fertilise both zona-intact and zona-free eggs; furthermore, the fusion ability of Crisp1-KO spermatozoa is reduced by addition of Crisp1 and Crisp2 during gamete co-incubation. This raises the possibility that Crisp2 may be upregulated in the Crisp1-KO mouse, explaining the fertility of these animals.

The druggable signature of this protein and its interaction with spermatozoa remains to be determined, but the fertility of the KO males is discouraging.

4.4 Infertility in Mice Involving Blockage of the Distal Duct

4.4.1 Juvenile Steatosis

Defects in the carnitine transporter OCTN2 (*slc22a5*) lead to primary carnitine deficiency in mice. At 8–9 weeks of age the epididymis becomes deformed with a greater weight than that of the WT as the proximal duct becomes dilated with accumulated spermatozoa. As these are extravasated into the stroma, immune responses follow, leaving the distal duct void of spermatozoa and the males infertile because of azoospermia (Toshimori et al. 1999). In the mutant males, the carnitine transporter is found on the apical side of the epididymal epithelium distal to the site of sperm accumulation (Yakushiji et al. 2006).

Interference in the function of L-carnitine in the epididymis has been a challenge because it has been difficult to deplete completely the normal very high intraluminal concentrations in the epididymis (Hinton et al. 1979). Chemical depletion does not lead to male infertility in rats (Cooper et al. 1997) or hamsters (Lewin et al. 1997). Further, L-carnitine plays a major role in lipid metabolism in many other tissues and specificity maybe an issue. However, several L-carnitine transporters have been identified (Tamai et al. 2000; Eraly et al. 2004; Koepsell et al. 2007) and although some have overlapping tissue expression, some may be unique to the male reproductive tract.

Organic transporters such as *slc22a5* are druggable targets and are also excellent vehicles for transporting potential contraceptives into the epididymis (see below).

4.4.2 RARα-Deficient Mice

The males of retinoic acid receptor-α-KO mice are either infertile or have reduced fertility, as a consequence of the epithelia lining the ducts of the epididymis and vas deferens exhibiting squamous metaplasia. Although spermatozoa develop normally in the testis, they degenerate in the epididymis and vas deferens because inspissated ductal fluid blocks the normal passage of the spermatozoa (Costa et al. 1997).

These models of highly disturbed epithelial function are not useful as contraceptive paradigms, since inspissation of spermatozoa and immunological sequelae make any contraception irreversible.

5 The Blood–Epididymis Barrier as a Hurdle and an Opening to the Administration of Putative Male Contraceptives

5.1 A Physical Barrier

The blood–epididymis barrier comprises more than just tight junctions. Cell–cell contacts such as tight junctions found in many epithelia are effective in preventing the passage of molecules from entering into a lumen or other specialised compartments. Tight junctions between epididymal epithelial cells are no exception and Friend and Gilula (1972) wrote; "Among the various epithelial cell contacts examined, the zonula occludens of the epididymis is the most highly developed". Later studies by Suzuki and Nagano (1978) and more recently by Cyr and colleagues (Gregory and Cyr 2006; Dubé et al. 2007; Cyr et al. 2007) have shown the extensive and complex nature of these junctions. As one might expect, the classic tracer lanthanum does not pass between the tight junctions if the tracer is injected into animals (Hoffer and Hinton 1984). Therefore, the tight junctions form a formidable hurdle for putative male contraceptives entering the epididymal lumen to affect the maturing spermatozoa.

The permeability of the tight junctions (paracellular pathway) has been extensively studied using micropuncture studies (Hinton and Howards 1981, 1982; Turner et al. 1981; Yamamoto and Turner 1990) and low molecular weight molecules such as water and urea pass into the lumen readily from blood. Any molecule larger than 160 kDa fails to enter, or only a very small amount enters, the lumen. At first glance, these findings would suggest that it would be challenging to identify a putative low molecular weight male contraceptive compound that would readily enter the epididymal lumen at high enough concentrations to affect the maturing spermatozoa. The answer to this dilemma is that the blood–epididymis barrier comprises more than just tight junctions.

5.2 A Physiological Barrier

The blood–epididymis barrier can also be considered to be a physiological barrier and clues to this originated from some of the micropuncture studies described above. If a non-metabolizable form of glucose, L-glucose, is injected into blood, it does not readily enter the lumen of the epididymis. If a non-metabolizable form of glucose, 3-*O*-methyl-D-glucose, the D-isomer, is injected, it is readily transported into the epididymal lumen (Hinton and Howards 1981). This would suggest that the blood–epididymis barrier is not an absolute, but a restrictive barrier, in that it only allows certain molecules to enter into its cells and lumen. The restrictive nature of the blood–epididymis barrier is a reflection of the permeability properties of the basolateral and apical membranes, which in turn reflect the transporting properties of various transporters and channels within them.

Therefore, transporters can either be targets themselves for contraceptive development or their transporting or permeability properties can be used to move contraceptive agents into the epididymis. An example of the latter, albeit for the testis, is when doxorubicin (adriamycin), an entineoplastic drug used for the treatment of many cancers, is administered to males an infertility phenotype is observed. This was shown to be due to germ cell decline in phospholipids and subsequent germ cell loss from the epithelium (Meistrich et al. 1985; Zanetti et al. 2007). Later studies showed that the transporter *slc22a16* (CT1; Enomoto et al. 2002), an organic cation transporter that transports L-carnitine, was identified as the primary candidate regulating the influx of doxorubicin (Okabe et al. 2005). The transporter *slc22a16* is highly expressed in the testis and to a lesser degree in the human epididymis (Enomoto et al. 2002). Therefore, the transporting properties of epididymal organic solute transporters could be exploited in a similar manner.

5.3 *Epithelial Transporters as Targets or Vehicles for Male Contraceptive Development*

Several transporters have been identified in the epididymis from gene microarray results (Jervis and Robaire 2001; Cornwall and Hann 1995; Cooper et al. 2004; Johnston et al. 2005; Jelinsky et al. 2007) but very few have been studied to any significant degree. The most studied series of transporters in the epididymis are the ion and water transporters (see reviews by Leung et al. 2004; Pastor-Soler et al. 2005) and a clue to their importance in male fertility came from the *Foxi1*-null mutation described earlier. Transporters are excellent targets for male contraceptive development because they are amenable to small molecular weight inhibitors and some, for example, those located on the basolateral membrane, e.g. OCTN2 (Rodríguez et al. 2002), are easily accessible to inhibitors present in the blood. Several inhibitors have already been designed that interfere with organic solute transporter activity and such inhibitors have proven useful in the clinic (Sweet et al. 2001; Ohtsuki 2004; Sai and Tsuji 2004; El Elwi et al. 2006; Koepsell et al. 2007).

6 Conclusion

Despite the many transgenic models reviewed above that are associated with male-selective fertility impairment, not all can serve as paradigms for post-testicular contraceptive development. Some do not bring consistent infertility whereas others are associated with spermatogenic damage. The combination of epididymal epithelial defects and sperm angulation inherent in c-*ros*-KO males is not echoed in all the transgenic models: flagellar angulation is more associated with infertility than a morphological expression of epididymal abnormality, although anatomically

invisible physiological deficiencies in the epididymal epithelium may well underlie the susceptibility of the sperm tail towards angulation.

Current knowledge on the role of epididymal osmolytes in sperm volume regulation suggests targets that could induce angulated spermatozoa: channels or transporters involved in the transport, uptake and efflux of osmolytes by the epididymis and spermatozoa. They all need to be characterised and their modes of regulation determined; in some areas, a start has been made, in others research needs to be initiated. To cover the possibility that epididymal- and sperm-specific drugs are not found, research on targeting of drugs to the epididymis needs to be started. In this regard, some epithelial channels involved in osmolyte provision could be hijacked for surreptitious entry of inhibitors into the epididymal lumen.

Research into factors affecting the initial segment, regulators of epithelial channels and transporters and inhibitors of sperm osmolyte influx and efflux should proceed together so as to be able to target inhibitors of sperm function to the epididymal lumen.

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Sperm–Zona Pellucida Interaction: Molecular Mechanisms and the Potential for Contraceptive Intervention

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Contents

Abstract At the moment of insemination, millions of mammalian sperm cells are released into the female reproductive tract with the single goal of finding the oocyte. The spermatozoa subsequently ignore the thousands of cells they make contact with during their journey to the site of fertilization, until they reach the surface of the oocyte. At this point, they bind tenaciously to the acellular coat, known as the zona pellucida, which surrounds the oocyte and orchestrate a cascade of cellular interactions that culminate in fertilization. These exquisitely cell- and species- specific recognition events are among the most strategically important cellular interactions in biology. Understanding the cellular and molecular mechanisms that underpin

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them has implications for the etiology of human infertility and the development of novel targets for fertility regulation. Herein we describe our current understanding of the molecular basis of successful sperm–zona pellucida binding.

Keywords Capacitation · Fertilization · Spermatozoa

1 Introduction

The continuation of all mammalian species relies on the cellular interactions that occur between gametes during the process of fertilization. These remarkable cell- and species-specific interactions are initiated by recognition and binding of a spermatozoon to the zona pellucida (ZP), a thick extracellular matrix that surrounds and protects the ovulated oocyte. Understanding the basic biology of this interaction has profound implications for the diagnosis of human infertility and the development of novel targets for fertility regulation. Accordingly, considerable research effort has been devoted to investigating the molecular mechanisms that underpin sperm–oocyte interaction. Nonetheless, this fundamental interaction remains poorly understood and is the subject of considerable controversy.

Studies of sperm–zona pellucida adhesion conducted during the last 60 years have led to the advancement of a widely accepted paradigm that sperm–ZP interaction is mediated by a single sperm receptor that engages with a complementary ligand within the ZP. While such a model holds obvious appeal, it fails to account for the fact that targeting of individual sperm proteins through inhibition studies (e.g., competitive substrates and mono-specific antibodies) and/or genetic deletion has failed to elicit the anticipated block to sperm–ZP interaction. Against this background we have introduced a novel hypothesis which states that sperm–ZP interaction requires the coordinated action of several sperm proteins, each of which contributes to the high affinity and specificity of this fundamental cellular interaction. Furthermore, we have also suggested that these discrete zona recognition proteins are assembled into a multimeric receptor complex during sperm capacitation. Throughout this review, we have chosen to focus on the mouse model as it represents the most widely studied of all laboratory animals with respect to mammalian fertilization. However, the authors encourage caution in extrapolating these data for cross-species comparison.

2 Sperm–Zona Pellucida Interaction

2.1 The Zona Pellucida

The zona pellucida (ZP) is synthesized during oogenesis and is located between the oocyte and the innermost layer of granulosa cells (Wassarman and Albertini 1994). The mature ZP is a porous matrix whose functions include the mediation of

species-specificity in gamete interaction (Vieira and Miller 2006), prevention of polyspermy, and protection of the developing embryo prior to implantation (McLeskey et al. 1998). The importance of these functions has been underscored by studies involving the targeted deletion of the genes encoding ZP proteins (Rankin and Dean 2000). Such studies have shown that the ZP is essential in maintaining the physiological status of the oocyte and in regulating successful growth and development of the embryo. Furthermore, it has been demonstrated that oocytes failing to correctly translate and assemble a ZP are unable to be fertilized (Liu et al. 1996; Rankin et al. 1996). Indeed, this structure represents the first barrier that mammalian sperm must encounter and breach to achieve fertilization.

In the mouse, the ZP is assembled as a trimeric protein matrix composed of long ZP2 and ZP3 heterodimer filaments that are cross-linked by homodimers of the third zona protein, ZP1 (Bleil and Wassarman 1980b; Greve and Wassarman 1985; Wassarman and Mortillo 1991) in a molar ratio of 4:4:1, respectively (Green 1997) (Fig. 1). The three mouse ZP proteins were initially characterized by Bleil and Wassarman (1980a, b, c) and have subsequently been shown to be encoded by single-copy genes located on chromosomes 19, 7, and 5 (Epifano et al. 1995). Determination of the primary structure of the ZP proteins revealed considerable divergence between their predicted molecular weight and that determined experimentally via SDS-PAGE (ZP1, ~200 kDa; ZP2, ~120 kDa; and ZP3, ~83 kDa). Such differences are accounted for by dramatic posttranslational modification of the mature proteins, primarily in the form of glycosylation (Ringuette et al. 1986; Kinloch et al. 1988; Liang et al. 1990; Epifano et al. 1995), a feature which appears critical for their biological activity. The oligosaccharides are in turn modified by sulfation, sialylation, and the addition/removal of other moieties (Liu et al. 1997).

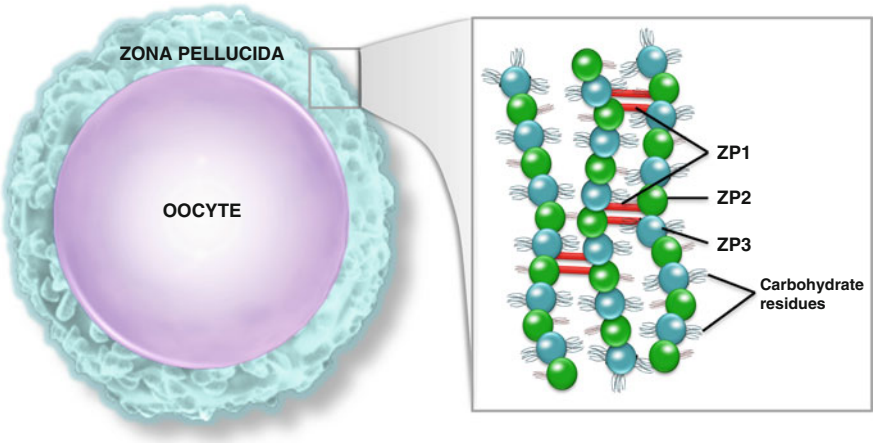

Fig. 1 *The mouse zona pellucida*. The ovulated oocyte is surrounded by the ZP, an acellular matrix whose functions include the mediation of species-specificity in gamete interaction, prevention of polyspermy, and protection of the developing embryo prior to implantation. The mouse zona pellucida is a fibrillar structure, the major strands of which are composed of repeating dimers of ZP2 and ZP3 glycoproteins. These strands are crosslinked by ZP1 to form a mesh-like network

As well as being essential structural components of the zona pellucida, ZP2 and ZP3 possess specific functions during the sequence of sperm–oocyte interactions that culminate in fertilization. The balance of evidence indicates that mouse ZP3 functions as both the primary sperm receptor, preferentially binding the plasma membrane region overlying the acrosome of acrosome-intact sperm, and as an inducer of the acrosome reaction following recognition of the zona matrix (Bleil and Wassarman 1986; Vazquez et al. 1989). For instance, it has been demonstrated that solubilized ZP3 is able to competitively inhibit sperm–ZP binding, whereas ZP1 and ZP2 do not elicit a similar response (Bleil and Wassarman 1980a). Similarly, female mice bearing a null mutation for ZP3 are infertile (Liu et al. 1996). It is noteworthy, however, that ZP3 is not uniquely responsible for facilitating sperm interaction in all mammalian species. In the pig for instance, sperm-binding activity resides in a heterodimer formed between the ZP1 and the ZP3 orthologues (Yurewicz et al. 1998). Similarly, the ZP1 orthologue, ZPB, has been shown to play a major role in sperm binding to the bovine zona (Yonezawa et al. 2001), while in humans and the bonnet monkey there is also compelling evidence that a fourth zona glycoprotein, ZP4, which is thought to be dysfunctional in the mouse (Lefievre et al. 2004), participates in primary sperm adhesion (and the induction of acrosomal exocytosis) (Gupta et al. 2007). The biological significance of this interspecies complexity in ZP structure and function is presently unknown. Nonetheless, given that the mouse remains the most widely studied model for understanding sperm–ZP interaction, this species will serve as the focus for the following discussion.

The mouse ZP3 glycoprotein comprises a number of domains including an N-terminal signal sequence, a large ZP domain, a consensus furin cleavage site (CFCS), and a hydrophobic transmembrane region located near the C-terminus. The ZP domain is in fact common to all ZP proteins and consists of a 260 amino acid sequence with eight conserved cysteine residues. This domain is believed to be responsible for the polymerization of the ZP proteins into the extensive lattice-like network that enables it to surround the oocyte (Jovine et al. 2002). Mouse ZP3 is initially synthesized as a 424 amino acid polypeptide, but is subject to dramatic posttranslation modification resulting in the addition of complex N-(asparagine) and O-linked (serine/threonine) carbohydrates. The sperm-binding domain of the ZP3 glycoprotein has been mapped to the C-terminus of the protein and encompasses both the ZP and CFCS domains (Litscher et al. 2009). This region of the polypeptide is commonly referred to as the sperm combining site and contains O-linked sugar residues that putatively interact with complementary receptors on the surface of acrosome intact spermatozoa (Wassarman et al. 2004). However, as discussed below, this model of sperm–ZP interaction is not universally accepted.

2.1.1 The Role of O-linked ZP3 Sugars in Mouse Sperm–ZP Interaction

Prevailing evidence indicates that primary sperm–oocyte interaction is mediated by binding between ZP3 carbohydrates (Gwatkin et al. 1977; Hoodbhoy and Dean 2004) and complementary lectin-like proteins located on the surface of the sperm

(see Sect. 2.2 and Table 1). The most widely accepted model of this interaction emphasizes the importance of O-linked carbohydrate moieties (Florman and Wassarman 1985; Litscher et al. 1995; Wassarman et al. 1999). For instance, it has been demonstrated that small glycopeptides derived from mouse ZP3 retain full sperm receptor activity (Florman et al. 1984). Conversely, the enzymatic and chemical removal or modification of O-linked oligosaccharides from ZP3 abolishes its sperm receptor activity whereas the removal of N-linked oligosaccharides elicits only negligible effects (Florman and Wassarman 1985). The importance of O-linked glycans has been further advanced by the demonstration that genetically engineered chimeric mouse oocytes expressing human ZP3 acquire the same O-linked glycans as mouse ZP3 and bind mouse, rather than human, spermatozoa (Rankin et al. 1996; Hoodbhoy and Dean 2004). Furthermore, the results of targeted mutagenesis studies indicate that the key O-linked carbohydrates responsible for sperm-binding activity most likely reside within the C-terminal portion of the ZP3 polypeptide chain (Kinloch et al. 1995).

However, while it is widely accepted that the principal bioactive component of ZP3 is associated with its O-linked carbohydrate moieties, the relative importance of the different oligosaccharide ligand(s) remains to be unequivocally established (Easton et al. 2000; Diekman 2003). This situation is due in part to the complexity of O-linked glycans, with recent mass spectrometry analysis of mouse ZP3 glycosylation revealing that the predominant core type 2 sequences are terminated with sialic acid, lacNAc (Galβ1–4GlcNAc), lacdiNAc (Gal–NAcβ1–4GlcNAc), Galα1–3Gal, and NeuAcα2–3[GalNAcβ1→4]Galβ1→4 (Sda antigen) (Dell et al. 2003). Early studies suggested that galactose, located in an α-linkage at the nonreducing terminus of O-linked oligosaccharides served as a critical determinant of sperm binding to ZP3 (Florman and Wassarman 1985; Bleil and Wassarman 1988; Litscher et al. 1995). However, such claims have since been refuted (Nagdas et al. 1994; Thall et al. 1995) in favor of terminal β-linked galactose (Yonezawa et al. 2005) in addition to *N*-acetylglucosamine (Miller et al. 1992), mannose (Tulsiani et al. 1989; Cornwall et al. 1991), *N*-acetylgalactosamine, and fucose residues (Johnston et al. 1998; Kerr et al. 2004), each of which have been demonstrated to inhibit sperm–zona binding (reviewed in Benoff 1997). Arguments against the involvement of α-linked galactose residues include the demonstration that sperm–ZP interaction is inhibited by pretreatment of oocytes with β-galactosidase but not α-galactosidase (Mori et al. 1997). Furthermore, female transgenic mice bearing a null mutation for α1→3 galactosyltransferase (and therefore terminal Galα1→3Gal residues) produce oocytes that display normal sperm-binding characteristics (Thall et al. 1995). These data are further supported by evidence from a novel heterologous cell-adhesion assay between mouse spermatozoa and rabbit erythrocytes. The precocious binding of these two cell types appears to be attributed to the presence of multiple branches of Galα1→3Galβ1→4GlcNAcβ1→6 linked to a linear polylactosamine backbone in the erythrocytes (Clark et al. 1996; Clark and Dell 2006; Sutton-Smith et al. 2007). However, pretreatment of the erythrocytes with α-galactosidase fails to elicit the anticipated reduction in sperm adhesion (Clark and Dell 2006), thereby suggesting sperm

Table 1 Putative sperm–ZP receptor candidates

Name (synonyms)	Species	Evidence	References
α-D-mannosidase (MAN2B2)	Mouse Rat Hamster Human	• Integral plasma membrane protein that may participate in sperm–ZP interaction by binding to mannose-containing ZP saccharides • Treatment of sperm with either D-mannose or an antibody raised against the protein elicit a dose-dependent inhibition of sperm–ZP binding	(Tulsiani et al. 1989, 1993; Cornwall et al. 1991; Pereira et al. 1998; Yoshida-Komiya et al. 1999; Akama et al. 2002)
Arylsulfatase A (AS-A; ARSA)	Mouse Human Boar	• Acquired from epididymal fluid via interaction with SGG and localizes to the membrane overlying the acrosome • A dose-dependent decrease in sperm–ZP binding is observed following pretreatment of sperm with ARSA antibodies • ARSA null males are fertile	(Hess et al. 1996; Tantibhedhyangkul et al. 2002; Weerachatyanukul et al. 2003)
β-1,4-galactosyltransferase (GalTase; GALT; B4GALT1)	Mouse Rat Human Guinea pig Rabbit Bull Boar Stallion	• Transmembrane protein located on dorsal surface of the anterior sperm head overlying the intact acrosome • Acts as a receptor for terminal GlcNAc residues on ZP3 • ZP3-induced GalTase aggregation triggers a pertussis toxin-sensitive G-protein cascade leading to induction of acrosomal exocytosis • Transgenic mice overexpressing GalTase are hypersensitive to ZP3 and undergo precocious acrosome reactions • Sperm from mice bearing targeted deletions in GalTase are unable to bind ZP3 nor undergo ZP3-dependent acrosomal exocytosis • However, GalTase-null sperm retain the ability to bind to the egg coat	(Shur and Hall 1982; Lopez and Shur 1987; Lu and Shur 1997; Shi et al. 2001)
Fertilization antigen 1 (FA1)	Mouse Human Bull	• Localized to postacrosomal region of sperm head • Immunization of mice with recombinant FA-1 antigen induces a strong immunocontraceptive response • Anti-FA-1 antibodies have been implicated in immune infertility in humans • No recorded knockout mouse	(Naz et al. 1984, 1992; Coonrod et al. 1994; Naz and Zhu 1998; Menge et al. 1999)
Glutathione S-transferase (GST)	Mouse Rat	• Two isoforms (Pi and Mu) localized to periphery of plasma membrane over anterior head, postacrosomal region and principal piece	(Shaha et al. 1988; Aravinda et al. 1995; Gopalakrishnan et al. 1998; Hemachand et al. 2002)

	Human Goat	• Selective inhibition of Pi , but not Mu, isoform with specific antisera leads to a reduction in fertilization rate • Both isoforms bind to ZP3	
Milk fat globule-EGF factor 8 (MFGE8; P47; SED1)	Mouse Boar	• An EGF repeat and discoidin domain protein that coats sperm (plasma membrane overlying the acrosome) within the epididymis • Binds specifically to the ZP of unfertilized but not fertilized eggs • Recombinant MFGE8 and anti-MFGE8 antibodies competitively inhibit sperm-egg binding • MFGE8 null males are subfertile and their sperm are unable to bind to the ZP in vitro	(Ensslin et al. 1995, 1998; Ensslin and Shur 2003)
Proacrosin (acrosin)	Mouse Boar	• Localizes to acrosome and inner acrosomal membrane from where it is thought to mediate secondary binding to ZP2 following acrosomal exocytosis • Binding to ZP is nonenzymatic and thought to involve recognition of polysulphate groups on ZP glycoproteins • Acrosin null males are fertile but show compromised ZP penetration	(Urch and Patel 1991; Baba et al. 1994; Howes et al. 2001; Howes and Jones 2002)
Sperm adhesion molecule 1 (SPAM1; PH-20)	All mammals	• Widely conserved sperm surface protein • Localized to plasma membrane over anterior head of mouse sperm • Possesses hyaluronidase activity that assists in the digestion of cumulus cells • Relocalizes to inner acrosomal membrane following acrosome reaction where it is putatively involved in secondary ZP binding • SPAM1 null males are fertile but their sperm are less efficient in dispersal of cumulus cells	(Lin et al. 1994; Hunnicutt et al. 1996; Myles and Primakoff 1997; Baba et al. 2002; Morales et al. 2004)
Sperm autoantigenic protein 17 (SPA17, SP17)	Mouse Rabbit Human Primates	• SPA17 is a conserved, highly antigenic protein variously localized to acrosome and flagellar fibrous sheath • Has been implicated in regulation of sperm maturation, capacitation, acrosome reaction and ZP binding • Shown to bind specific carbohydrate components (mannose) of the ZP	(Yamasaki et al. 1995; Grizzi et al. 2003; Chiriva-Internati et al. 2009)

(*continued*)

Table 1 (continued)

Name (synonyms)	Species	Evidence	References
Spermadhesins (AWN; AQN-1; AQN-3)	Boar Stallion Bull	• Spermadhesin represents a major component of seminal plasma and is deposited over the sperm head via interaction with plasma membrane phospholipids • Multifunctional lectins able to bind to carbohydrates, sulfated glycosaminoglycans, phospholipids and protease inhibitors • May therefore participate in several sequential steps of the fertilization process	(Sanz et al. 1993; Sinowatz et al. 1995; Topfer-Petersen et al. 1998; Petrunkina et al. 2000)
Sulfogalactosylglycerolipid (SGG)	Mouse Rat Human Boar	• Localizes to plasma membrane overlying acrosome and postacrosomal regions of mouse and human spermatozoa. Restricted to anterior head plasma membrane of boar sperm • SGG is a major sperm sulfoglycolipid that putatively facilitates the uptake of sulfolipid-immobilizing protein-1 (SLIP1) and ARSA • Following capacitation, SGG is predominantly found in membrane rafts, microdomains that possess ZP affinity • Pretreatment of sperm with monovalent anti-SGG Fab fragments markedly inhibits sperm binding to the ZP	(Kornblatt 1979; Tanphaichitr et al. 1990, 1993; White et al. 2000; Weerachatyanukul et al. 2001; Bou Khalil et al. 2006)
Zonadhesin (ZAN)	Mouse Hamster Rabbit Boar Bull Horse Primates	• ZAN localizes to the apical head overlying the acrosome following spermatogenesis and epididymal maturation • The protein displays testis-specific expression • Zan features a mosaic protein architecture with several domains (MAM, mucin, D-, and EGF) that putatively enable the protein to participate in multiple cell adhesion processes including ZP binding	(Hardy and Garbers 1994, 1995; Hickox et al. 2001; Bi et al. 2003; Olson et al. 2004; Gasper and Swanson 2006; Herlyn and Zischler 2008)

Zona pellucida binding proteins 1 & 2 (ZPBP1; Sp38; IAM38; ZPBP2)	Mouse Human Boar	• Both ZPBP1 and its paralog, ZPBP2, localize to the sperm acrosome and are expressed exclusively in the testes of mice and humans • Both proteins have been implicated in secondary ZP binding • Preincubation of boar spermatozoa with anti-ZPBP1 antibodies block in vitro fertilization • ZPBP1 null males produce sperm with abnormal head morphology and no forward motility and are consequently sterile • ZPBP2 null males produce dysmorphic sperm with reduced ability to penetrate zona pellucida	(Mori et al. 1993, 1995; Yu et al. 2006; Lin et al. 2007)
ZP3 receptor protein (ZP3R; SP56; AM67)	Mouse Rat Guinea pig	• ZP3R is an intraacrosomal protein but may be presented to surface of the apical region of the sperm head region following capacitation • Evidence exists that ZP3R may be involved in either primary and/or secondary ZP interactions • Oocytes pretreated with recombinant ZP3R show a dose dependant decrease in sperm affinity • No recorded knockout mouse	(Bleil and Wassarman 1990; Cheng et al. 1994; Foster et al. 1997; Cohen and Wassarman 2001; Kim et al. 2001a, b; Kim and Gerton 2003; Buffone et al. 2008b)

instead interact with β1→4-linked glycans. These results indicate that sperm can recognize terminal Galβ1→4GlcNAc sugars (Mori et al. 1997), which interestingly, are essentially the same structures in both ZP3 and ZP2 (Noguchi and Nakano 1993).

2.1.2 The Role of N-Linked ZP3 Sugars in Sperm–ZP Interaction

In addition to the classes of O-linked oligosaccharides described above, murine ZP3 is also known to be furnished with both high mannose and complex-type N-glycans (Easton et al. 2000). The predominant high mannose-type glycan is composed of Man5GlcNAc2, whereas the array of biantennary, triantennary, and tetraantennary complex-type N-glycans have been shown to be terminated with the following antennae: Galβ1–4GlcNAc, NeuAcα2–3Galβ1–4GlcNAc, NeuGcα2–3Galβ1– 4GlcNAc, the Sda antigen, and terminal GlcNAc (Easton et al. 2000). Interestingly, with the exception of the latter sugar, these N-glycan sequences resemble those that terminate the β1–6-linked branches of ZP3 O-glycans. Such findings raise the prospect that N-linked glycans may also contribute to sperm adhesion. Indeed, in species such as the pig, N-linked and not the O-linked carbohydrates appear to mediate sperm–ZP interaction (Yonezawa et al. 1995; Nakano et al. 1996). It may therefore be argued that carbohydrate moieties of the ZP glycoproteins may be underpinning the species-specificity associated with sperm–ZP interaction.

2.1.3 Carbohydrate-Independent Models of Sperm–ZP Interaction

Notwithstanding the compelling evidence in favor of carbohydrate residues as the main determinant in mediating sperm–ZP interaction, the production of transgenic mice bearing null mutations for key glycosyltransferases has also raised some doubt regarding the overall necessity of ZP carbohydrates for binding sperm. For instance, female mice singly deficient in any one of the three known glycosyltransferases that generate core 2 O-glycans (C2GnT1, C2GnT2, and C2GnT3), and therefore many of the O-glycans normally found in the zona are fertile (Ellies et al. 1998; Stone et al. 2009). Remarkably, elimination of all three C2GnTs is also permissive of fertility (Ellies et al. 1998). Similarly, mice that do not possess MGAT-I, the enzyme that initiates complex and hybrid-type glycan synthesis, produce oocytes that retain the ability to be fertilized (Shi et al. 2004).

Among the carbohydrate-independent models that have been proposed, recent analyses conducted by Tanphaichitr and colleagues have raised the interesting prospect that the sulfation of ZP glycans may play a key role in sperm adhesion (Tanphaichitr et al. 2007). Specifically, it has been postulated that sulfated sugar residues of ZP3 serve as a ligand for a sulfatase enzyme, arylsulfatase-A (ARSA),

that is added to the sperm surface during post-testicular maturation and becomes annexed within the apical region of the sperm head following capacitation (Tanphaichitr et al. 1993; White et al. 2000; Weerachatyanukul et al. 2001, 2003; Carmona et al. 2002; Tantibhedhyangkul et al. 2002) (see Table 1). Support for this model rests with the demonstration that the components of the zona pellucida (Prasad et al. 2000), as well as the sperm surface (Murray et al. 1980), are highly sulfated in nature. Furthermore, it has been shown that a range of synthetic sulfated substrates (including arylsulfates, sulfated monosaccharides, and ascorbate 2-sulfate) are capable of competitively inhibiting the fertilization of hamster oocytes in vitro at the level of sperm–zona binding (Ahuja and Gilburt 1985). In addition, the exposure of spermatozoa to exogenous enzymes capable of desulfating biological macromolecules (such as cerebrosides, glycosaminoglycans and glycoproteins), significantly inhibits their zona binding affinity (Ahuja and Gilburt 1985). We have recently observed comparable levels of inhibition following treatment of mouse spermatozoa and oocytes with a similar range of reagents (Nixon et al., unpublished).

At present, the nature of sulfated zona binding sites on spermatozoa remains to be fully elucidated; however, these findings take on added significance in light of the recent report that male mice bearing a targeted deletion of the gene for protein-tyrosine sulfotransferase 2 (TPST2) are infertile (Borghei et al. 2006). TPST2 is one of two closely related isoenzymes that mediate the tyrosine O-sulfation of a myriad of substrates such as adhesion molecules, G-protein-coupled receptors, coagulation factors, serpins, extracellular matrix proteins, and hormones, in both mice and humans (Beisswanger et al. 1998). TPST2 null mice have normal spermatogenesis and produce normal numbers of epididymal sperm that appear indistinct from their wild-type counterparts in terms of their morphology, motility, ability to capacitate in vitro, and undergo acrosome exocytosis in response to an agonist (Borghei et al. 2006). However, they are severely defective in terms of their ability to fertilize ZP-intact eggs. The substrates for tyrosine O-sulfation in spermatozoa await further investigation.

Interestingly, in addition to the carbohydrate and sulfate residues that adorn the mature ZP proteins, a small number of studies have also suggested that sperm–ZP interaction may be facilitated, at least in part, by the core polypeptide backbone of ZP3 (Florman et al. 1984; Chapman et al. 1998; Hinsch et al. 2005). Specifically, the polypeptide backbone has been implicated in the induction of acrosomal exocytosis (Chapman et al. 1998; Hinsch et al. 2005). This notion is consistent with the demonstration that although sperm are able to bind to the glycocalyx of rabbit erythrocytes in a manner that appears analogous to that of ZP binding, this interaction fails to elicit the signaling cascades required to induce an acrosome reaction (Clark et al. 1996; Clark and Dell 2006). Notably, phenotypic analysis of a number of transgenic mouse models have also raised the prospect that the three-dimensional structure of the zona matrix, rather than a single protein (or carbohydrate), may be central to mediation of sperm binding (Dean 2004, 2005; Hoodbhoy and Dean 2004).

2.1.4 Models of ZP3 Independent Sperm–ZP Interaction

The debate regarding the nature of the ZP3 ligand(s) responsible for initiating sperm–egg interaction has been overshadowed by recent evidence that sperm may be able to resolve gamete recognition into at least two distinct binding events. In this context, the work of Shur and colleagues has raised the interesting prospect that prior to engaging in interaction with ZP3 ligands, sperm are able to be tethered to the oocyte via adhesion to an oviduct-derived glycoprotein (oviduct-specific glycoprotein, OGP) (Rodeheffer and Shur 2004; Lyng and Shur 2009). OGP is a high molecular weight glycoprotein synthesized and secreted by oviductal cells within the fimbriae and infundibulum and apparently coats the periphery of the ZP in addition to permeating the perivitelline space of ovulated mouse oocytes (Rodeheffer and Shur 2004; Lyng and Shur 2009). Support for this ZP3 independent model has been advanced by the demonstration that both immunoprecipitated and natively purified OGP are able to competitively inhibit sperm–egg binding (Lyng and Shur 2009). The sperm-binding activity of OGP appears to be carbohydrate-dependent since denatured OGP retains the ability to inhibit binding, and interestingly is restricted to a relatively minor peanut agglutinin (PNA)-binding glycoform (Lyng and Shur 2009). While such findings may initially appear to be at odds with data from competitive inhibition assays indicating that mouse sperm–oocyte interaction is potently inhibited by preincubation of the sperm with either solubilized ZP or purified mouse ZP3 (Bleil and Wassarman 1980a), it must be remembered that these latter experiments were conducted in vitro and therefore may not be entirely physiologically relevant. It is also noteworthy, however, that ectopic ovarian pregnancies, although rare, have been recorded in humans (Cabero et al. 1989). These pregnancies take place without the oocyte ever reaching the oviduct and therefore would be unlikely to have been exposed to OGP.

2.1.5 The Role of ZP2 in Sperm–ZP Interactions

The role of ZP2 during gamete interaction has traditionally been viewed as that of a secondary ligand that possesses the ability to bind to the inner acrosomal membrane of acrosome-reacted sperm, thus ensuring close contact between the penetrating spermatozoon and the zona matrix (Bleil et al. 1988). This sperm-binding affinity of ZP2 is abrogated by the proteolytic modification of the protein that accompanies cortical granule exocytosis at the moment of fertilization (Moller and Wassarman 1989). The modification of ZP2 facilitates an increase in the interaction between the ZP filaments, in turn promoting the hardening of the ZP (Moller and Wassarman 1989) and thus producing one of two blocks to polyspermy. An interesting caveat to this model has recently been advanced by the work of Dean and colleagues using transgenic mice expressing a chimeric zona pellucida, containing human ZP2 (Dean 2004). The oocytes of these mice bind mouse but not human spermatozoa. Surprisingly, however, this sperm-binding activity persists even after fertilization of the oocytes. This phenomenon is not completely understood, since the ZP2

proteolytic cleavage domain is conserved between species and the human ZP2 would therefore have been expected to be digested following cortical granule exocytosis (Dean 2004, 2005). Interestingly, similar results were also observed in mice expressing human ZP3. One possible explanation for these anomalous results is that sperm binding is modulated by the overall supramolecular structure of the zona pellucida rather than relying on individual proteins and/or oligosaccharides (Dean 2004).

2.2 Sperm Receptor Molecules Involved in Zona Pellucida Interaction

In accordance with the complexity of the various models for sperm–zona interaction, it has proven difficult to identify definitively the corresponding sperm surface molecules that mediate primary recognition and adhesion to the ZP. A multiplicity of putative ZP receptors have been postulated on the basis of a range of experimental techniques including analysis of mutations influencing fertility, development of inhibitory monoclonal antibodies, analysis of sperm autoantigens, ZP affinity columns, photoaffinity crosslinking, and binding of radiolabeled ZP to sperm lysates (reviewed by McLeskey et al. 1998; Table 1). Consistent with the model of primary sperm–egg interaction being initiated by defined carbohydrate structures on ZP3 (Sect. 2.1.1), a number of these putative receptors possess lectin-like affinity for specific sugar residues (Table 1). However, notwithstanding compelling in vitro data implicating these molecules in zona adhesion, no single candidate has been identified that is uniquely responsible for directing the interaction between sperm and the ZP. Such findings fuel speculation that this fundamental cellular interaction may require the coordinated action of several sperm proteins. Indeed, emerging evidence supports the concept that sperm–zona interaction is mediated by a multimeric complex incorporating several discrete molecular entities, each of which may have a specific role at different stages of the recognition process (see Sect. 2.2.2). Furthermore, in recognition of the fact that gamete interaction is predicated on spermatozoa acquiring a state of functional maturation during their post-testicular development, it has been suggested that the assembly of this complex may be causally linked to membrane remodeling events associated with epididymal maturation and/or capacitation (Sect. 2.2.1).

2.2.1 Acquisition of the Ability to Engage in Sperm–ZP Interaction

Mammalian spermatozoa are produced by spermatogenesis, a prolonged, inordinately complex process that culminates in the generation of a morphologically mature, yet functionally incompetent sperm cell (reviewed by Eddy and O'Brien 1994). During this process, spermatids undergo a dramatic metamorphosis from a rounded shape into an elongated cell consisting of a number of highly specialized

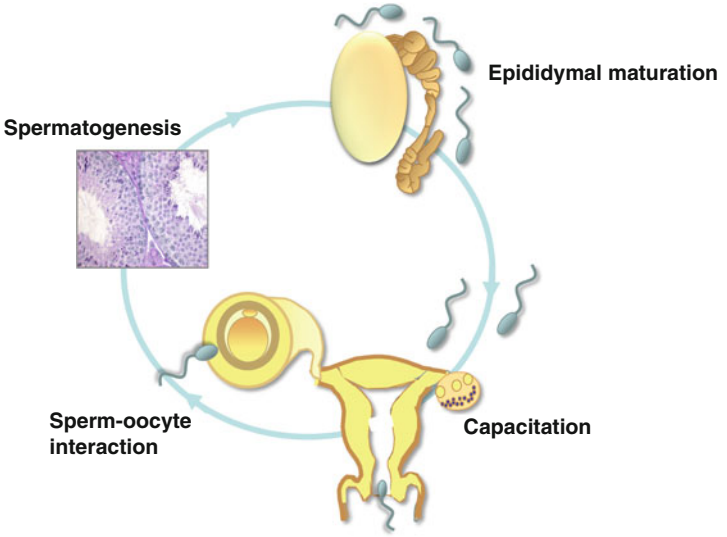

Fig. 2 *Phases of sperm maturation required for successful sperm–oocyte interaction*. Following their production in the testes (spermatogenesis), mammalian spermatozoa enter the male reproductive tract (epididymis) as functionally incompetent cells. Exposure to the intraluminal milieu of the epididymis results in acquisition of the potential for forward progressive motility and the ability to engage in interaction with the ZP. However, these functional attributes are only expressed after a final phase of maturation (capacitation) as the spermatozoa ascend the female reproductive tract

regions: a head comprising the acrosomal vesicle, nucleus, cytoskeletal structures, and cytoplasm; a midpiece which houses the mitochondria; and a flagellum that is used for locomotion. The final phase of cytodifferentiation, spermiogenesis, is also characterized by the repackaging of the chromosomes in preparation for their delivery to the oocyte (Eddy and O'Brien 1994). As a consequence of these events, it is widely held that spermatozoa leave the testes in a transcriptionally silent state and similarly lack the capacity for de novo protein synthesis (Engel et al. 1973). The post-testicular functional transformation of these cells that ensues is therefore reliant upon protein changes (loss, acquisition and post translational modification) driven by exposure to the external environment, as these cells move through the male and female reproductive tracts (Fig. 2).

Epididymal Maturation

Notwithstanding the high degree of morphological specialization that is achieved during spermatogenesis, spermatozoa enter the epididymis without the capacity to exhibit forward progressive motility nor to recognize and engage in interaction with the ZP (reviewed by Yanagimachi 1994). Spermatozoa acquire the potential to express these functional attributes during their transit of the male reproductive tract,

particularly the epididymis (reviewed by Cooper 1986). Elegant ligation and epididymostomy studies have provided compelling evidence that the accompanying changes are not intrinsic to spermatozoa, but rather appear to be driven by dynamic changes in the ambient intraluminal milieu as they pass along the length of the epididymal tubule (Cooper 1986). Indeed, the exposure of spermatozoa to the microenvironment created by the combined secretory and resorptive functions of the epididymal epithelial cells has been variously correlated with the addition, repositioning, removal, and/or modification of specific proteins and lipids within the sperm membrane (Jones 1998, 1999; Jervis and Robaire 2001; Chaurand et al. 2003; Johnston et al. 2005; Turner et al. 2006).

The ability of epididymal spermatozoa to bind to the ZP is first observed in the proximal corpus epididymis and achieves maximal levels in the caudal region in virtually all species studied to date (Cooper 1986). Interestingly, the acquisition of zona binding competence coincides with the attainment of the potential for movement (Aitken et al. 2007). However, it is considered unlikely that these two events are causally related since, unlike motility, sperm–zona interaction is dependent on the ability of the spermatozoa to undergo capacitation, with noncapacitated cells proving largely refractory to zona adhesion (Asquith et al. 2004). Additionally, zona binding ability is retained in immobilized caudal epididymal spermatozoa (Saling 1982). The acquisition of zona binding also appears temporally associated with the exposure of spermatozoa to two distinct subsets of macromolecular structures in the epididymal lumen: the first being amorphous, chaperone laden "dense bodies" (Asquith et al. 2005) and the second being membrane-bound prostasome-like particles known as epididymosomes (Saez et al. 2003). It has been hypothesized that together, these epididymal granules facilitate the bulk transfer of proteins to the sperm surface during their transit of the organ. This idea is consistent with the demonstration that biotinylated proteins are able to be transferred between epididymosomes and the acrosomal cap and midpiece of spermatozoa (Saez et al. 2003). However at present, neither the molecular mechanisms that underpin protein transfer nor the identity of the transferred protein(s) has been fully elucidated. Similarly, the causative nature of this relationship remains the subject of ongoing investigation.

Sperm Capacitation

Following their passage through the epididymis spermatozoa must complete an additional phase of maturation, termed capacitation, before realizing their full potential for fertilization. Capacitation occurs in vivo as spermatozoa ascend the female reproductive tract and encompasses a series of elaborate cellular modifications. Indeed, in the 60 years that have elapsed since capacitation was first described (Austin 1951; Chang 1951), a number of changes have been correlated with this process, including extensive remodeling of the sperm plasma membrane and the posttranslational modification of intrinsic sperm proteins (Visconti et al. 1995a, b; Gadella and Van Gestel 2004; Boerke et al. 2008; Gadella 2008; Gadella et al. 2008).

Among the posttranslational modifications that have been documented to date, a global upregulation of phosphotyrosine expression has emerged as a critical factor in regulating the ability of spermatozoa to hyperactivate, bind to the zona pellucida, undergo an acrosome reaction, and ultimately fertilize the oocyte (Visconti et al. 1995a, b, 2002; Luconi et al. 1998; Urner et al. 2001; Sakkas et al. 2003; Asquith et al. 2004; O'Flaherty et al. 2005; Baker et al. 2006; Mitchell et al. 2007).

The induction of tyrosine phosphorylation appears to be modulated predominantly by a unique soluble adenylyl cyclase/cAMP/PKA axis (Visconti et al. 1995a, b; Aitken et al. 1998). However, in addition to PKA-dependent phosphorylation of targets which for the most part appear to reside within the sperm flagellum (Visconti et al. 1997; O'Flaherty et al. 2005; Baker et al. 2006; Mitchell et al. 2007), an alternative subset of tyrosine phosphorylated proteins have been detected on the surface of live, capacitated mouse spermatozoa (Asquith et al. 2004; Piehler et al. 2006). Furthermore, the expression of these proteins appears confined to the plasma membrane overlying the acrosomal domain of the sperm head – an ideal position from which to orchestrate the membrane remodeling events associated with sperm–ZP recognition. In contrast to the aforementioned flagellar proteins, we have recently secured evidence that the phosphorylation of these sperm surface proteins is largely insensitive to inhibition with specific antagonists of the canonical PKA pathway (Nixon et al. 2010). Rather our findings suggest that an alternative signaling pathway involving the classical MAP kinases may underpin this capacitation-associated surface exposure of phosphotyrosine residues in mouse spermatozoa (Nixon et al. 2010). These findings take on added significance in light of the demonstration that the inhibition of the MAP kinase pathway, and hence sperm surface phosphotyrosine expression, induces a concomitant reduction in sperm–zona pellucida interaction (Nixon et al. 2010).

Although surface phosphotyrosine expression does not appear to be a universal correlate of capacitation in all species (Liu et al. 2006), it is not unique to mouse spermatozoa. For instance, recent quantitative studies of surface phosphotyrosine expression in boar spermatozoa have revealed a significant increase in phosphotyrosine-associated fluorescence following capacitation (Piehler et al. 2006). This increase coincides with the exposure of several tyrosine phosphorylated proteins on the outer leaflet of the boar sperm plasma membrane, at least two of which possess high affinity for the ZP (Flesch et al. 1999, 2001). In contrast, plasma membrane proteins isolated from freshly ejaculated boar spermatozoa did not exhibit any ZP binding proteins, likely because these proteins were not tyrosine phosphorylated (Flesch et al. 1999, 2001). Unfortunately however, the identity of these proteins remains to be elucidated.

Interestingly, our own analysis of the repertoire of phosphoproteins that are uniquely expressed on the surface of capacitated mouse spermatozoa, identified a subset of molecular chaperone proteins including heat shock 60 kDa protein 1 (HSPD1) and heat shock protein 90, beta 1 (HSP90B1) (Ecroyd et al. 2003; Asquith et al. 2004). Both of these proteins have in turn been localized to dense bodies within the proximal corpus epididymis (see Sect. 2.2.1) and to the sperm surface overlying the anterior acrosome, the precise location where sperm–zona interaction is initiated (Asquith et al. 2004, 2005). Although such findings invite speculation

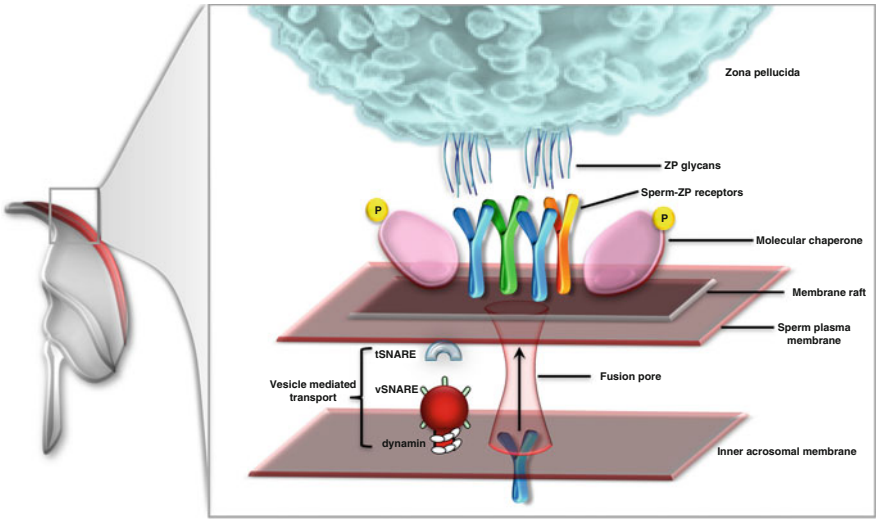

Fig. 3 *Model for mouse sperm–zona pellucida interaction*. We propose that sperm membrane rafts serve as a platform for the recruitment of key zona adhesion molecules and promote their delivery to the sperm head. This process coincides with the capacitation-associated phosphorylation of a subset of molecular chaperones and their exposure on the sperm surface. The chaperones subsequently provide the molecular machinery to assemble a functional receptor complex, rendering the sperm competent to bind to the glycans that furnish the zona pellucida. Based on emerging evidence, we also propose that capacitation-associated sperm surface remodeling may be underpinned by the translocation of a subset of ZP receptors from the acrosomal vesicle to the sperm membrane. At least two mechanisms, i.e., vesicle-mediated transport and the formation of small fusion pores, have been postulated to account for the incremental exposure of these proteins

that these chaperones may directly mediate sperm–egg interaction, our cumulative evidence argues against such a conclusion (Walsh et al. 2008). Rather, it is suggested that these proteins are responsible for chaperoning key recognition molecules to the site of sperm–oocyte interaction and/or orchestrating their assembly into a multimeric zona–receptor complex on the sperm surface (Nixon et al. 2005) (see Sect. 2.3.1; Fig. 3). In agreement with this model, we have recently employed the novel technique of blue native polyacrylamide gel electrophoresis (BN-PAGE) to provide the first direct evidence for the expression of chaperone laden complexes on the surface of capacitated mouse spermatozoa. Interestingly, a subset of these complexes also harbor putative ZP receptor proteins and possess strong affinity for solubilized zonae (Dun et al., unpublished).

2.2.2 ZP Receptor Candidates

Considerable research has been devoted to investigating the identity of the individual proteins in spermatozoa that facilitate the binding of this specialized cell to the ZP (reviewed by Nixon et al. 2007). On the basis of varying degrees of

circumstantial and direct evidence in excess of ten different candidate proteins have been proposed to participate in different aspects of this interaction in the mouse model alone (Table 1). Consistent with the notion that primary sperm–zona pellucida binding is a carbohydrate-mediated event, a number of these candidate sperm proteins are either glycoenzymes or possess the requisite lectin-like affinity for ZP3 sugars.

Perhaps the most widely studied of the putative ZP3 receptor candidates is mouse β-1,4-galactosyltransferase (GalTase). This enzyme normally resides within the Golgi apparatus, where it functions in the biosynthesis of complex glycoconjugates on secretory and membrane-bound glycoproteins (Nixon et al. 2001). However a novel, functionally distinct, isoform of GalTase has been shown to be expressed during spermatogenesis and localized to the dorsal, anterior aspect of the membrane overlying the intact acrosome (Shur and Neely 1988). From this position, GalTase is thought to function as a gamete receptor by binding to complementary terminal *N*-acetylglucosamine (GlcNAc) residues that furnish the Sperm Combining Site of the ZP3 protein (Shur and Hall 1982; Shur and Neely 1988; Shur 1989; Miller et al. 1992). Furthermore, aggregation of GalTase by ZP3 oligosaccharides activates a heterotrimeric G-protein coupled signaling cascade that culminates in the induction of the acrosome reaction (Macek et al. 1991; Miller et al. 1993). Accordingly, overexpression of GalTase on the sperm surface leads to increased ZP3 binding, accelerated G-protein activation, and precocious acrosome reactions (Youakim et al. 1994).

The expression of GalTase isoforms in the anterior portion of the sperm head of a variety of mammalian species raises the possibility that the zona receptor activity of the protein may be widely distributed (Humphreys-Beher and Blackwell 1989; Sullivan et al. 1989; Larson and Miller 1997). Surprisingly, however, targeted mutations of mouse GalTase do not induce the anticipated infertility phenotype (Lu and Shur 1997). Rather, spermatozoa from GalTase null males retain their fertility despite a marked reduction in their ZP3 binding affinity and an inability to undergo a ZP3 induced acrosome reaction (Lu and Shur 1997). Thus, although the ZP3–GalTase receptor–ligand complex may confer a physiological advantage on fertilizing spermatozoa, its expression is dispensable for fertilization. Similarly, definitive studies examining the effects of null mutations on additional sperm surface components that have been implicated in ZP adhesion have shown that the majority are also superfluous (see Table 1). Collectively these findings raise the intriguing possibility, discussed below, that the zona receptor is in fact a multimeric complex incorporating several discrete molecular entities.

Molecular Basis for Multiple Sperm–ZP Receptor Candidates

Despite the significant advances in our understanding of the initial interaction between sperm and the oocyte, it is clear that this fundamental recognition event remains largely enigmatic. The large number of sperm molecules that possess affinity for the ZP (Table 1) challenges the concept of a simple lock and key

mechanism to account for gamete interaction. Indeed, despite the wealth of in vitro data implicating various ZP receptor candidates, the prevailing evidence now indicates that none are uniquely responsible for directing the interaction between sperm and the ZP (Nixon et al. 2007). The fact that spermatozoa are adorned with a multiplicity of ZP receptor candidates could afford the cells with a level of functional redundancy commensurate with the overall importance of this fundamental cellular interaction. However, it is also possible that the individual receptors function in a coordinated fashion, each with unique role(s) during the multifaceted ZP recognition process. The latter model is consistent with biochemical and biophysical studies of sperm–ZP binding that indicate it comprises both low and high affinity interactions (Thaler and Cardullo 1996, 2002). Indeed, prior to penetration of the ZP, spermatozoa first adhere loosely to the zona matrix in a manner that is easily disrupted by repetitive pipetting or density gradient centrifugation (Bleil and Wassarman 1980a). The promiscuous nature of this initial binding event contrasts with the high affinity, comparatively species-specific, interaction that follows. The former adhesion event is therefore likely to employ sperm surface molecules that are conserved across species, while those involved in the latter binding event are, instead, expected to be species- and/or order-specific (Tanphaichitr et al. 2007). In addition to those sperm proteins required for zona adhesion, alternative candidates may be engaged in the activation of ZP-induced sperm signaling events that culminate in the acrosome reaction. If this model holds true, it inevitably raises questions regarding how the presentation of such a large number of putative ZP receptor and signal transduction candidates is coordinated. As discussed below, recent analyses have led to the proposal of at least two complementary mechanisms involving molecular chaperones and membrane rafts, the relative contribution of which may vary depending on the species.

2.3 Toward an Integrated Model of Sperm–Zona Interaction

2.3.1 The Role of Molecular Chaperones in Sperm–Zona Pellucida Interaction

The term molecular chaperone denotes a large family of highly conserved proteins that form a ubiquitous defense system within cells. However, in addition to their archetypal role of protecting cells from the adverse effects of stress, it has become increasingly apparent that chaperones play additional roles in diverse cellular phenomena under normal physiological conditions. Of particular note is the recognized ability of molecular chaperones to direct the assembly of oligomeric protein complexes and mediate their transport across the plasma membrane (Voos and Rottgers 2002). In addition, emerging evidence indicates that certain members of the chaperone family exert a necessary, but still poorly understood, role in the recruitment and clustering of specific receptors on the cell surface and in signal transduction (Triantafilou et al. 2001; Triantafilou and Triantafilou 2003, 2004).

Consistent with such roles, chaperones have been identified within a number of divergent subcellular compartments including the plasma membrane of a wide variety of cell types (Soltys and Gupta 1996, 1997, 1999; Shin et al. 2003). Interestingly, the chaperoning activity of the plasma membrane resident chaperones appears to be regulated by their phosphorylation status (Khan et al. 1998). The significance of this finding is underscored by the demonstration that at least two key chaperone proteins are phosphorylated during the capacitation of mouse spermatozoa (Asquith et al. 2004) (see Sect. 2.2.1).

Although several molecular chaperones, including calmegin, calnexin, and members of the HSP60, 70, and 90 families, have been identified in spermatogenic cells (Tanaka et al. 1997; Zhu et al. 1997; Eddy 1998; Ohsako et al. 1998; Ogi et al. 1999; Yoshinaga et al. 1999), the functional significance of many of these proteins remains unclear. However, at least one member of this family, calmegin, has been implicated in sperm–ZP interaction (Ikawa et al. 1997, 2001; Yamagata et al. 2002). Despite the fact that calmegin is not expressed in mature spermatozoa, it has been identified as a critical determinate in the functioning of these cells on the basis of its role in ensuring the correct folding of endoplasmic reticulum glycoproteins destined for the acrosomal matrix and the plasma membrane. Targeted disruption of the calmegin gene compromises male fertility due to impaired sperm transport in the female reproductive tract in vivo (Ikawa et al. 2001) and the loss of sperm–zona binding ability (Ikawa et al. 1997). An absence of signaling proteins or antigenic determinants from the surface of sperm has been proposed as the mechanism to explain these defects in sperm function. Interestingly, in this regard, sperm from calmegin$^{-/-}$ mice also lack fertilin beta (ADAM2), a protein implicated in sperm–egg plasma membrane binding and fusion (Ikawa et al. 2001). Thus, the chaperone function of calmegin may regulate the correct processing of a variety of sperm molecules. Collectively, such observations invite speculation that chaperones direct the assembly of key recognition molecules on the sperm surface.

Support for this hypothesis rests with the demonstration that mouse spermatozoa express a subset of molecular chaperones (including: HSPE1, HSPD1, HSP90, and HSP90B1) within the periacrosomal region of their head (Ecroyd et al. 2003; Asquith et al. 2004; Walsh et al. 2008). Interestingly, the surface expression of these proteins increases dramatically in populations of sperm in which capacitation has been actively driven. Nonetheless, a direct role for the chaperones in sperm–oocyte interaction has been discounted on the basis that incubation of sperm with anti-chaperone antibodies does not significantly compromise their ability to bind to the ZP (Walsh et al. 2008). Rather it appears that chaperones play an indirect role possibly in the assembly of multiple zona adhesion molecules into a functional receptor complex (Fig. 3). An alternative possibility is that molecular chaperone proteins participate in the active translocation of sperm proteins to their site of action.

Since spermatozoa lack the molecular machinery for protein synthesis, these proteins must be either unmasked or held cryptic within the cell prior to their surface presentation. A growing body of evidence favors the latter interpretation, indeed many of the putative ZP receptors are proteins one would normally

associate with the sperm acrosome (Tulsiani and Abou-Haila 2001, 2004). The zona pellucida 3 receptor (ZP3R; formerly sperm protein 56 or SP56) provides an interesting example of one such protein. ZP3R was originally identified on the basis of elegant photoaffinity crosslinking studies as a primary receptor for ZP3 (Bleil and Wassarman 1990; Cheng et al. 1994). This role was subsequently discounted on the basis of immunoelectron microscopy evidence that revealed the protein was enclosed within the acrosomal matrix (Foster et al. 1997; Kim et al. 2001a). Since such a location is incompatible with the mediation of ZP3 binding in acrosome intact spermatozoa, it was postulated that the ZP3R was likely to participate in secondary sperm–ZP interactions. Resolution of this apparent discrepancy has recently been afforded by the demonstration that ZP3R, in addition to other acrosomal matrix proteins, are progressively released to the sperm surface during capacitation through the formation of small fusion pores (Fig. 3) (Kim et al. 2001b; Kim and Gerton 2003; Buffone et al. 2008b). This evidence not only challenges the widely held view of acrosomal exocytosis as an all or none reaction but also raises the intriguing possibility that the acrosome may fulfill a secondary role as a reservoir for key ZP recognition molecules (Buffone et al. 2008a). This interpretation may explain why uncapacitated mammalian sperm are unable to engage in high affinity interaction with the ZP. However, while it is tempting to speculate that chaperones mediate the relocalization of these proteins and hence prime the sperm surface for ZP adhesion, direct evidence in support of this model has yet to be furnished.

Among the main challenges that remain in establishing the definitive role of molecular chaperones in mature spermatozoa is the characterization of the client proteins with which they associate in both noncapacitated and capacitated spermatozoa (Nixon et al. 2007). Unfortunately, the use of conventional techniques such as affinity purification and immunoprecipitation has proven largely unsuccessful in this regard (Walsh et al. 2008). This lack of success may reflect the fact that chaperones generally form only weak, transient interactions with their client proteins. Among the alternative strategies that could prove informative in this regard are the isolation and detailed proteomic characterization of the repertoire of membrane-associated proteins that populate the region of the sperm head that interacts with the oocyte. Such an approach has been published by Myles and colleagues (Stein et al. 2006). Following vectorial labeling of the mouse sperm surface, the authors conducted a comparative analysis of the profile of surface exposed proteins with that of proteins recovered in hybrid membrane vesicles released from the anterior sperm head following the acrosome reaction (Stein et al. 2006). This approach has helped define the basic proteomic inventory of the anterior sperm head, the significance of which is highlighted by the fact that among the 85 proteins identified were at least three molecular chaperones (including HSP90B1) in addition to nine proteins that have been implicated in fertilization in vivo on the basis of gene knockout studies (Stein et al. 2006). One limitation of this approach, however, was that it did not address the important question of the temporal and spatial organization of membrane-associated proteins in relation to the dynamic cellular changes that accompany capacitation.

A complementary strategy has recently been published by Gadella and colleagues (van Gestel et al. 2007). In this study, the authors isolated the apical plasma membrane from porcine sperm by nitrogen cavitation, achieving an approximate 20-fold enrichment in plasma membrane markers compared with that of contaminating membrane markers. These membrane preparations were then coincubated with isolated zona ghosts and sperm–ZP binding proteins were identified by tandem mass spectrometry (van Gestel et al. 2007). This study confirmed the involvement of multiple sperm proteins in ZP binding, with 24 sperm proteins reproducibly remaining associated with zona ghosts under conditions of low stringency. As anticipated, a subset of these proteins was identified as previously characterized ZP-binding receptors including: spermadhesin (AQN-3), P47 (SED1), and fertilin beta (ADAM2). Remarkably, the majority of the zona ghost-binding proteins were also detected in lipid ordered membrane microdomains (membrane rafts) that are assembled in the apical ridge area of the sperm head plasma membrane during in vitro capacitation (Boerke et al. 2008). On the basis of such evidence, it has been postulated that the study of membrane rafts may provide novel insights into the molecular mechanisms that underpin sperm–ZP interaction (Tanphaichitr et al. 2007; Gadella 2008; Gadella et al. 2008; Nixon and Aitken 2009).

2.3.2 The Role of Membrane Rafts in Sperm–Zona Pellucida Interaction

Membrane rafts (formerly lipid rafts) are generally defined as small, heterogeneous domains that serve to compartmentalize cellular processes (Pike 2006). The unique, ordered properties of these domains reflect the stabilizing influence of hydrogen bonds and hydrophobic interactions between their resident saturated fatty acids and the rigid structure of intercalated cholesterol. These properties also result in the resistance of lipid rafts to solubilization by a number of nonionic detergents (Schuck et al. 2003) and hence they are often referred to as detergent resistant membranes (DRMs). Despite their stability, rafts remain highly dynamic and have been observed to display considerable lateral movement in various cell types in response to appropriate physiological stimuli or cellular activation events (Simons and Vaz 2004). The significance of these structures is highlighted by the myriad of cell adhesion, signaling and trafficking molecules that have been found to preferentially associate with isolated membrane rafts (Foster et al. 2003). Indeed membrane rafts are now considered as platforms for mediating membrane trafficking, cellular signal transduction, and cellular adhesion events as diverse as viral entry and fertilization (Nixon and Aitken 2009).

It has been demonstrated that liquid-ordered domains analogous to the membrane rafts observed in somatic cells are present in the spermatozoa of all mammalian species studied to date, albeit at a larger scale (Cross 2004; Shadan et al. 2004; Sleight et al. 2005; Bou Khalil et al. 2006; Selvaraj et al. 2006, 2009; Weerachatyanukul et al. 2007; Boerke et al. 2008; Asano et al. 2009; Nixon et al. 2009). Indeed, the

size and stability of sperm membrane rafts appear quite excessive, raising the possibility that they may represent "super-rafts" consisting of stably segregated smaller subdomains (Selvaraj et al. 2006, 2009). This is consistent with a recent demonstration that a number of subtypes of membrane raft domains are likely to exist in these cells (Asano et al. 2009). With the recognition that mammalian spermatozoa possess membrane rafts, two lines of enquiry have predominated. First, whether the physical and biochemical properties of the membrane rafts are influenced by the capacitation status of spermatozoa, and second, whether the rafts modulate important aspects of sperm function (Tanphaichitr et al. 2007). Paradoxically, two conflicting views have emerged from the former studies, with evidence suggesting that these domains may either be compromised by the capacitation-associated loss of cholesterol (Sleight et al. 2005), or alternatively may cluster within the sperm head and coalesce to form larger ordered membrane microdomains during capacitation (Shadan et al. 2004; Bou Khalil et al. 2006; Boerke et al. 2008; Nixon et al. 2009). It is still unclear what functions might underlie such distinct membrane remodeling; however, the focal enrichment of membrane rafts within the sperm head encourages speculation that they may serve as platforms for modulating oocyte interaction (Fig. 3) (Tanphaichitr et al. 2007). This hypothesis is commensurate with the demonstration that DRMs isolated from both boar and mouse spermatozoa possess the ability to bind with high affinity and specificity to the zona pellucidae of homologous oocytes (Bou Khalil et al. 2006; Boerke et al. 2008; Nixon et al. 2009).

Consistent with these findings, comprehensive proteomic profiling of isolated sperm DRMs has confirmed the anticipated presence of the majority of molecules that have been implicated in sperm–zona pellucida binding (Table 1) in addition to many of those involved in downstream interaction with the oolemma (Sleight et al. 2005; Nixon et al. 2009). Although caution is required in equating DRM association with a protein's residence in membrane raft domains in situ (Foster et al. 2003; Munro 2003), such findings suggest that sperm membrane rafts serve as constitutive platforms for the spatial constraint of key recognition molecules and that the remodeling events associated with capacitation lead to their assembly and presentation on the outer leaflet of the sperm plasma membrane (Nixon et al. 2005, 2007).

Such a conclusion is supported by the demonstration that the proteomic composition of membrane rafts undergoes substantial changes in response to the induction of capacitation (Thaler et al. 2006). Among the various models that could account for such changes, Tulsiani and colleagues (Abou-Haila and Tulsiani 2003) have postulated that capacitating spermatozoa undergo a progressive priming that results in the exposure of intraacrosomal enzymes (see Sect. 2.3.1). Their model is based on the precept that as capacitation proceeds, the outer acrosomal membrane evaginates, forming a vesicle that enlarges and becomes tethered to the plasma membrane through complementary vesicle-associated (v-) SNARE and target membrane (t-) SNARE proteins residing within the two membranes (Fig. 3). Although the movement of vesicles between the sperm acrosome and plasma membrane has not previously been documented during capacitation, many of the components of the molecular machinery necessary for coordinating the assembly and trafficking of

exocytotic vesicles are present in spermatozoa. For instance, dynamin, an enzyme that forms restriction collars around budding vesicles and promotes their release, has recently been identified within the acrosome of mouse spermatozoa (Zhao et al. 2007). Furthermore, the complementary SNARE proteins, VAMP, SNAP, and syntaxin have been localized to the outer acrosomal membrane and apical sperm head plasma membrane, respectively, of mouse spermatozoa (Brahmaraju et al. 2004; Tsai et al. 2007) in addition to that of other species (Schulz et al. 1998; Tomes et al. 2002; De Blas et al. 2005). Notably these SNARE proteins share the lateral redistribution properties of both ZP-binding proteins and raft marker proteins, each of which are able to be recovered within DRMs prepared from capacitated spermatozoa (Boerke et al. 2008). Collectively, these data invite speculation that key components of a zona adhesion complex are conveyed from the acrosomal vesicle into membrane rafts during capacitation. This notion is supported by the demonstration that antibodies to VAMP and SNAP inhibited mouse sperm–zona pellucida interaction (Brahmaraju et al. 2004).

It is also noteworthy that sperm membrane rafts are laden with a subset of molecular chaperone proteins (Nixon et al. 2009), at least two of which, HSP90B1 and HSPD1, have previously been implicated in remodeling the sperm surface and enhancing oocyte interaction (Asquith et al. 2004, 2005; Sect. 2.2.1). The identification of a number of constitutively expressed molecular chaperones (HSP90B1, HSPA8, HSPD1, and DNAJB1) as integral components of membrane rafts in other cell types (Broquet et al. 2003; Chen et al. 2005) suggests that these proteins may fulfill an important general mechanism operating at the level of the plasma membrane through which cellular signaling/adhesion complexes are sorted and assembled. In this regard, previous studies have demonstrated that chaperones play important roles in maintaining the stability of lipid raft-associated signal transduction complexes (Chen et al. 2005). Conversely, it has also been demonstrated that lipid rafts regulate the functions of resident chaperones through the spatial constraint of their substrates (Elhyany et al. 2004). Taken together these results suggest that sperm membrane rafts provide a favorable environment for chaperones to mediate the conformational conversion and assembly of functional zona receptor complexes. Furthermore, the aggregation of such microdomains during capacitation may facilitate the recruitment of these complexes to the site of engagement with the ZP.

3 Potential for Contraceptive Intervention

In addition to fundamental benefits in terms of understanding causes of male infertility, the molecular dissection of sperm–ZP interaction also promises to inform the development of novel approaches for contraceptive intervention. Indeed, it has long been held that the identification of sperm proteins involved in ZP recognition and binding events could provide a range of candidates that, by virtue of their specificity, location, and susceptibility to suppression, would exhibit

potential as contraceptive targets with equal effectiveness for both males and females. Such contraceptives would be of considerable benefit for the control of both captive and feral animal species and thus contribute to ameliorating global problems associated with a lack of habitat, overcrowding, and disease (Hardy and Braid 2007; Kirkpatrick 2007; Fayrer-Hosken 2008). The realization of such technology may also contribute to the development of novel, safe, effective measures to fill the void in the current contraceptive armory for our own species, the population of which continues to grow at an alarming rate (McLaughlin and Aitken in press). Contraceptive vaccines, for example, have the potential to provide safe, effective, prolonged, reversible protection against pregnancy in a form that can be easily administered in the Third World. However, in order to meet the above criteria, the target antigen must be an essential component of fertility and must be inhibitable.

3.1 Target Antigens of the Zona Pellucida

The zona pellucida glycoproteins are among the most widely investigated candidate targets for immunocontraceptive vaccines. The ZP proteins afford the advantage of being a female organ-specific antigen and an immune response elicited against these proteins could in principle block sperm–ZP interaction. In practice, however, the reduction in fertility achieved following immunization with homologous or heterologous zona proteins appears to be primarily attributed to either the loss of endogenous antigen and/or the induction of ovarian-specific autoimmune disease (Paterson et al. 2000). This is highlighted by fertility trials involving whole native porcine zona pellucida (pZP), a popular heterologous antigen in current use for feral and exotic animal fertility control (Hardy and Braid 2007). Such studies have demonstrated that pZP is a potent heteroimmunogen in most species and is efficacious in the control of fertility in horses, white tailed deer, bonnet monkeys, wallaby, bears, and elephants (Bagavant et al. 1994; Fayrer-Hosken et al. 1999; Miller et al. 2000; Kitchener et al. 2002, 2009; Turner et al. 2002; Delsink et al. 2007; Lane et al. 2007; Locke et al. 2007). However, the use of this material as an immunogen is problematical as it has been shown to induce ovarian pathology, the loss of hormone-dependent behavior, and permanent sterility (Dietl et al. 1982; Drell et al. 1984; Bhatnagar et al. 1992). The isolation of a consistent native pZP product, free of viral contamination, for immunization purposes has also proven technically challenging (Kaul et al. 1996). While the latter problem may be alleviated by the production of glycosylated porcine ZP recombinant protein in a defined mammalian cell line, the permanent ovarian pathology that accompanies the active immunity against ZP antigens represents a significant barrier to their clinical use (McLaughlin and Aitken in press). Until researchers separate the immunocontraceptive effect from the unwanted pathology induced by immunodominant epitopes, ZP proteins will remain unlikely target antigens for a human immunocontraceptive vaccine. Furthermore, the fact that ZP immunogens lack

species-specificity imposes restraints on their mode of delivery and hence overall applicability in free-ranging wildlife species. Other antigenic targets are clearly required.

3.2 *Target Antigens of Spermatozoa*

The demonstration that spermatozoa are highly immunogenic in both females and males presents a strong rationale for the development of contraceptive technologies centered on a defined sperm-specific antigen. This is emphasized by the fact that the presence of antisperm antibodies in the male or female partner has been identified as a causative agent in the infertility associated with a relatively large number (9–36%) of couples seeking recourse to assisted conception (Menge et al. 1982; Collins et al. 1993; Ohl and Naz 1995). Furthermore, the development of antisperm antibodies that occurs in over 70% of vasectomized men limits the potential for recovery of fertility even after successful vaso-vasostomy surgery (Hull et al. 1985).

Notwithstanding the award of a US patent for a spermatoxic vaccine based around the injection of whole semen (Baskin 1932), this approach has limited utility. Among the obvious problems is the fact that spermatozoa express numerous antigens that are shared with somatic cells, thus raising the prospect of potentially severe immunopathological side effects. Attention has instead focused on the identification of individual sperm proteins capable of eliciting a contraceptive response. The appropriate sperm antigen should display sperm-specific expression, surface accessibility and have a pivotal role in fertilization. In principle, sperm proteins involved in ZP interaction therefore represent ideal candidates. Accordingly, a myriad of these proteins, including: SP17 (O'Rand and Widgren 1994), SPAM1 and ADAM1/2/3 (Primakoff et al. 1987, 1988; McLaughlin et al. 1997, 2001), LDHC4 (Goldberg 1973; Goldberg et al. 1981; Chen et al. 2008), SP10 (Srinivasan et al. 1995), ZPR3 (Hardy and Mobbs 1999), FA1 (Naz and Wolf 1994; Naz and Zhu 1998), SOB2 (Lefevre et al. 1997), a novel form of CD52 (Diekman et al. 1999), human sperm-associated antigen 9 (hSPAG9) (Jagadish et al. 2006), and nuclear autoantigenic sperm protein (tNASP) (Wang et al. 2009), have been investigated as the basis for a fertility-regulating vaccine. Nevertheless, while some of these antigens have shown promise in animal trials with notable inhibition of sperm–ZP interaction and concomitant subfertility, such studies have failed to deliver on the objective of identifying a single, suitable target that induces a 100% block to fertility.

Collectively, this lack of success highlights the naivety of the paradigm that sperm–ZP interaction is regulated by a single molecular entity that is constitutively expressed on the cell surface. Rather, it is likely that multiple sperm receptors are required to achieve high affinity binding to the complex multivalent polysaccharide ligands present within the ZP (see Sect. 2.2.2). The growing acceptance of this model is demonstrated by the fact that researchers have recently opted for construction of multiantigen vaccines. Examples include multiantigen recombinant

polypeptides comprising the mouse reproductive antigens SP56, ZP3, ZP2, and ZP1 administered to female mice (Hardy et al. 2008). This resulted in significantly reduced fertility without significant ovarian pathology (Hardy et al. 2008). Using six sperm-specific antigens (mFA-12,19, mFA-1117136, YLP12, P10G, A9D, and SP56) also resulted in reduced fertility in multipeptide vaccination studies (Naz and Aleem 2007). A third vaccine formulation comprised of five recombinant human intraacrosomal sperm proteins (ESP, SLLP1, SAMP32, SP10, and SAMP14) was used to immunize female cynomolgus monkeys, all of which developed IgG and IgA serum responses to each immunogen, indicating that a multivalent contraceptive vaccine may be a viable alternative in primates (Kurth et al. 2008).

4 Summary

Despite the myriad of putative sperm–zona pellucida adhesion molecules that have been reported, no single candidate appears uniquely responsible for mediating this important interaction. Rather than this simple lock and key mechanism, the balance of evidence favors the novel hypothesis that sperm–egg interaction is mediated by the coordinated action of several sperm receptors, each of which contribute to the high affinity and specificity of the recognition process. Furthermore, it appears that these discrete receptors are either constitutively or inducibly associated with membrane rafts following the process of sperm capacitation. The fact that these specialized membrane microdomains also accommodate a family of molecular chaperones raises the intriguing possibility that spermatozoa express a multimeric zona receptor complex that is assembled into a functional unit during capacitation. The examination of this hypothesis will provide informed insights into the molecular basis of sperm–zona pellucida interaction and may pave the way for the development of novel contraceptives for feral animals and humans and the diagnosis and treatment of infertility.

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Mouse Models as Tools in Fertility Research and Male-Based Contraceptive Development

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Contents

Abstract The production of functional spermatozoa is a complex process requiring the coordinated expression of thousands of genes. It is likely that the intricate nature of these interactions contributes to the large number of idiopathic male infertility cases seen in humans. Conversely, the complexity of the highly regulated and interconnected processes of spermatogenesis and posttesticular sperm maturation events offers opportunities for the development of male-based contraceptive targets.

The recent advances in genetic manipulation technologies and the completion of the human and mouse genome sequencing programs have provided scientists with sophisticated ways to generate mouse models for the study of basic biological mechanisms, in order to understand disease pathology and develop novel therapeutic approaches. The three common types of mouse model used for medical research

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are transgenic, knockout/knockin, and chemical-induced point mutant mice. Each type has relative strengths and weaknesses with respect to its fidelity to the disease processes in humans. In this chapter, we focus on the utility of the different types of mouse model in obtaining a better understanding of the mechanisms that control spermatogenesis and developing male-based contraceptive regimens.

Keywords ENU · Infertility · Knockout · Sperm · Testis · Transgenic · Whole genome

1 Mammalian Spermatogenesis

In adult mammals, the production of sperm (spermatogenesis) encompasses the renewal and differentiation of spermatogonial stem cells into rapidly proliferating spermatogonia, meiosis of spermatocytes, and, finally, the terminal differentiation of spermatids into spermatozoa in a process known as spermiogenesis (Clermont 1972; Fawcett 1975; de Kretser et al. 1998; McLachlan et al. 1998). Spermatogenesis relies upon coordinated interactions between the somatic, i.e., Sertoli, Leydig, and other interstitial cells, and the spermatogenic germ cells within the seminiferous tubules of the testis, as well as upon hormonal stimulation via the hypothalamic–pituitary–gonadal axis. Sertoli cells provide both physical and paracrine support for the developing germ cells. They also form the blood–testis barrier through the presence of intercellular tight junctions, thus providing an immunologically isolated environment for developing spermatocytes and spermatids. Leydig cells in the interstitial tissue of the testis provide testosterone, which acts via the Sertoli cells to drive spermatogenesis. Endocrine factors secreted by the hypothalamus (GnRH, gonadotrophin-releasing hormone), pituitary gland (LH, luteinizing hormone; FSH, follicle stimulating hormone), and many autocrine factors are involved in the regulation of spermatogenesis (Clermont 1972; Fawcett 1975; de Kretser et al. 1998; McLachlan et al. 1998).

In the process of spermiogenesis, haploid round spermatids differentiate into highly polarized cells specialized for motility and fertilization. This differentiation process involves a series of complex morphological alterations, including chromatin reorganization and condensation, sperm tail assembly, and acrosome formation (Fawcett 1975). Spermatozoa ultimately detach from Sertoli cells and are released into the seminiferous tubule lumen by a hormonally regulated process termed spermiation (Jones 1998; Aitken et al. 2007).

Spermatozoa appear morphologically mature following spermiation; however, they do not gain the capacity for fertilization until they have transited through the epididymis. As discussed elsewhere in this book, the process of epididymal maturation involves sperm plasma membrane remodeling (Jones 1998), the adsorption of several epididymal-derived proteins and the progressive acquisition of the potential for forward motility and oocyte binding (Aitken et al. 2007). Following the completion of epididymal maturation, sperm remain incapable of fertilization

until they have undergone an additional maturation process, capacitation, in the female reproductive tract (de Lamirande et al. 1997). Capacitation is a complex process that is required for the activation of ejaculated sperm and gives them the ability to bind to the zona pellucida of the oocyte and undergo the acrosome reaction. Several changes have been associated with the capacitation process, including an efflux of cholesterol, an increase in membrane fluidity, an increased influx of calcium, an increase in the intracellular concentration of cAMP, the tyrosine phosphorylation of many proteins, and manifestation of oocyte-binding ability and hyperactivated motility (de Lamirande et al. 1997).

To achieve fertilization, a number of biological changes are required in both sperm and eggs. Steps in fertilization include (1) sperm penetration of the cumulus cells surrounding ovulated eggs, (2) sperm binding to the zona pellucida, (3) sperm acrosome reaction and penetration of the zona pellucida, and (4) sperm–egg plasma membrane binding and fusion (Primakoff and Myles 2002).

2 Mouse Models for Fertility Research and Male-Based Contraceptive Development

The production of functional spermatozoa is a complex process that requires the coordinated expression of a large number of genes. In simple eukaryotes, such as flies and worms, it has been estimated that spermatogenesis encompasses the coordinated activation of >1,000 transcripts (Andrews et al. 2000; Reinke et al. 2000), whereas in mammals ~4% of all genes are proposed to be specially expressed within the male germ cells (Schultz et al. 2003). Defects in all aspects of spermatogenesis can, at least in theory, contribute to human infertility, but few have been modeled *in vitro*. The complexity of the tightly regulated and interconnected spermatogenic process offers opportunities for the development of male-based contraceptives.

The use of animal models is a powerful tool in the analysis of many aspects of medicine, from the study of basic biological mechanisms to the understanding of disease pathology and the development of novel therapeutic regimes. The mouse has become the major mammalian model of human disease as it offers several advantages over other species including a short reproduction cycle, relatively cheap housing conditions and their embryos are amenable to manipulation. Moreover, over 90% of the mouse genome lies in the conserved syntenic segments in the human genome (Waterston et al. 2002; Church et al. 2009), thus making them an ideal human surrogate in the study of most diseases.

Strategies used to identify and evaluate the *in vivo* function of a defined gene in biomedical research may be divided into two major categories: reverse genetic and forward genetic approaches. The reverse genetic approach (also known as a candidate gene or gene-driven approach) is based on alterations, i.e., ectopic expression or abolished expression (targeted deletion) of a selected candidate gene followed by an assessment of the physical manifestation (phenotype) of that

genetic trait. In contrast, a forward genetic approach (also known as a phenotypic-driven approach) relies on the observed phenotypic defects then defining the underlining genetic defect.

In this chapter, we discuss the utility, strengths, and weaknesses of the different types of mouse models (summarized in Table 1) with an emphasis on contraceptive target discovery.

3 Transgenic Mice: Ectopic Expression Models

A transgenic animal is one that carries a foreign piece of DNA (usually a gene) that has been deliberately inserted into its genome. Two common approaches are used for the generation of transgenic mice (1) Transformation of a transgene in embryonic stem (ES) cells and (2) Pronuclear injection of a transgene into one-cell stage mouse embryos (Gardiner and Teboul 2009). In the ES cells approach, ES cells are harvested from the inner cell mass (ICM) of mouse blastocysts after which a transgene is electroporated or transfected in, i.e., they are "transformed." ES cells carrying the desired transgene are selected for, then injected into the ICM of additional embryos, and implanted into the uterus of pseudo-pregnant recipient mice. In the pronuclear microinjection method, transgene fragments are directly injected into one-cell stage mouse embryos followed by embryo transfer into a pseudo-pregnant recipient.

The transgene is constructed using recombinant DNA techniques. In addition to a sequence encoding a protein of interest, the transgene usually contains regulatory sequences, e.g., a promoter and a polyadenylation signal, to enable the transgene to be expressed appropriately by the cells of the host. In general, mice resulting from this technique are called "over-expressors." Transgenes can, however, be constructed in more sophisticated ways, e.g., carrying a dominant negative form of the protein which will interfere with the native protein function, thus resulting in a lack of function phenotype. In order to visualize transgene expression, transgene fragments can be engineered to also contain markers e.g., fluorescence markers [yellow florescence protein (YFP), green fluorescence protein (GFP)] and/or enzymatic-based marker (β-galactosidase).

The integration of a transgene cassette into the ES cell host genome usually occurs randomly, and the level of transgene expression is affected by the integration site and number of the integrated transgene copies (Giraldo and Montoliu 2001; Chandler et al. 2007). The host genome sequences surrounding the site of transgene integration can modify transgene expression and may result in ectopic, weak, or undetectable expression. This effect is known as a chromosomal position effect (Hogan 1983) and can limit the creation of gain-of-function or dominant-negative alleles. The use of small DNA fragments is often the cause of positional effects in transgenic mice (Teboul 2009).

The use of a large transgene, up to 300 kb, carrying regulatory elements (located distantly from the protein coding of the gene e.g., enhancer and insulator) derived

Table 1 Strengths and weakness of different mouse models

Mouse model	Strengths	Weaknesses
Transgenic mice	• Markers can be incorporated into the transgenes to localize *in vivo* sites of expression • Large transgenes that contain essential regulatory elements are possible (enabling normal genomic architecture)	• Mouse lines are produced and evaluated one at a time • Large variations in transgene copy number, and thus expression levels • Integration site effects • Levels of transgene expression can limit the generation of gain-of-function alleles or dominant-negative alleles • Random integration may inactivate endogenous host genes
Conventional knockout mice	• Allow the possibility to define a null allele phenotype • Some targeted ES cells are available from the International Mouse Knockout program repositories	• Mouse lines are produced and evaluated one at a time • Homologous recombination efficiency in ES cells is generally low, thus necessitating the screening of large number of ES clones • Some genes are difficult to target due to the nature of sequence within or surrounding the gene • Null alleles can result in an absence of a phenotype due to functional redundancy with related genes
Conditional knockout mice	• Allow possibility to define function of gene in specific cell/tissue and/or a specific time of development • Some targeted ES cells are available from the International Mouse Knockout program repositories	• Targeting construct must be designed carefully to avoid disruption of the endogenous allele prior to activation of the *Cre* recombinase activity • Requires an appropriated *Cre* transgenic mouse line • Poorly characterized *Cre*-expressing line may result in residual expression of the gene within some cells or deletion in additional cell types
Gene-trap mice	• High throughput approach • Publically available through consortiums	• Can result in the production of hypomorphic, rather than null alleles • A large up-front investment to establish the mouse colony and mapping/sequencing technologies • A trapped ES clone may contain an unidentified trapped gene(s)
ENU mutagenised mice	• Requires no foreknowledge about gene function • Novel genes can be identified • Novel functional domains within genes can be identified • Mutations are already in the germline, thus avoiding the need to produce chimeric mice to obtain germ line transmission • Can sometimes circumvent functional redundancy from related genes during development	• Researchers may have to screen the entire library to obtain mouse lines with specific phenotype of interest • Maintaining the mouse colony can be challenging, as there is no precise way to genotype mice prior to the identification of the causal mutation • The identification of the causal mutation is labor and cost intensive • A large up-front investment to establish the mouse colony and mapping/sequencing technologies

from Yeast Artificial Chromosome (YAC) or Bacterial Artificial Chromosome (BAC) constructs have been shown to confer endogenous-like transgene expression patterns and are less susceptible to positional effects (Giraldo and Montoliu 2001; Chandler et al. 2007). Due to several limitations of YACs, e.g., insert chimaerism, insert instability, rearrangements, and potential endogenous yeast chromosome contamination (Monaco and Larin 1994), BACs have become the preferred choice for the generation of transgenic mice (Heintz 2001; Van Keuren et al. 2009).

In addition, the integration of a transgene into a host genome may disrupt endogenous genes, which could result in loss or gain of function of the host gene. In order to minimize these effects, recent studies have made significant progress on the development of site-specific integration of transgene fragments into the host genome (Brough et al. 2007; Howden et al. 2008; Wigley et al. 1994). Alternatively, researchers should examine at least two independently generated transgenic models before concluding a specific effect of a transgene.

Examples of transgenic mouse models that have revealed valuable information about male fertility and insights relevant to contraceptive design include: the overexpression of transgenic *Nanos2* in spermatogonia has revealed the role of NANOS2 in stem cell renewal (Sada et al. 2009); the exogenous expression of FSH in the hypogonadal (*Hpg*) background has been used to reveal the relative contribution of testosterone and FSH signaling on particular aspects of male fertility (Allan et al. 2004; Allan and Handelsman 2005); and FGFR1 dominant-negative mice have revealed the role of FGF signaling in establishing pathways involved in sperm capacitation (Cotton et al. 2006).

4 Knockout Mice: Loss of Function Models

Targeted gene ablation is the most common approach used to assess the *in vivo* function of a gene. It relies upon abolishing the expression of a gene then observing the phenotype. These types of mice are referred to as "knockout mice" and are based on targeted deletion/disruption of an endogenous gene by homologous recombination in ES cells (Smithies et al. 1985; Thomas and Capecchi 1987) (Fig. 1). ES cells carrying the desired ablated gene are microinjected into blastocyst stage embryos of a different mouse strain and transferred into pseudo-pregnant host females to produce chimeric offspring. Chimeric mice are subsequently bred with wild-type mice to establish a heterozygous knockout mouse, i.e., carrying one deleted allele and one wild-type allele. Through the breeding of heterozygous carriers, mice with two completely inactivated alleles can be generated, i.e., homozygous null mice (Capecchi 1989a, b). Targeting constructs can be designed by replacing the whole protein coding region of small genes (<10 kb) with a drug-selection marker, to knock out exons containing the ATG start codon (Fig. 1) or a key functional domain, or to result in the generation of a sequence frame-shift that leads to the production of truncated nonfunctional protein. Generally, knockout constructs contain a bacterial/mammalian selectable marker, e.g., a Kan/Neo

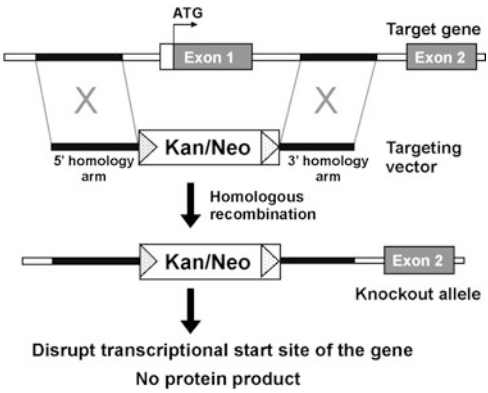

Fig. 1 Conventional knockout strategy for the generation of null alleles. The generation of null alleles in the genome of ES cells is based on the introduction of a targeting vector that carries pieces of DNA that contain identical, or highly homologous, sequences to both upstream (5′ homology arm) and downstream regions of the target gene (3′ homology arm). For screening/positive selection purposes, the homology arms are usually designed to flank a drug-selectable marker (e.g., Kan/Neo). Following the introduction of the targeting construct into the ES cell, recombination machinery within the cells recognizes the identical stretches of sequence and swaps out the nonhomologous sequence between the homology arms, thus replacing part of the endogenous gene with an artificial piece of DNA from the targeting vector. This results in a complete or partial deletion of the target gene that disrupts protein synthesis or creates an unstable protein product. As shown in this case, the first exon of the target gene is knocked-out resulting in a complete disruption of mRNA transcription, thus no protein from the knockout allele is produced

cassette, to allow manipulation of the construct in bacteria and to preferentially isolate targeted ES cells in culture. The Kan/Neo cassette can also be modified to contain markers for expression analysis within the genetically modified mouse.

In additional to the generation of null alleles, specific human alleles carrying mutations/SNPs of interest can be introduced to replace the orthologous mouse gene via homologous recombination. Such mice are referred to as "Knockin mice" and are particularly useful for the recapitulation of known human disease-causing mutations (Sotillo et al. 2001; Gong et al. 2002; Menalled et al. 2002; Lute et al. 2005; Yu et al. 2009).

There have been several hundred knockout mouse lines that have revealed valuable information on the role of genes in male fertility (Cooke and Saunders 2002; Matzuk and Lamb 2002; Escalier 2006; O'Bryan and de Kretser 2006; Matzuk and Lamb 2008; Naz et al. 2009; Yan 2009), but models with particular relevance to contraceptive design include: the *Catsper* knockout lines which have revealed a role for this ion channel in the manifestation of hyperactivated motility and thus fertility (Ren et al. 2001; Quill et al. 2003; Jin et al. 2007; Qi et al. 2007); the soluble adenylate cyclase (*sAC*) knockout mouse line which revealed a role for this enzyme in the initiation of sperm motility (Esposito et al. 2004); the *Calmegin* knockout line which revealed a role for this chaperone in the packing or presentation of sperm zona receptor molecules related to oocyte binding (Ikawa et al. 1997)

and the *Izumo* knockout lines in establishing the role for this molecule in sperm–oocyte binding (Inoue et al. 2005).

5 Conventional and Conditional Knockout Mice

In the conventional gene knockout approach described above, the null allele will be present in every cell in the animal throughout its life. Frequently, this results in a severe and early phenotype, e.g., embryonic or perinatal death of the homozygotes. While the embryonic or peri-natal lethality phenotype indicates a vital role for the gene in development, it limits the utility of the mouse model in the analysis of later stages of life. In the case of male infertility and contraceptive research, this is a serious limitation. To overcome this limitation, a conditional knockout approach has been developed (Lewandoski 2001). This approach allows a tissue-specific and/or time-specific inactivation of the gene of interest. This added feature of reversibility provides greater control over the target cell/tissue, timing, and duration of a resulting phenotype, and more closely mimics the effect of pharmacological intervention, and is thus of particular relevance in contraceptive research where the ultimate goal is to give compounds to adults and affect fully established spermatogenesis.

The ability to switch on or off a particular gene in conditional knockout models relies upon the use of the bacteriophage P1-derived *Cre*/*lox*P system (Sternberg and Hamilton 1981; Orban et al. 1992). The system is based on site-directed recombination of sequences between a short *Cre* recombinase recognition site (*lox*P) mediated by the enzyme, *Cre* recombinase, and utilizes the ability of *Cre* recombinase to excise a DNA segment flanked by a 34 bp *lox*P site in the same orientation. Mice carrying a *Cre* allele (referred to as a "*flox*" allele) are generated by homologous recombination in ES cells (Fig. 2) in the same manner as generation of a conventional knockout allele. The generation of a conditional knockout allele is achieved by crossing *flox* allele mice with transgenic mice expressing *Cre* recombinase under the control of a tissue-specific promoter and/or a binary inducible promoter e.g., tetracyclin-based transcription transactivation (Quwailid et al. 2004).

In contrast to a conventional knockout strategy, in the absence of *Cre* recombinase activity, the expression of the *flox* allele should be expressed normally as in the wild-type allele. However, to avoid the effect of *lox*P site interference on gene expression prior to exposing to *Cre* recombinase, the position of *lox*P sites must be chosen carefully to avoid the destruction of regulatory elements or pre-mRNA splice sites (Betz 1997). Another alternative/supplementary system to the *Cre*/*lox*P system is the *Flp* (Flippase)/FRT system (Rodriguez et al. 2000). The most important factors to be considered prior to choosing a *Cre* (and *Flp*) transgenic mouse line are (1) the cell/tissue target specificity, (2) the efficiency of the system, and (3) control over the timing of gene activation/inactivation (Papathanasiou et al. 2003). Examples of common *Cre* transgenic mice used in male fertility studies are protamine 1 (*Prm1*)-*Cre*, synaptonemal complex protein 1 (*Sycp1*)-*Cre*, cAMP

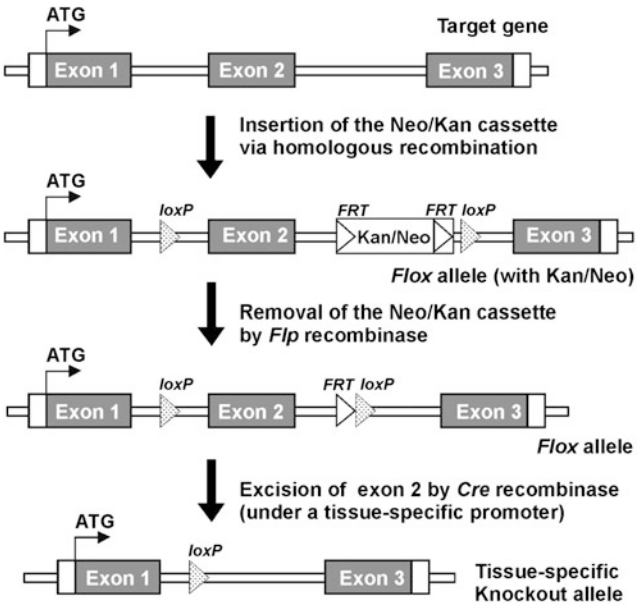

Fig. 2 Conditional knockout strategy. The generation of conditional knockout alleles is based on the use of intrinsic homologous recombination machinery within the ES cells and an artificial site-specific recombination system (*Cre*/*lox*P or FRT/*Flp*) introduced during the mice breeding program. In the first instance, a targeted *flox* allele carrying a positive selectable marker (e.g., Kan/Neo) is generated via homologous recombination in ES cells in a similar manner to that used in the conventional knockout approach, with the exception that the *flox* allele is designed to contain *lox*P sites flanking the region (e.g., exon) of the gene a researcher wishes to remove. Generally, the expression of the *flox* allele should resemble a wild-type allele. The insertion of a selectable cassette can, however, potentially interfere with the expression of the *flox* allele prior to excision, thus creating unwanted null or hypomorphic alleles. To avoid this, the selectable cassette may be designed to include FRT sites to facilitate removal of the cassette upon successful ES cells screening (e.g., via the introduction of *Flp* recombinase into the targeted ES cells). Following the generation of heterozygous mice carrying the targeted *flox* allele, the excision of a *lox*P flanking exon in a tissue-specific or developmental stage-specific manner can be achieved by crossing the heterozygous *flox* mice with transgenic mice carrying a *Cre* recombinase driven by an appropriated promoter. The tissue-specific expression of the recombinase allows the inactivation of the gene of interest only in the tissue where the recombinase is expressed

responsive element modulator (*Crem*)-*Cre*, stimulated by retinoic acid gene 8 (*Stra8*)-*Cre*, and anti-Mullerian hormone (*Amh*)-*Cre*. A list of available *Cre* transgenic mouse lines can be found at http://jaxmice.jax.org/index.html. An example of the power of such approaches in the field of male fertility and contraceptive research is seen in the systematic dissection of the role of androgen receptor (AR) signaling in testicular cells and their effect on male fertility (Wang et al. 2009).

Researchers should be aware of the existence of the International Mouse Knockout Program which is a European Union, National Institutes of Health, and Canadian Government funded initiative to systematically produce *flox* alleles for the majority of genes in the mouse genome (Austin et al. 2004; Auwerx et al. 2004;

Collins et al. 2007). This resource, and the subsequent analysis of several hundreds of the resultant knockout mouse lines (Mallon et al. 2008; Beckers et al. 2009), promises to add greatly to the fields knowledge of many biological processes including male fertility.

6 Knockout Generated by Gene Trapping

Although conventional and conditional knockout mice are extremely powerful tools for ascertaining gene function, these approaches are limited by the fact that only one gene can be knocked out at a time. To overcome this limitation, a high throughput insertional null allele mutagenesis approach referred to as "gene trapping" was developed. This approach is based on the utility of a gene trapping vector to randomly integrate into a genomic locus of an ES cell and interrupt transcription of a trapped gene. Through a high throughput screening approach, ES clones containing thousands of different trapped genes have been produced by several consortiums. The collection of ES gene trap cell lines has been made publically available and centralized through the International Gene Trap Consortium (IGTC), which aims to generate a library of mouse mutant ES cells covering most of the genes in the mouse genome (Stanford et al. 2001; Stryke et al. 2003; Nord et al. 2006).

The simplest type of gene trap vector contains a promoterless selectable genetic marker (e.g., Neo) and/or reporter gene (e.g., β-galactosidase) flanked by an upstream 3′ splice site (splice acceptor; SA) and a downstream transcriptional termination sequence (polyadenylation sequence; polyA) (Fig. 3). When inserted into an intron of a gene, transcription (under the endogenous promoter) of the trapped gene is terminated prematurely at the polyadenylation site of the gene trap vector, thus generating a fusion pre-mRNA carrying part of the endogenous mRNA sequence fused to the gene trap cassette. This fusion pre-mRNA subsequently undergoes alternative splicing at the splice acceptor site within the gene trap cassette and in most cases where the insertions occur within introns downstream of translational start sites, fusion mRNAs are generally produced that encode a truncated fusion protein (Fig. 3). Such fusion proteins can be hypomorphic if they retain partial functionality or if the insertion disrupts the translational start site or a critical functional protein domain, the fusion mRNA/protein will effectively be a null allele. Several types of gene trap vectors have been developed (see detail at http://www.genetrap.org) to allow cellular localisation of fusion trapped proteins with the animals.

7 Chemical-Induced Point Mutant Mice Generated by Random Whole Genome Mutagenesis

The ability to generate null alleles by gene targeting in ES cells either in germline or conditional null alleles will no doubt continue to be a powerful tool in defining *in vivo* gene function. However, the most common type of mutation in humans is

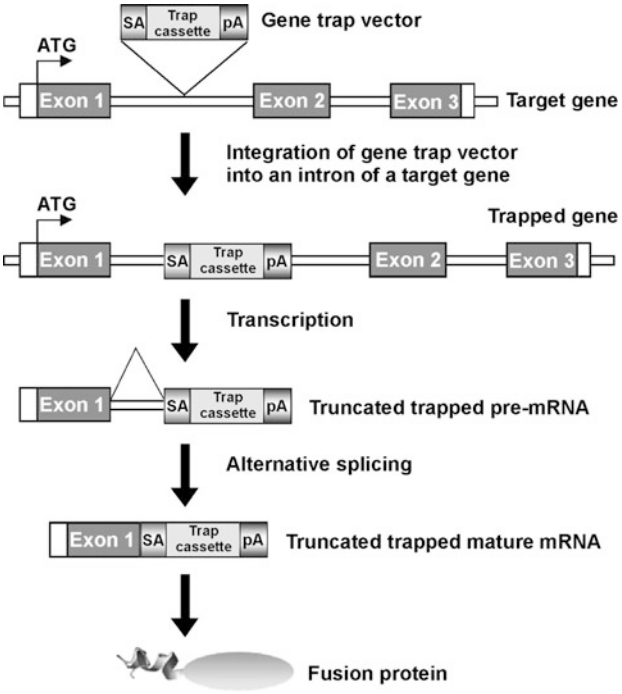

Fig. 3 Gene tapping strategy. This approach is based on the utility of a viral-based vector to randomly insert and disrupt genes throughout the ES cell genome. Gene trap vectors are designed to carry a promoterless selectable/reporter cassette (e.g., Neo/β-galactosidase) flanked by an upstream 3′ splice site (splice acceptor; SA) and a downstream transcriptional termination sequence (polyadenylation sequence; polyA). Insertion of the cassette occurs randomly; however, if it occurs within an intron of a gene it will result in the production of a fusion transcript carrying a portion of the endogenous mRNA and the gene trapping cassette. Depending upon the insertion site of the cassette; the fusion transcript can produce a hypomorphic allele or null allele mutation

not a null allele, but rather a single nucleotide variant that results in aberrant gene expression and/or compromised (but not ablated) protein function.

Random whole mouse genome mutagenesis has been developed to facilitate high throughput gene discovery and is a phenotypic-driven or forward genetic approach. The overall aim of such strategies is to induce a high rate of random point mutations throughout the entire genome of an animal, to filter out those animals (mice in this case) that contain a phenotype of interest, then to identify the underlying causal mutation. Such a strategy, in addition to avoiding misleading preconceptions of gene function, also has the advantage that it usually compromises protein function without eliminating the whole protein. In several instances, this has been shown to avoid redundancy, and the loss of a phenotype, during development from related family members (Papathanasiou et al. 2003). As mentioned above, such a strategy also holds the potential to identify key domains within a protein which may ultimately be inactivated using pharmaceutical strategies. In the following section,

we shall describe the use of the chemical mutagen *N*-ethyl-*N*-nitrosourea (ENU) as an example of the random mutagenesis approach. However, several other equivalent strategies have been employed.

ENU is an alkylating agent which can transfer its ethyl group to oxygen or nitrogen radicals in DNA. The transferred ethyl group forms a bulky lesion in the DNA strand, which can result in mispairing and base pair substitution during DNA replication, if not repaired. The treatment of inbred male mice with optimized dosages of ENU can induce nucleotide substitutions once in approximately 10^6 basepairs (Nelms and Goodnow 2001; Quwailid et al. 2004).

In order to set up a whole genome mouse ENU mutagenesis project, male mice are injected with a controlled dose of ENU that introduces point mutations in all

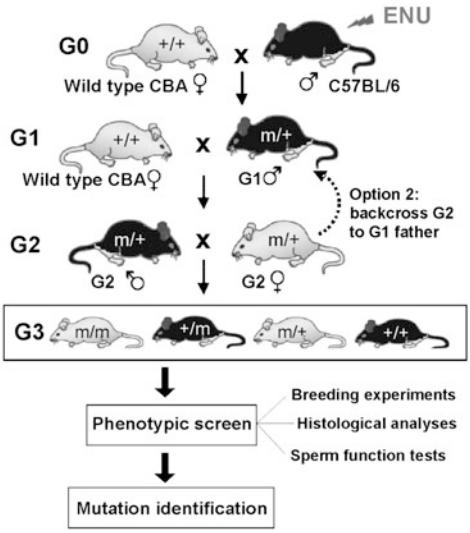

Fig. 4 ENU screens for recessive mutations causing male infertility phenotypes. The mutagenesis program involves injections of ENU into adult male mice followed by out-breeding to a different mouse strain, phenotypic screening, and linkage analysis for mutation identification. To screen for recessive mutations, a controlled three-generation breeding program is used. For example, founder ENU-treated mice (G0, C57BL/6) that carry many different mutations are crossed with wild-type female mice of a different strain (e.g., CBA). Generation 1 (G1) progenies are subsequently crossed with wild-type CBA females to generate G2 progeny. G3 mice, which are homozygous for a number of undefined point mutations, are generated either by intercrossing G2 siblings or backcrossing G2 females with G1 fathers. In order to identify which of the randomly mutated lines contains mutations in key fertility genes, G3 siblings are paired. Those pairs which did not produce pups are subsequently separated and paired with fertile wild-type mice to determine if the etiology of the infertility is male and/or female in origin. Lines with male infertility defects are further characterized using histological analyses and/or sperm function tests. To identify the causal mutations, genomic DNA from affected and unaffected mice is used in linkage analysis using whole genome microarrays based on single nucleotide polymorphism (SNP) differences between the mutated mouse strain and the strain of mouse used for out-breeding. Once a linkage interval has been narrowed to a relatively small interval, candidate mutated genes can be selected based on expression profile and/or proposed function, and their gDNA sequence determined; m represents a mutation introduced by ENU

dividing cells, but of importance, a high rate of mutations in spermatogonial stem cells (Nelms and Goodnow 2001; Quwailid et al. 2004). Following a transient period of infertility, the testes of treated mice are repopulated with sperm bearing the mutations introduced into spermatogonia and are bred with wild-type females to produce offspring carrying a subset of the mutations. Either these mice, if looking for dominant mutations, or subsequent inbred generations (Acevedo-Arozena et al. 2008; Georgel et al. 2008), if looking for recessive mutations, can be screened for phenotypes of interest including male infertility (Ward et al. 2003; Lessard et al. 2004, 2007; Kennedy and O'Bryan 2006) (Fig. 4). Once a line of interest has been identified, the region of the genome containing the underlying causal mutation can be identified using gene mapping techniques and ultimately the precise mutation is identified through candidate gene sequencing.

Through a number of centralized ENU programs around the world, several novel genes involved in male fertility, and thus potential contraceptive targets, have been identified including: *Capza3*, which is involved in cytoplasmic extrusion at the time of spermiation (Geyer et al. 2009); *Brwd1*, in spermiogenesis and oocyte maturation (Philipps et al. 2008); and GPX4, a selenium-dependent glutathione peroxidase that plays a key role in male fertility (Schneider et al. 2009).

8 Conclusions

Despite advances in assisted reproductive technologies, infertility remains a major health problem worldwide. Conversely, the desire to suppress fertility is of concern to the vast majority of other men during their life time. These points underscore the great need for research into the mechanisms of male fertility and the development of regimes to either enhance or suppress fertility.

The sequencing of the human, and many other species, genomes has focused attention onto functional genomics and defining the function of all genes. In this regard, the mouse is an attractive model system. Here we have discussed the utility, strengths, and weaknesses of mouse models with respect to their fidelity to disease states in the human. The greatest challenge for researchers will be the translation of the research findings from the mouse into clinical practice.

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Part III
New options: From target to product

Male Hormonal Contraception

E. Nieschlag

Contents

Abstract The principle of hormonal male contraception based on suppression of gonadotropins and spermatogenesis has been established over the last three decades. All hormonal male contraceptives use testosterone, but only in East Asian men can testosterone alone suppress spermatogenesis to a level compatible with contraceptive protection. In Caucasians, additional agents are required of which progestins are favored. Current clinical trials concentrate on testosterone combined with norethisterone, desogestrel, etonogestrel, DMPA, or nestorone. The first randomized, placebo-controlled clinical trial performed by the pharmaceutical industry demonstrated the effectiveness of a combination of testosterone undecanoate and etonogestrel in suppressing spermatogenesis in volunteers.

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1 Introduction

1.1 The Rationale for Hormonal Male Contraception

In view of the heightened attention politicians and the media are currently paying to the subject of declining birthrates in industrialized countries and especially in Germany, at first sight it would not seem appropriate to report on the development of new contraceptive methods. However, the decline in births is not a phenomenon that arose with the introduction of oral contraceptives at the beginning of the 1960s. In fact, birthrates declined parallel to the rise of industrialization. In Germany, in 1860 women bore an average of five children, four in 1874, three in 1881, and in 1904 the average rate was only two children per woman (Birg 2005). The trend then continued relatively slowly, marked by several ups and downs, and today it lies at ca. 1.4. Thus, contraceptives per se can hardly be considered the reason for limiting family size; they are only adjuncts enabling couples to achieve their ideal family size with greater ease and safety. In this sense, contraceptive methods also contributed to population stability in industrialized nations. Similarly, in developing countries contraceptives make a valuable contribution to limiting population growth which continues to remain high.

Female contraception is very effective. Nevertheless, 50% of the 1,000,000 conceptions occurring every day worldwide remain unplanned, of which 150,000 are terminated by abortion, an intervention that will end fatally for 500 of these women. Although improved distribution and utilization of female contraceptive methods might ameliorate this situation, the contribution of a male contraceptive is well worth considering. Men enjoy the pleasures of sex, but can do little to contribute to the tasks of family planning – a pharmacological male contraceptive is surely long overdue. Moreover, the risks of contraception would also be more fairly shared between women and men. Representative surveys have shown that a pharmacological male contraceptive would be acceptable to large segments of the population in industrial nations and would thus contribute to further stabilization of population dynamics. It might also help developing countries whose exponential population growth endangers economic, social, and medical progress. Last but not least, male contraception can be considered an outstanding issue in the political field of gender equality.

1.2 Choices for the Male

For the male there are ways to eliminate both procreation and sex at the same time. Such methods have been used in the past and are still being practiced on a limited

scale. *Castration* has been employed since ancient time to destroy enemies by abolishing their ability to reproduce and transmit their genes. Until the end of the imperial period in China (1912), men were willing to sacrifice their testicles (and often with them their lives) in return for high-ranking positions and political influence at the emperor's court. Meanwhile, in the West, up until almost the same time, some promising boys were forced to give up their manhood for the sake of preserving their prepubertal voice and achieving fame as singers, often without success (Nieschlag et al. 2009). *Abstinence* is a less bloody means of eliminating procreation, but few men are willing to give up both sex and procreation for extended periods of time, let alone their entire lives.

Traditional male methods of contraception such as *periodic abstinence* or *coitus interruptus* are associated with a relatively high rate of unwanted pregnancy and also cause a disturbance in sexual activity. *Condoms* are the oldest barrier method available. However, when using condoms conception rates are relatively high, with 12 out of 100 couples conceiving during the first year of use (Pearl index = 12). Condom use has increased since the beginning of the AIDS epidemic, but more for protection from HIV infection and other sexually transmitted diseases than for contraceptive purposes (April et al. 1993).

Vasectomy is a safe and relatively simple surgical method for male contraception. The rate of unwanted pregnancies after vasectomy is less than 1%. The drawback to vasectomy is that it is not easily reversible. Achieving fatherhood after vasectomy requires either surgical reversal or sperm extraction from a testicular biopsy and intracytoplasmatic sperm injection into the ovum. Only about 50% of these men will become fathers in the end (for review see Engelmann and Gralla 2010).

Given the disadvantages of these mechanical male methods, what then are the *prerequisites for an ideal male contraceptive?* It should:

- Be applied independently of the sexual act
- Be acceptable for both partners
- Not interfere with libido, potency, or sexual activity
- Have neither short- nor long-term toxic side effects
- Have no impact on eventual offspring
- Be rapidly effective and fully reversible
- Be as effective as comparable female methods

For the past 40 years, hormonal approaches to male contraception have been tested clinically. In the following, these developments will be reviewed taking the above prerequisites into account.

2 Principle of Hormonal Male Contraception

Of all the different experimental approaches and pharmacological methods tested so far for male contraception, hormonal methods come closest to fulfilling the criteria set out above. The endocrine feedback mechanism operating between

hypothalamus, pituitary, and testes is the basis on which hormonal approaches to male contraception rest. Its goal is to suppress spermatogenesis and to reduce sperm concentration, if possible to azoospermia or at least to a sperm concentration low enough to provide contraceptive protection (<1 Mio. sperm per ml ejaculate).

Sperm production and secretion of testicular testosterone are so closely interwoven that it has remained impossible to interrupt spermatogenesis by hormonal means without inhibiting androgen production (Fig. 1a). Inhibition of FSH alone, e.g., by antibodies, leads to reduction of sperm concentration but not to azoospermia, as monkey studies have shown. Suppression of both FSH and LH would indeed lead to azoospermia, but would also induce symptoms of androgen deficiency which affects libido, potency, male role behavior, and general metabolic processes (erythropoesis, protein, mineral, and bone metabolism). For this reason, inhibition of gonadotropins will always necessitate androgen administration.

Thus, the principle of hormonal male contraception is based on:

1. Suppression of LH *and* FSH
2. Depletion of intratesticular testosterone and atrophy of spermatogenesis
3. Substitution of peripheral testosterone to maintain androgenicity

At first sight testosterone itself would be the steroid of choice as it simultaneously suppresses the gonadotropins and maintains androgenicity. However, studies showed that by administration of testosterone alone azoospermia could only be achieved in two-thirds of Caucasian men, so that another gonadotropin-suppressing agent must be added to interrupt spermatogenesis as completely as possible. GnRH analogs and several different steroid combinations and delivery systems such as oral, transdermal, subcutaneous, and intramuscular have been examined. Each has its respective merits and drawbacks (Fig. 1b, c).

Table 1 provides an overview of clinical trials for male hormonal contraception based on steroids. At the outset of this summary it should be noted that in all these many clinical trials only very few untoward side effects were reported, including mild acne and moderate weight gain as a more frequent symptom, the latter due to the anabolic effect of testosterone. Hardly any serious adverse events occurred. A recently performed placebo-controlled clinical trial for male contraception demonstrated that several symptoms previously ascribed to the steroid regimen used also occured in the placebo group (Mommers et al. 2008). Hardly any serious adverse events were registered in this multitude of trials. In all studies, sperm counts returned to normal levels as also a review of major studies revealed (Liu et al. 2006) so that one of the prime goals of male hormonal contraception, i.e., reversibility is met. However, long-term studies extending over three or more years have not yet been performed.

Incidentally, it is worth mentioning that results from animal studies have contributed little to the development of male hormonal contraception, except in the case of GnRH analogs using nonhuman primates. On the contrary, humans have provided models for fertility control in wildlife (Barfield et al. 2006).

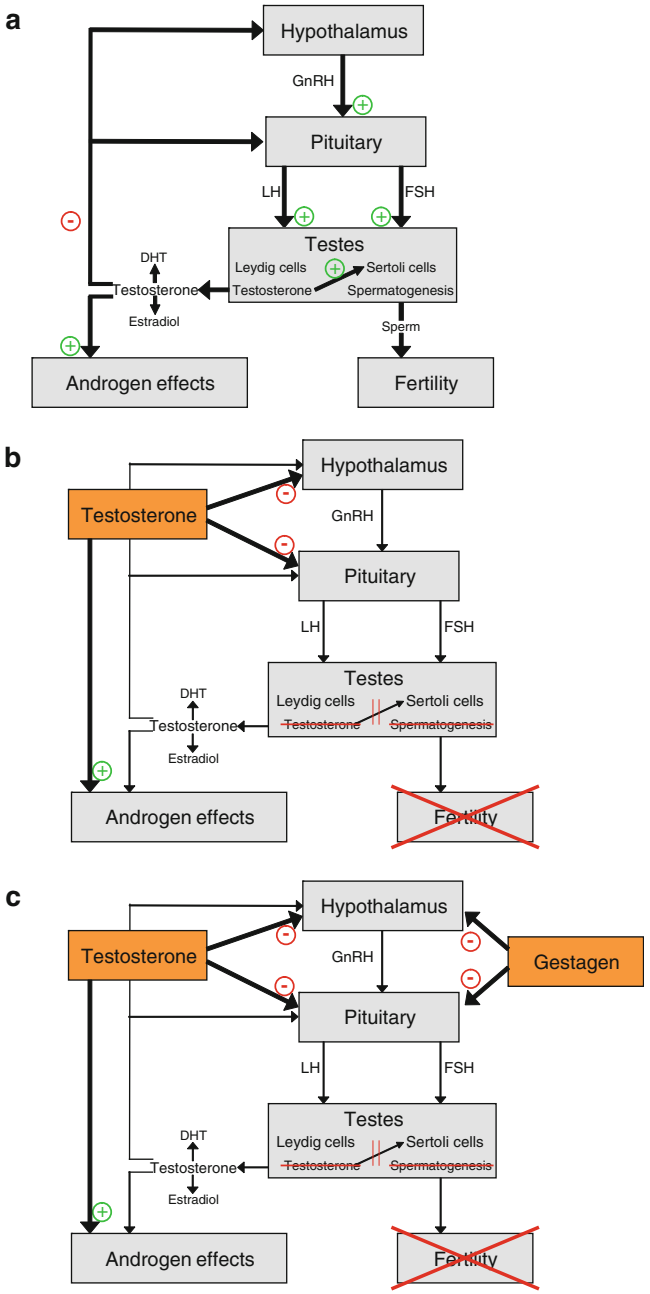

Fig. 1 Schematic representation of the endocrine mechanism controlling testicular function (**a**). (**b**) Shows the principle of hormonal male contraception using testosterone. (**c**) Shows the principle of hormonal male contraception using testosterone plus a gestagen (from Nieschlag and Behre 2010)

Table 1 Overview of studies on hormonal male contraception using either testosterone alone or in combination with progestins (updated from Kamischke and Nieschlag 2004)

References	Number of subjects	Ethnic origin	Androgen dose	Progestin dose	Azoospermia (n)	Severe oligozoospermia below 1 Mio/ml (n)	Oligozoospermia below 3 Mio/ml (n)
Testosterone alone							
WHO 1990	271	Mixed	TE 200 mg i.m./week	None	157	??	??
Behre et al. 1995	8	Caucasian	TB 1,000 mg i.m. one	None	3	–	–
Handelsman et al. 1992	9	Unknown	T-Pellets 1,200 mg	None	5	4	0
Handelsman et al. 1996	10	Unknown	T-Pellets 400 mg	None	0	0	0
Handelsman et al. 1996	10	Unknown	T-Pellets 800 mg	None	4	0	0
Meriggiola et al. 1996	5	Caucasian	TE 100 mg i.m./week	None	5	0	0
Bebb et al. 1996	18	Caucasian	TE 100 mg i.m./week	None	6	4	1
WHO 1996	225	Mixed	TE 200 mg i.m./week	None	157	29	8
Zhang et al. 1999	12	Chinese	TU* 500 mg i.m./4 weeks	None	11	1	0
Zhang et al. 1999	12	Chinese	TU* 1,000 mg i.m./4 weeks	None	12	0	0
Kamischke et al. 2000	14	Caucasian	TU 1,000 mg i.m./6 weeks	None	7	4	1
McLachlan et al. 2002	5	Not stated	TE 200 mg i.m./week	None	4 $\leq$ 0,1 or azoospermia	4	0
von Eckardstein et al. 2003	35	Caucasian	MENT implants, 3 doses	None	10	–	3
Gu et al. 2003	305	Chinese	TU* 500 mg i.m./4 weeks	None	284	6	6
Gu et al. 2009	898	Chinese	TU* 500 mg i.m./4 weeks	None	855	–	43
Depot medroxyprogesterone acetate (DMPA)							
Alvarez-Sanchez et al. 1977	8	Dominican Republic	TE 250 mg i.m./week	DMPA 150 mg/4 weeks	4	3	1
Alvarez-Sanchez et al. 1977	10	Dominican Republic	TE 250 mg i.m./week	DMPA 300 mg/4 weeks	7	2	0
Brenner et al. 1977	6	Caucasian	TE 200 mg i.m./week	DMPA 100 mg/4 weeks	1	2	1
Brenner et al. 1977	3	Caucasian	TE 200 mg i.m./week	DMPA 150 mg/4 weeks	1	0	0

Frick et al. 1977	12	Caucasian	TE 250 mg i.m./week	DMPA 100 mg i.m./4 weeks	6	4	0
Frick et al. 1977	6	Caucasian	T-Propionate 4 rods	DMPA 100 mg i.m./4 weeks	2	0	0
Melo and Coutinho 1977	11	Brasilian	TE 200 mg i.m./week	DMPA 100–150 mg i.m/ 4 weeks	11 $\leq$ 0,1 or azoospermia	0	???
Faundes et al. 1981	10	Dominican Republic	TE 500 mg i.m./week	DMPA 150 mg/4 weeks	8	1	0
Frick et al. 1982	4	Caucasian	TE 500 mg/4 weeks	150 mg/4 weeks	4	0	0
Frick et al. 1982	5	Caucasian	TE 250 mg/2 weeks	75 mg/2 weeks	5	0	0
WHO 1993	45	Indonesian	19-Nortestosterone 200 mg i.m./3 weeks	DMPA 250 mg i.m./6 weeks	44	1	0
WHO 1993	45	Indonesian	TE 200 mg i.m./3 weeks	DMPA 250 mg i.m./6 weeks	43	2	0
Knuth et al. 1989	12	Caucasian	19-Nortestosterone 200 mg i.m./3 weeks	DMPA 250 mg i.m./6 weeks	6	4	2
Wu and Aitken 1989	10	Caucasian	TE 250 mg i.m./week	DMPA 200 mg/4 weeks	6	0	4
Pangkahila 1991	10	Indonesian	TE 100 mg i.m./week	DMPA 100 mg/4 weeks	10	0	0
Pangkahila 1991	10	Indonesian	TE 250 mg i.m./week	DMPA 200 mg/4 weeks	10	0	0
Handelsman et al. 1996	10	Not stated	T-Pellets 800 mg	DMPA once 300 mg i. m.	9	0	1
McLachlan et al. 2002	5	Not stated	TE 200 mg i.m./week	DMPA once 300 mg i. m.	5 $\leq$ 0,1 or azoospermia	5	0
Turner et al. 2003	53	Unknown	T-Pellets 800 mg/16 weeks	DMPA 300 mg i.m./12 weeks	49	2	0
Gu et al. 2004	30	Chinese	TU*1,000 mg/4 weeks	DMPA 150 or 300 mg/ 8 weeks	28	1	1
Page et al. 2006	38	Not stated	T gel 100 mg/day	DMPA 300 mg/12 weeks $\pm$GnRH antagonist	31	2	3

(*continued*)

Table 1 (continued)

References	Number of subjects	Ethnic origin	Androgen dose	Progestin dose	Azoospermia (n)	Severe oligozoo-spermia below 1 Mio/ml (n)	Oligozoo-spermia below 3 Mio/ml (n)
Levonorgestrel (LNG)							
Fogh et al. 1980	5	Caucasian	TE 200 mg/4 weeks	LNG 250 µg p.o./day	1	?	1
Fogh et al. 1980	5	Caucasian	TE 200 mg i.m./4 weeks	LNG 500 µg p.o./day	2	?	?
Bebb et al. 1996	18	Caucasian	TE 100 mg i.m./week	LNG 500 µg p.o./day	12	2	3
Anawalt et al. 1999	18	Caucasian	TE 100 mg i.m./week	LNG 125 µg p.o./day	11	5	1
Anawalt et al. 1999	18	Caucasian	TE 100 mg i.m./week	LNG 250 µg p.o./day	14	2	0
Ersheng et al. 1999	16	Chinese	TU 250 mg i.m./4 weeks	Sino-Implant 2 rods	6	0	1
Kamischke et al. 2000	14	Caucasian	TU 1,000 mg i.m./6 weeks	LNG 250 µg p.o./day	8	4	2
Gaw Gonzalo et al. 2002	20	Mixed	Testoderm TTS 2 patches/ day	Norplant II 4 rods	7	5	2
Gaw Gonzalo et al. 2002	15	Mixed	Testoderm TTS 2 patches/ day	LNG 125 µg p.o./day	5	1	1
Gaw Gonzalo et al. 2002	14	Mixed	TE 100 mg i.m./week	Norplant II 4 rods	13	1	0
Pöllänen et al. 2001	5	Caucasian	DHT-Gel 250 mg/day	LNG 30 µg p.o./day	0	0	1
Pöllänen et al. 2001	5	Caucasian	DHT-Gel 250 mg/day	Jardelle (LNG) 1 rod	0	0	0
Pöllänen et al. 2001	8	Caucasian	DHT-Gel 500 mg/day	Jardelle (LNG) 2 rods	0	0	0
Pöllänen et al. 2001	7	Caucasian	DHT-Gel 250 mg/day	Jardelle (LNG) 4 rods	0	0	0
Gui et al. 2004	41	Chinese	TU* 500 or 1,000 mg/ 8 weeks	LNG 4 implants	31	5	4
Anawalt et al. 2005	41	Mixed	TE 100 mg/week	LNG 31 µg or 62 µg/day	25	13	2
Wang et al. 2006	19 21	Caucasian Chinese	T implants/15–18 weeks	LNG 4 implants	13 19	–	–
Wang et al. 2007	18	Chinese	TU* 500 mg/6 weeks	LNG 250 mg/p.o./day	17	–	1
Norethisterone enanthate (NETE)							
Kamischke et al. 2001	14	Caucasian	TU 1,000 mg i.m./6 weeks	NETE 200 mg/6 weeks	13	0	0
Kamischke et al. 2002	14	Caucasian	TU 1,000 mg i.m./6 weeks	NETE 200 mg/6 weeks	13	1	0

Kamischke et al. 2002	14	Caucasian	TU 1,000 mg i.m./6 weeks	NETE 400 mg/6 weeks	13	1	0
Kamischke et al. 2002	14	Caucasian	TU 1,000 mg i.m./6 weeks	NETA 10 mg p.o./day	12	2	0
Meriggiola et al. 2005	10	Caucasian	TU 1,000 mg/8 weeks	NETE 200 mg/6 weeks	9		
	8	Caucasian	TU 1,000 mg/12 weeks	NETE 200 mg/12 weeks	3		
Qoubaitary et al. 2006	10	Mixed	TU 750 mg/8 weeks	NETE 250 mg/8 weeks	5	2	1
	10	Mixed	TU 1,000 mg/8 weeks	NETE 250 mg/8 weeks	10	–	–
Cyproterone acetate							
Meriggiola et al. 1996	5	Caucasian	TE 100 mg i.m./week	CPA 50 mg p.o./day	3	0	1
Meriggiola et al. 1996	5	Caucasian	TE 100 mg i.m./week	CPA 100 mg p.o./day	5	0	0
Meriggiola et al. 1998	5	Caucasian	TE 100 mg i.m./week	CPA 12.5 mg p.o./day	3	2	0
Meriggiola et al. 1998	5	Caucasian	TE 100 mg i.m./week	CPA 25 mg p.o./day	5	0	0
Meriggiola et al. 2002	9	Caucasian	TE 100 mg i.m./week	CPA 5 mg p.o./day	6	3	0
Meriggiola et al. 2002	7	Caucasian	TE 200 mg i.m./week	CPA 5 mg p.o./day	0	4	2
Meriggiola et al. 2003	24	Caucasian	TU 1,000 mg/6 weeks	CPA 20 and 2 mg/p.o./day	13	11	–
Desogestrel (DSG) or etonorgestrel							
Wu et al. 1999	8	Caucasian	TE 50 mg i.m./week	DSG 300 μg p.o./day	8	0	0
Wu et al. 1999	7	Caucasian	TE 100 mg i.m./week	DSG 150 μg p.o./day	4	3	0
Wu et al. 1999	8	Caucasian	TE 100 mg i.m./week	DSG 300 μg p.o./day	6	0	1
Anawalt et al. 2000	7	Caucasian	TE 50 mg i.m./week	DSG 150 μg p.o./day	4	1	0
Anawalt et al. 2000	8	Caucasian	TE 100 mg i.m./week	DSG 150 μg p.o./day	8	0	0
Anawalt et al. 2000	8	Caucasian	TE 100 mg i.m./week	DSG 300 μg p.o./day	7	1	0
Kinniburgh et al. 2001	8	Caucasian	T-Pellets 400 mg/12 weeks	DSG 150 μg p.o./day	6	2	0
Kinniburgh et al. 2001	7	Caucasian	T-Pellets 400 mg/12 weeks	DSG 150 μg p.o./day	5	1	0
Anderson et al. 2002b	9	Black	T-Pellets 400 mg/12 weeks	DSG 150 μg p.o./day	9	0	0
Anderson et al. 2002b	11	Mixed	T-Pellets 400 mg/12 weeks	DSG 150 μg p.o./day	9	0	1
Anderson et al. 2002b	8	Black	T-Pellets 400 mg/12 weeks	DSG 300 μg p.o./day	8	0	0
Anderson et al. 2002b	12	Mixed	T-Pellets 400 mg/12 weeks	DSG 300 μg p.o./day	8	0	0
Anderson et al. 2002a	14	Caucasian	T-Pellets 400 mg/12 weeks	Implanon (ENG) 1 rod	9	1	3
Anderson et al. 2002a	14	Caucasian	T-Pellets 400 mg/12 weeks	Implanon (ENG) 2 rods	9	4	0
Kinniburgh et al. 2002	15	Caucasian	T-Pellets 400 mg/12 weeks	DSG 300 μg p.o./day	15	0	0
Kinniburgh et al. 2002	18	Chinese	T-Pellets 400 mg/12 weeks	DSG 300 μg p.o./day	18	0	0

(*continued*)

Table 1 (continued)

References	Number of subjects	Ethnic origin	Androgen dose	Progestin dose	Azoospermia (n)	Severe oligozoo-spermia below 1 Mio/ml (n)	Oligozoo-spermia below 3 Mio/ml (n)
Kinniburgh et al. 2002	18	Chinese	T-Pellets 400 mg/12 weeks	DSG 150 μg p.o./day	11	2	2
Kinniburgh et al. 2002	13	Caucasian	T-Pellets 400 mg/12 weeks	DSG 150 μg p.o./day	11	2	0
Brady et al. 2004	9	Not stated	T pellets 400 mg/12 weeks	Etonogestrel implants	9	2	–
Walton et al. 2007	16	Caucasian	T pellets 600 mg/12 weeks	Etonogestrel implants	11	2	–
	10	Caucasian	MENT implants	Etonogestrel implants	3	5	–
Mommers et al. 2008	134	Caucasian	TU 750 mg/12 weeks	Etonogestrel implants high dose	–	≈125	–
	112	Caucasian	TU 750 mg/10 weeks TU 1,000 mg/12 weeks	Etonogestrel implants low dose	–	≈100	–
Self applicable							
Nieschlag et al. 1978	7	Caucasian	Andriol 240 mg p.o./day	None	1	0	0
Guerin and Rollet. 1988	13	Caucasian	Andriol 160 mg p.o./day	NETA 10 mg p.o./day	7	2	3
Guerin and Rollet. 1988	5	Caucasian	T gel 250 mg/day	NETA 5 mg p.o./day	4	1	0
Guerin and Rollet. 1988	5	Caucasian	T gel 250 mg/day	NETA 10 mg p.o./day	5	0	0
Guerin and Rollet. 1988	8	Caucasian	T gel 250 mg/day	MPA 20 mg p.o./day	5	0	1
Meriggiola et al. 1997	8	Caucasian	Andriol 80 mg p.o./day	CPA 12.5 mg p.o./day	1	3	2
Hair et al. 1999	4	Caucasian	Andropatch 2 patches/day	DSG 75 μg p.o./day	0	1	0
Hair et al. 1999	6	Caucasian	Andropatch 2 patches/day	DSG 150 μg p.o./day	3	0	0
Hair et al. 1999	7	Caucasian	Andropatch 2 patches/day	DSG 300 μg p.o./day	4	1	0
Büchter et al. 1999	12	Caucasian	Testoderm TTS 2 patches/day	LNG 250 μg p.o. later 500 μg	2	3	0
Gaw Gonzalo et al. 2002	19	Mixed	Testoderm TTS 2 patches/day	None	5	0	1
Pöllänen et al. 2001	2	Caucasian	DHT-Gel 250 mg/day	None	0	0	0

TE testosterone enanthate, *TB* testosterone buciclate, *TU** testosterone undecanoate in tea seed oil, *TU* testosterone undecanoate in castor oil, *MENT* 7α-methyl-19 nortestosterone

3 Clinical Trials to Date

3.1 Androgens Alone

3.1.1 Testosterone Enanthate

Soon after testosterone was synthesized and became available for clinical use in the late 1930s, its spermatogenesis-suppressing effect was recognized (Heckel 1939), but not until the 1970s did investigations start to exploit this phenomenon for male contraception. As in most hormonal male contraceptive studies to date, in the early studies sperm concentrations and counts were used as surrogate parameters for efficacy.

The *first efficacy study* of testosterone-based hormonal male contraception was sponsored by the WHO (1990) and included ten centers on four continents. Healthy fertile participants were given 200 mg of the longer-acting testosterone enanthate weekly by intramuscular injection. One hundred and fifty-seven men (70%) reached azoospermia after 6 months of treatment and entered the efficacy phase for a further year, during which no other contraceptive was used by the couple. Only one pregnancy was reported in this first proof-of-principle study. Although the efficacy of this study was very high, it cannot be used to determine the overall efficacy of testosterone alone as a contraceptive because only men who became azoospermic could enter the efficacy phase while the others were excluded.

In order to clarify the question whether men developing oligozoospermia can be considered infertile, a *second worldwide multicenter efficacy study* involving 357 couples followed (WHO 1996). In this study, azoospermia again proved to be a most effective prerequisite for contraception. If sperm concentration, however, failed to drop below 3 Mio/ml ejaculate, resulting pregnancy rates were higher than when using condoms. When sperm concentrations decreased below 3 Mio/ml, which was the case in 98% of the participants, then protection was not as effective as for azoospermic men, but was better than that offered by condoms (Fig. 2).

Even if these WHO studies represented a breakthrough by confirming the principle of action, they did not offer a practicable method because a method requiring weekly i.m. injections is not acceptable for broad use. Moreover, several months, often up to 1 year, are required before sperm production reaches significant suppression. For this reason, current research is concentrating on the development of long-acting testosterone preparations and on methods to hasten the onset of effectiveness.

3.1.2 Testosterone Buciclate

As long-acting testosterone preparations appeared more promising in terms of practicability and acceptability, WHO and the NIH initiated a synthesis program for such preparations (Waites 2003), through which the long-acting testosterone

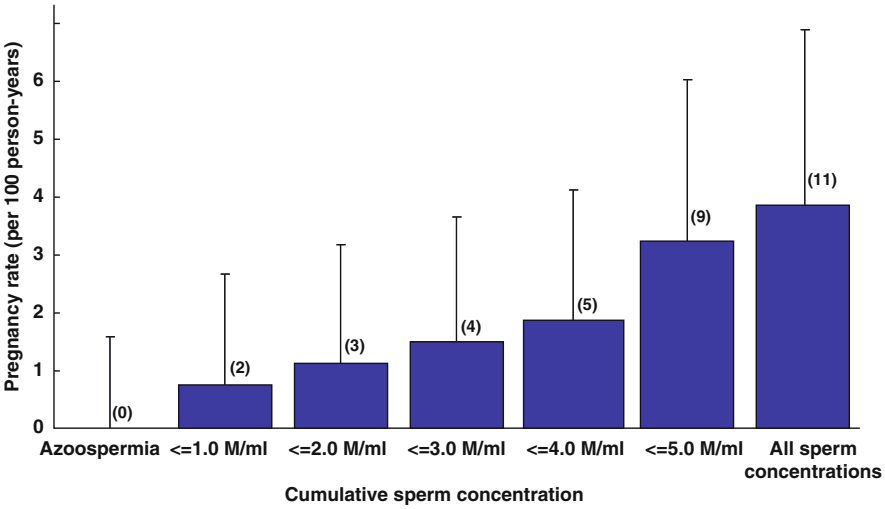

Fig. 2 Contraceptive efficacy of testosterone enanthate (250 mg/weekly) in 364 volunteers: pregnancy rates per 100 person/years in relation to sperm concentration (WHO 1996)

ester *testosterone buciclate* was identified. This molecule showed a half-life of 29.5 days when tested in hypogonadal men, much longer than the 4.5 days of testosterone enanthate (Behre et al. 2004). Suppression of spermatogenesis was comparable to that of weekly testosterone enanthate injections, reaching azoospermia in three out of eight volunteers after a single injection of 1,200 mg of testosterone buciclate (Behre et al. 1995). Despite its promising pharmacokinetic profile, no industrial partner could be found to undertake development of this preparation.

3.1.3 Testosterone Undecanoate

Initially, *testosterone undecanoate* was studied *as an oral preparation* in volunteers of Caucasian origin (Nieschlag et al. 1978). Subjects were given a daily dose of 240 mg over a period of 12 weeks, but only one out of seven volunteers reduced sperm output sufficiently for contraception. This low effectiveness is probably due to the short half-life of testosterone undecanoate when given orally. Even if administered four times a day, the peaks are not sufficient to suppress gonadotropins consistently and thereby to achieve azoospermia.

While testosterone undecanoate had been developed as an oral preparation in Europe it was turned into an *injection in China*, using *tea seed oil* as a vehicle and is used as such in China for hypogonadism and in trials for male contraception. Back in Europe, the half-life of this Chinese preparation could be extended even further when dissolved in *castor oil* and is now available for clinical use in 1,000 mg depot injections (Nieschlag 2006).

In the clinical trials in China, testosterone undecanoate alone administered every 4 weeks resulted in azoospermia in all Chinese men who received a dose of 1,000 mg and in azoospermia or severe oligozoospermia in 95% of Chinese men who received a dose of 500 mg during a 4–6-month suppression phase (Zhang et al. 1999). In the ensuing *Phase III study* involving 305 couples an efficacy phase followed the suppression phase and no pregnancies were initiated by men exhibiting azoospermia or severe oligozoospermia (Gu et al. 2003). However, reappearance of sperm occurred in six men during the efficacy phase; one pregnancy was attributed to "sperm rebound." Side effects observed in subjects were all typical of elevated testosterone serum levels. The largest efficacy study to date was also performed in China, based on a loading dose of 1,000 mg followed by monthly injections of 500 mg testosterone undecanoate. 898 men entered the efficacy phase during which only 9 pregnancies were recorded. This represents a pregnancy rate of 1.1/100 person years (Gu et al. 2009). *Thus, in China testosterone undecanoate provides better protection against pregnancy than condom use*. Although injection intervals of 4 weeks appeared to be an achievement over the weekly injections of testosterone enanthate, the participants in a Chinese study considered the frequency of injections the most inconvenient part of this regimen (Zhang et al. 2006). Would testosterone undecanoate in castor oil also be used in China, this complaint could certainly be overcome.

In a first contraceptive trial of testosterone undecanoate in castor oil 1,000 mg were injected into 14 Caucasian volunteers at 6-week intervals. 8/14 men achieved azoospermia (Kamischke et al. 2000). Although this rate of azoospermia is not different from that achieved with testosterone enanthate alone, the longer injection interval represents a significant advantage. A later pharmacokinetic study concluded that 8-week intervals of 1,000 mg injections would be sufficient for contraceptive purposes (Qoubaitary et al. 2006).

Considering that 10–14-week intervals of 1,000 mg testosterone undecanoate are required for substitution of hypogonadal men, about 1/3 more testosterone is required for contraception in normal volunteers.

3.1.4 Testosterone Pellets

Pellets consisting of pure testosterone are used for substitution in hypogonadism in some countries. In male contraceptive studies, the sperm-suppressing effect was comparable to weekly testosterone enanthate injections (McLachlan et al. 2000). The disadvantage of minor surgery required for insertion under the abdominal skin is compensated for by their low price. Spontaneous extrusion may be a disadvantage.

3.1.5 19-Nortestosterone

When searching for preparations with longer lasting effectiveness *19-nortestosterone-hexoxyphenylpropionate* was tested whose spectrum of effects is very similar to

that of testosterone and which had been used as an anabolic steroid since the 1960s. The 19-nortestosterone ester injected every 3 weeks enabled azoospermia to be reached by as many men as by testosterone enanthate. Thus, the 19-nortestosterone ester is as effective as testosterone enanthate but allows a longer interval between injections (Knuth et al. 1985).

Although effective in suppressing spermatogenesis and without any notable side effects in the studies, it could not be determined whether this synthetic androgen would have any unwanted effects under long-term use. The lack of negative reports from widespread use of 19-nortestosterone in athletics cannot be taken as evidence for its clinical application as systematic evaluations in athletes have not been published.

3.1.6 7α-Methyl-19-Nortestosterone (MENT)

The synthetic androgen 7α-methyl-19-nortestosterone (MENT) offers an approximately tenfold higher potency to suppress pituitary gonadotropins than does testosterone. In contrast to testosterone there is no 5α-reduction so that effects on the prostate could be minimal. A first dose-finding study showed that MENT administered in subcutaneous implants was as effective as testosterone given alone (von Eckardstein et al. 2003). The potential of these implants either alone or in combination with gestagen implants is currently being investigated by the Population Council.

3.2 Androgens Combined with GnRH Analogs

3.2.1 GnRH Agonists

The pituitary-inhibiting effects of *GnRH agonists* are well known from their use in females and in the therapy of prostate cancer. After an initial phase of gonadotropin stimulation, they suppress gonadotropins and, consequentially, intratesticular testosterone by GnRH receptor down regulation. However, trials for hormonal male contraception in which mostly testosterone was added showed that sperm numbers were only insufficiently reduced, thus rendering these agonists unsuitable as male contraceptives (Behre et al. 1992).

3.2.2 GnRH Antagonists

GnRH antagonists lack the effect of initial gonadotropin release as they competitively inhibit pituitary GnRH receptors, thus leading to a more immediate onset of azoospermia. This could be demonstrated by small clinical studies using various GnRH antagonists in addition to a testosterone preparation (summarized in Nieschlag et al. 2004). Out of 47 volunteers participating in various clinical trials with different GnRH-antagonists, azoospermia was achieved in 39 subjects and oligozoospermia (<1 Mio sperm per ml ejaculate) occurred in one further volunteer,

while only three men maintained sperm concentrations above 3 Mio/ml ejaculate. Of the more recently developed GnRH antagonists, Acycline has been tested in male contraceptive trials. Although Acycline given alone had a potent gonadotropin-suppressing effect (Page et al. 2008), the addition of Acycline to a combination of testosterone gel plus DMPA did not increase the suppression of sperm production achieved by steroids alone (Page et al. 2006).

Despite these encouraging results, the requirement for daily or weekly injections and the high costs of the available preparations have hindered the further development of GnRH antagonists for hormonal male contraception. Promising attempts to use the GnRH antagonists only to initiate azoospermia and then maintain this by androgens alone were not pursued further (Swerdloff et al. 1998; Behre et al. 2001).

3.3 Androgens Plus Gestagens

The potency of gestagens to suppress gonadotropins is well known from female contraceptives where gestagens effectively supplement estrogens. Numerous studies combining androgens (mainly testosterone) with various gestagens have been performed over the past four decades to identify a regimen suited for male contraception (Fig. 3). Unfortunately, a systematic comparison of the different gestagens with regard to their contraceptive potency in males has never been performed. Even worse, a Cochrane Review analyzing 45 clinical trials came to the conclusion that the studies comprised too small numbers of volunteers so that significant differences between the various steroid combinations could not be detected (Grimes et al. 2007). Moreover, not all studies observe strict criteria for randomized controlled trials. However, it should be kept in mind that many of these trials were performed as proof of principle and not necessarily as trials for registration with the regulatory authorities. In addition, single centers are financially and logistically unable to cope with the numbers of volunteers and criteria demanded by regulatory agencies. Stimulated by researchers and by public demand, the pharmaceutical industry finally performed a trial fulfilling the Cochrane criteria – and left the field! (Mommers et al. 2008). In the following, important studies are briefly summarized to highlight the cumbersome and often frustrating pathway of development (Table 1).

The gestagens used in these studies derive either from *19-nortestosterone* or from *17-hydroxyprogesterone* and are all being used in female contraceptives (Fig. 3).

3.3.1 Depot Medoxyprogesterone Acetate (DMPA)

From early studies in the 1970s initiated by the WHO and the Population Council, DMPA emerged as a gestagen with great potential in male contraception (Barfield et al. 1979). The combination of DMPA with 19-nortestosterone in 3-week intervals first tested in Caucasians (Knuth et al. 1989) was especially promising in Indonesian men (WHO 1993).

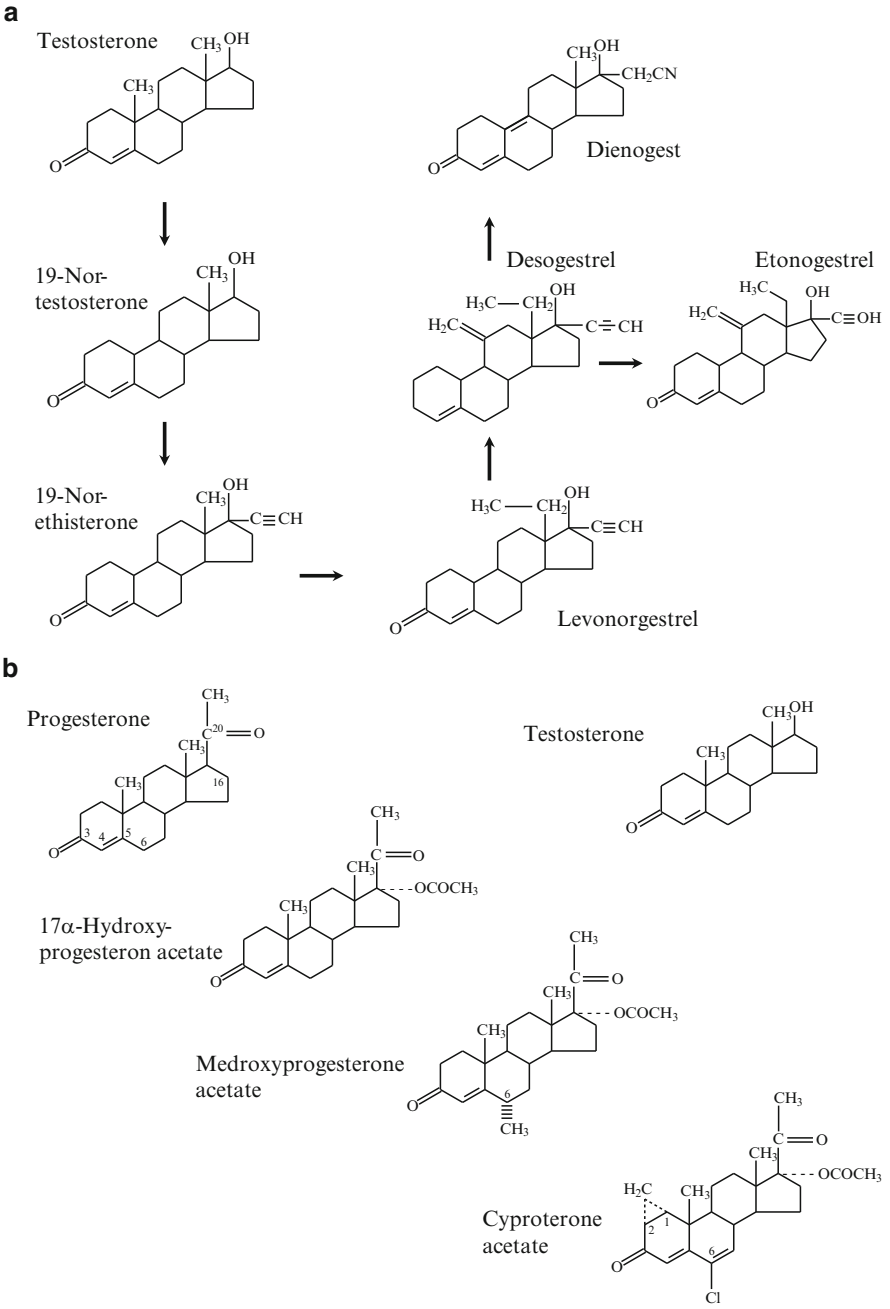

Fig. 3 Gestagens derived from either testosterone (**a**) or progesterone (**b**) in trials for male hormonal contraception

One of the very few efficacy studies aiming at pregnancy rates also used DMPA, however, in combination with testosterone pellets (Turner et al. 2003). In this Australian study, 53/55 volunteers suppressed to azoospermia and during the 1-year efficacy phase no pregnancy occurred. However, the discontinuation rate in this study was high and onset of and recovery from azoospermia took several months.

In order to test whether one of the two steroid entities could be self-administered, the addition of a testosterone transdermal gel to the DMPA injections (300 mg/3 months) was tested (Page et al. 2006). The results were comparable to those from trials where DMPA was combined with injectable testosterone.

3.3.2 Levonorgestrel

Oral levonorgestrel, when combined with testosterone enanthate i.m. slightly enhanced the effect of testosterone enanthate alone (Bebb et al. 1996). Similarly, when combined with testosterone undecanoate i.m. the additional effect of oral levonorgestrel remained marginal in Caucasian men (Kamischke et al. 2000), but seemed to increase effectiveness in Chinese men (Wang et al. 2007).

In a comparative study, when *levonorgestrel implants* were combined with testosterone pellets, an additive effect of levonorgestrel was seen in Caucasian men, but not in Chinese men who responded equally well to testosterone pellets alone (Wang et al. 2006).

When MENT implants were combined with levonorgestrel implants in different doses, a clear dose-dependent effect could be observed, but it remains undetermined whether implants with sufficiently long duration can be manufactured; nonbiodegradable implants that have to be removed surgically from the implantation site when contraceptive protection is no longer required appear impractical for widespread use unless they can be left in situ for long periods (Wang et al. in preparation).

3.3.3 Norethisterone

The injectable depot preparation *norethisterone enanthate (NETE)* and the orally effective *norethisterone acetate (NETA)* are hydrolysed to release the active compound *norethisterone*, which can be 5α-reduced to 5α-norethisterone and aromatized to ethinyl estradiol. While norethisterone has strong androgenic activity (~10% of testosterone), 5α-noresthisterone also shows antiandrogenic properties.

13/14 men who received 200 mg NETE combined with 1,000 mg testosterone undecanoate every 6 weeks achieved azoospermia (Kamischke et al. 2001). Further investigations showed that the injection intervals could be extended to 8 weeks (Meriggiola et al. 2005) or that testosterone undecanoate could be combined with oral NETA without loss of effectiveness (Kamischke et al. 2002). Based on these findings, together with CONRAD WHO is planning a Phase II efficacy study involving 400 couples in eight centers worldwide (WHO 2005).

3.3.4 Cyproterone Acetate

The orally effective *antiandrogen cyproterone acetate (CPA)* has strong gestagenic properties. In early studies combining oral CPA with testosterone enanthate injections, the sperm-suppressing effects were considerable, but the antiandrogenic effects (e.g., reflected by decreased hematocrit) were undesirable. However, when combining 1,000 mg testosterone undecanoate every 6 weeks with 20 mg CPA daily initially, followed by only 2 mg CPA/day, the initial suppression of spermatogenesis could be maintained and antiandrogenic effects prevented (Meriggiola et al. 2003).

3.3.5 Desogestrel and Etonogestrel

Desogestrel is an orally effective gestagen which becomes active after conversion to *etonogestrel*. Etonogestrel can be administered directly as an implant (Implanon®). In combination with testosterone enanthate or testosterone pellets desogestrel showed good suppression of spermatogenesis (Wu et al. 1999; Kinniburgh et al. 2001).

Etonogestrel implants combined with testosterone pellets s.c. resulted in a high azoospermia rate, although it took up to 28 weeks to reach this goal in individuals (Brady et al. 2004).

In the first (and so far last) *industry-sponsored trial*, Organon and Schering decided to test etonogestrel implants with testosterone undecanoate injections in various combinations (Mommers et al. 2008). This study involved 354 volunteers in seven treatment groups receiving either placebo or 750–1,000 mg testosterone undecanoate every 10–12 weeks with two doses of etonogestrel for 42–44 weeks. 90% of treated men suppressed spermatogenesis to $\leq$1 Mio/ml ejaculate (Fig. 4). Although the combination of an implant with injections may not appear too attractive for practical use, the study had a high success rate and could have formed the basis for a Phase III efficacy study. Unfortunately, both companies discontinued their male contraception programs when they were taken over by other firms who were at that stage not interested in male contraception.

3.4 Differences Between Responders and Nonresponders

As outlined above, testosterone alone would seem to be the ideal male contraceptive as it suppresses pituitary gonadotropin secretion while at the same time it replaces all extratesticular functions of testosterone to maintain androgenicity. However, only about two-thirds of Caucasian men and over 90% of Chinese men react to testosterone with azoospermia. The rest requires an additional agent to achieve this goal. Not only the degree, but also the dynamics of suppression of spermatogenesis show great interindividual variations. Investigators are puzzled by this heterogeneous response, but to date could not find a uniform explanation for this variability.

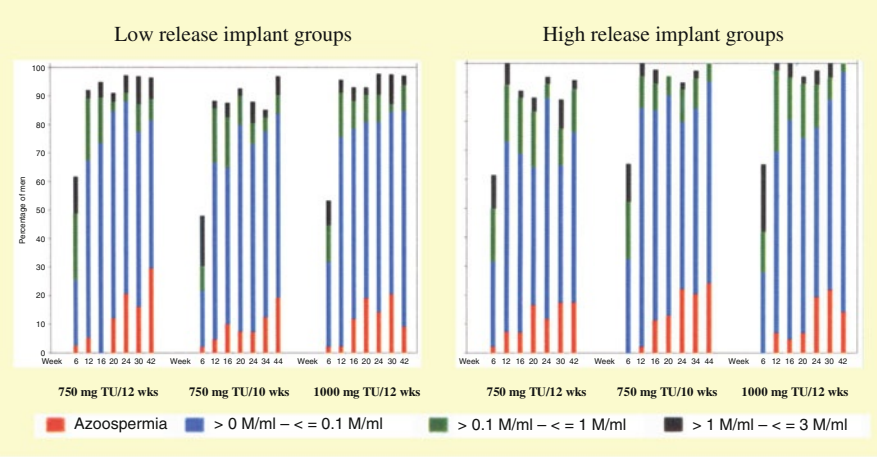

Fig. 4 Percentage of 333 men with sperm concentration below or equal to different cut of levels, participating in a double-blind, randomized, placebo-controlled multicentre trial using various combinations of etonogestrel implants and testosterone undecanoate injections (from Mommers et al. 2008)

As a result of the Summit Meetings on Hormonal Male Contraception (1997–2009), the participating investigators provided data from their various clinical trials for a joint analysis. Results from 30 trials on male hormonal contraception performed between 1990 and 2006 were combined, comprising a total of 1,756 volunteers, 18–51 years old, representing 85% of all published data (Liu et al. 2008). The analysis showed that younger age as well as lower serum testosterone levels and sperm concentrations lead to faster sperm suppression. Statistically, the differences only became evident in this large data set and showed little predictive value for the individual case so that no clear practical consequences for the identification of good or bad responders resulted.

Earlier, in an analysis of 85 Caucasian volunteers from several single-centre contraceptive trials von Eckardstein et al. (2002) had found that the rate of suppression of spermatogenesis correlated positively with the rate of suppression of gonadotropins. While this confirmed findings from several other clinical trials, the investigators additionally found that the suppression of spermatogenesis in those volunteers with incomplete gonadotropin suppression correlated with the number of CAG repeats in the androgen receptor, i.e., the longer the CAG repeats, the better the sperm suppression, despite incomplete gonadotropin suppression. Li and Gu (2008) specified that in Chinese volunteers with incomplete suppression of FSH, the chances of reaching azoospermia were higher in those volunteers with more than 22 CAG repeats. The androgen receptor polymorphism requires further investigation before it can be considered a clear predictor for responders and nonresponders.

In early clinical trials for male contraception, analysis of anthropometric characteristics failed to contribute to solving the responder:nonresponder problem. However, recently the importance of body fat for the suppressibility of gonadotropins has been emphasized (Kornmann et al. 2009). When 40 healthy volunteers receiving equal doses of testosterone undecanoate intramuscularly were divided into groups according to their suppression of gonadotropins, those with consistent suppression had the lowest body fat content (10.3 ± 1.5 kg) and those with no suppression had the highest fat content (23.2 ± 6.4 kg). As suppression of gonadotropins is a prerequisite for suppression of spermatogenesis, more attention should be paid to the individual phenotypes; perhaps the dosing of contraceptive steroids needs to be adjusted to individual requirements. The influence of body fat may also explain the differences in response to suppression of spermatogenesis between Chinese and Caucasian men, as the former are lighter and have less fat than the latter.

4 Acceptability of Male Contraception

One of the reasons why the pharmaceutical industry has not continued to further develop a male contraceptive at this stage may be doubts about the possible acceptability of such a pharmacological method. However, recently public interest in male methods for contraception has notably grown. It is increasingly expected that men share with their partners not only the advantages but also the risks of family planning. As risks tend to increase with duration of use, sharing contraception between men and women would reduce dangers for each partner. Population conferences and women's world forums have explicitly called for new male contraceptive methods.

Worldwide *one quarter of all couples practicing contraception rely on male methods*, albeit with varying preferences and the proportion of men practicing contraception is increasing. Thus, in the Netherlands the percentage of vasectomized men whose wives were of reproductive age rose from 2 to 10.5% from 1975 to 2008 and from 8 to 12.2% in the United States; the highest rates of vasectomized men are found in the United Kingdom and in New Zealand. Worldwide, however, only 2.7% of men are vasectomized. Similarly, the use of condoms for contraception varies from country to country with a worldwide average of 5.7%. It is to be expected that the percentage of men willing to practice contraception varies between cultures and with methods available. According to a survey in Hongkong and Shanghai 10 years ago, half the men interviewed were willing to take a daily contraceptive pill; in Edinburgh and Capetown two-thirds were willing to do so (Anderson and Baird 1997; Martin et al. 2000). After almost 50 years of female oral contraception, the attitude of men toward *new* methods of male contraception has changed. Worldwide surveys showed men willing to use pharmacological contraceptive methods (Heinemann et al. 2005) (Fig. 5).

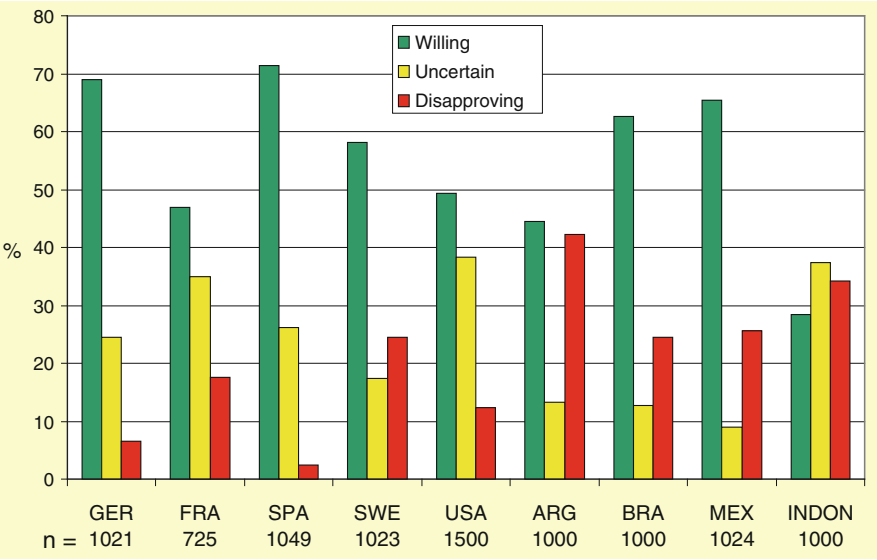

Fig. 5 Multinational male fertility control survey among 9,342 men who were asked the question: "If available, would you be willing to use the new male fertility control method?" (Heinemann et al. 2005)

5 Responsibility for the Development of Contraceptives

The world population has tripled in the last 50 years and is approaching seven billion. Less developed countries bear the onus of this enormous population growth while the population in industrialized nations is largely stable – due to use of contraceptive methods. The population explosion creates hardly surmountable ecologic and economic problems. Medical progress has decisively lowered mortality, particularly of children, so that life expectancy worldwide is currently 64.2 years for men and 68.6 years for women. Ever more people reach reproductive age. If medical progress allows an increasing number of people to achieve reproductive age, causing overpopulation, then medicine must also provide contraceptive methods to maintain or restore a balance between reproduction and death. It has become clear that the Millennium Development Goals cannot be achieved with the current level of population growth (All Party Parliamentary Group 2007). It goes without saying that a newly developed male contraceptive would not suddenly resolve population problems, but a male method could contribute to the resolution, especially as research into female methods for contraception is similarly on the decline (Strauss and Chaudhuri 2007). In addition, women increasingly demand that men share the responsibility and risks of family planning and men, on the other hand, want to regain some of the reproductive power they surrendered to women since the advent of modern female contraceptives (Darroch 2008; Meriggiola et al. 2006).

This should be reason enough for the pharmaceutical industry to actively develop male contraceptives.

While a large proportion of clinical research is driven by the pharmaceutical industry, in the case of male contraception industry fails. Without the long-range perspective and endurance of institutions and organizations such as NICHD, CONRAD, Population Council, WHO, some medical research councils and few foundations, male contraception would long have been abandoned (Nieschlag 2009). The principle and effectiveness of hormonal male contraception has been demonstrated in many studies. The fact that the majority of clinical trials on hormonal male methods, have been published in high-ranking journals emphasizes the high priority the scientific community attributes to these endeavors. Investigators are so convinced of the validity of the concept of hormonal male contraception that they drafted recommendations for regulatory approval for male hormonal contraception at their annual summit meetings (since 1997) (Nieschlag and 10th Summit Meeting Group 2007). Little more is required to convince industry to bring this development to fruition. Comparing the situation with the development of the female pill, the lack of public advocacy for male contraception is striking. Male contraception lacks prominent advocates as the development of female contraception benefited from personalities such as Margaret Sanger (1879–1966) and Katherine McCormick (1875–1967). Hormonal male contraception requires similar advocacy to finally result in a marketable product (Nieschlag 2009).

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Family Planning: Today and in the Future

Michael J.K. Harper

Contents

Abstract This review covers the state of contraceptive development noting new entries in the clinic (mainly steroidal and different delivery methods) and novel leads for nonsteroidal female- and male-methods in the pipeline. The time taken to market and the absence of partnerships with industry are stressed as major factors for the slow progress in their development.

Keywords Contraception · Devices · Drugs · Family Planning · Maternal Reproductive Health · Nonsteroids · NSAIDs · Pipeline Leads · Research and Development · Steroids

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1 Background

1.1 Why Do We Need Them?

In the 1960s, USAID made substantial efforts to increase international family planning programs: these efforts were strongly criticized by Davis (1967) and rebutted by Ravenholt (1969) who felt that if women reproduced only when they wanted, the family and social problems of unplanned and unwanted children would be eased and population growth reduced. This disagreement between those advocating increased access and those increased socio-economic development as the way to achieve reduced population growth has bedeviled the international efforts to achieve this nirvana. For the last 40 plus years, this sterile debate has raged and been a major issue at every international conference on Population and Development (Bucharest 1974, Mexico City 1984 and Cairo 1994). In a recent discussion of what has happened to family planning since Cairo, Sinding (2008) has defined family planning as both "the individual act of avoiding a pregnancy and organized efforts to make contraception and contraceptive services available" to individuals who lack information or access. He was also of the opinion that the Cairo conference was the end of the family planning movement which was replaced by the reproductive health and rights movement (Sinding 2008). Similarly, Potts and Campbell (2008) posit that it is now a given that "modern contraception improves the health of women and their families and is central to the autonomy of women" and conclude from the available evidence that increased access is the major factor in increasing the contraceptive prevalence rate, perhaps ending this debate. It is time to move on and accomplish what all agree is the endpoint, reduction of population growth where it is needed.

In September 2000, the *United Nations General Assembly* adopted Resolution 55/2. UN Millennium Declaration. This included eight Millennium Development Goals (UN MDGs) which ranged "from halving extreme poverty to halting the spread of HIV/AIDS and providing universal primary education" by the year 2015 (UN Millennium Goals: http://www.un.org/milleniumgoals). Three of these goals impinge on maternal reproductive health. Number 4 seeks to reduce child mortality, number 5 to improve maternal health, and number 7 to ensure environmental sustainability, but "the Cairo goal of universal access to reproductive health services is missing" (Sinding 2008).

A report by the United Kingdom All Party Parliamentary Group on Population, Development and Reproductive Health concluded that some of these objectives cannot be achieved within the proposed time frame because of the high levels of population growth in some of the poorest countries (All Party Parliamentary Group on Population, Development and Reproductive Health Report 2007). The report sees a solution in ensuring greater accessibility of existing contraceptives and more widespread use of family planning. The need to address this issue of unsustainable population growth is stressed in a commentary on this report by Campbell et al. (2007), who indicate that some countries have explosive and unsustainable

population growth. Earlier, Speidel and Grossman (2007) had argued that provision of "family planning and access to safe and legal abortion are vital to safeguard the environment." Thus, reduction of population growth is still needed. It has been calculated by Collumbien et al. (2004) that globally, maternal conditions arising from unwanted births, caused by failure to use contraception or use of ineffective traditional methods, resulted in a loss of 51,000 lives and 4.4 million disability adjusted life years. In the two African subregions loss of lives was almost double that of other regions.

1.2 Who Needs Them?

In a recent report from the Guttmacher Institute on international family planning efforts, Sedgh et al. (2007) address "the measurement of unmet need, the obstacles faced by women with unmet need and the potential impact of meeting unmet need." Demographic and Health Surveys show unmet need ranges from 10 to 12% in most regions, but is much higher in Sub-Saharan Africa (Sedgh et al. 2007). Figures for unmet need in 2008 were 24% in Sub-Saharan Africa and 17% in South Central and Southeastern Asia (J.E. Darroch and G. Sedgh, personal communication, Guttmacher Institute 2010). The main reasons for nonuse among married women are lack of access, health effects, and inconvenience, but most women with unmet need stated that they intended to use contraceptives in the future (Sedgh et al. 2007). This 2007 report provided six recommendations to address the unmet need. These were to focus on Sub-Saharan Africa, concentrate on those populations with the greatest need in each country, provide a range of services, provide counseling, improve contraceptive technologies, and educate women about risks of becoming pregnant (Sedgh et al. 2007).

The need for male contraceptives has been propounded in a recent review by Darroch (2008). The number of men presently using contraceptives has remained fairly stable at about 165 million from 1994 to 2005, but since total contraceptive use has increased, this actually reflects a decreased use by men (United Nations 2005). This may be because of the lack of substantial choice of male methods, i.e., condoms, withdrawal, and vasectomy. Variations in method use are due to differences in age, marital status, birth spacing, and national and cultural characteristics (United Nations 2005). Nevertheless, Darroch (2008) feels that there is scope for greater use of contraception by men based on results of recent surveys (Ringheim 1995; Martin et al. 2000; Heinemann et al. 2005). Sixty-six percent of visitors to the International Male Contraception Web site in 2006 wanted new male contraceptives, preferably nonhormonal (Thompson 2006). By 2007, 1,300 individuals had signed up on the Web site urging funding for new male contraceptives (Thompson 2007). Other studies, the largest of which surveyed 9,000 men in nine countries on four continents, also showed high acceptance for the development of new male methods of contraception (Heinemann et al. 2005).

There seems to be a consensus among the experts in the field that despite the plethora of hormonal methods for women and lesser nonhormonal options (e.g., barriers, implants, copper T IUD, and sterilization), there is still a need for new methods for women and, given the very limited and poorly acceptable methods for men, for men also (Harper 2005, 2007).

2 Methods of Contraception in the Clinic

2.1 Female Methods

2.1.1 Steroidal

In 1983, it was concluded that certain new contraceptives would be available by the year 2000 (Harper 1983). These conclusions were arrived at by taking into account various factors, such as rate of innovation, base of knowledge in reproductive physiology, extent of translational research in the area, and expert (soi-disant) opinion. In the most advanced grouping thought to be "highly likely by 1990" were safer oral contraceptives, improved IUDs, improved barrier contraceptives for women, improved long-acting steroid injections, improved ovulation-detection methods for use with periodic abstinence, steroid implants, steroid vaginal rings, GnRH analogs for female contraception, and prostaglandin analogs for self-administered induction of menses. In fact, none were available by 1990, but were by 2000. Methods possible by 1990 or 2000 were even further delayed or still not available. This proves that despite the wisdom of experts, development of radically new methods takes much longer and more funding than anticipated, as compared with "me-too" variations on a theme.

Contraceptive products recently introduced or in late stage clinical trials are shown by category in Table 1. Products in all the categories identified in the 1983 review are now available. Oral contraceptives resulted from the original discoveries by Pincus and associates (Pincus and Chang 1953; Chang et al. 1956; Garcia et al. 1956; Rock et al. 1957) of the utility of progestin/estrogen combinations for contraception through blockade of ovulation. This is achieved by suppressing gonadotropin release from the pituitary, thereby preventing follicular growth and rupture.

There have been many variations on this theme with different progestins (but usually the same estrogen, ethinyl estradiol except for a recent addition using the natural estradiol – NOMAC/E2. (See: http://www.medicalnewstoday.com/articles/71276.php). These variations of new steroids, new formulations and new combinations have been aimed at reducing side effects and having different hormonal activities, e.g., progestogenic (chlormadinone acetate), androgenic (levonorgestrel and its esters), antiandrogenic (nomegestrel acetate and cyproterone acetate), and antimineralocorticoid and antiandrogenic (drospirenone). Certain of these activities

Table 1 Examples of products for women recently introduced in the clinic and in clinical trials

Product	Method of use	Effect and/or benefit	Stage of development
OCs			
Seasonale®	84 tablets containing ethinyl estradiol (EE; an estrogen) and levonorgestrel (LNG; a progestin), and 7 placebo tablets	Menstrual periods are reduced from one per month to one every 3 months	Marketed
Seasonique™	84 tablets containing EE and LNG, and 7 tablets containing 10 μg EE	Menstrual periods are reduced from one per month to one every 3 months The 7 tablets containing 10 μg EE reduce breakthrough bleeding	Marketed
Lybrel®	28 tablets containing 90 μg of LNG and 20 μg of EE, to be taken continuously	No scheduled bleeding	Marketed
Femcon Fe™	NET/EE/Ferrous fumarate 21 active pills and 7 placebo with iron	Mint-flavored and chewable; contains iron	Marketed
NOMAC E2	Nomegestrol acetate and estradiol: 24 active and 4 placebo pills	NA has strong antiandrogenic effect Natural estradiol used instead of usual EE	Phase III trial started 2006 and ended Q4 2009
Pill-Plus™	Progestin/estrogen and androgen	Contains androgen to increase sex-drive in women	In the clinic since 2008
Emergency contraceptives			
CDB-2914: VA-2914	50 mg taken within 5 days of unprotected intercourse	Synthetic selective PRM that binds to the progesterone receptor but does not have progestational activity (Orihuela 2007)	Efficacy in USA (Creinin et al. 2006): Approved for sale in Europe
Meloxicam	Dosage to be determined. Could be used alone or with LNG (Massai et al. 2007; Jesam et al. 2010)	Nonsteroid that might expand the window during which emergency contraception is effective by 24 h or as monthly OC Prevents follicular rupture even after the LH surge (Bata et al. 2006; Jesam et al. 2010)	Inexpensive and widely available over-the-counter (OTC); could possibly be available OTC for EC
Nonoral steroidal contraceptives			
Ortho Evra® patch	Releases 20 μg of EE and 150 μg norelgestromin daily	Prevents the need for daily dosing In 2005, the FDA required a new bolded warning that users are exposed to about 60% more total estrogen in their blood	Marketed

(*continued*)

Table 1 (continued)

Product	Method of use	Effect and/or benefit	Stage of development
		than women taking a daily OC containing 35 μg of estrogen because one of two studies found an increased risk of venous thromboembolic events for current users of Ortho Evra compared with current users of OCs (Cole et al. 2007; Jick and Jick 2007; Jick et al. 2007)	
NuvaRing®	Releases 150 μg etonogestrel and 15 μg EE per day	Prevents the need for daily dosing In 2005, FDA required labeling to reflect an increased risk of thromboembolic and thrombotic disease	Marketed
Nestorone ring	Releases 150 μg nestorone (synthetic progestin that is not active when taken orally) and 15 μg EE per day	Prevents the need for daily dosing	Phase 3 clinical trial in progress: enrolment complete: preparing for launch
Nestorone spray	Dosage to be determined, will be combined with estrogen	Spray avoids side effects common to oral dosing	Early trials
Nestorone rod	Single rod uterine implant releasing nestorone for 2 years; daily transdermal spray	Implant prevents the need for daily dosing	Phase II trials of implant completed
Implanon™	Single-rod uterine implant that contains 68 mg of etonogestrel, released over 3 years	Prevents the need for daily dosing	Marketed
Depo-Provera	150 mg IM every 3 months	Prevents the need for daily dosing In 2004, the FDA required "black-box" warning regarding bone mineral density (BMD) as two studies suggested users could lose significant BMD (Shaarawy et al. 2006; Clark et al. 2006; Beksinska et al. 2007) FDA warning states that Depo-Provera should not be used for more than 2 years unless other contraceptive methods are inadequate	Marketed in many countries

		Other studies have generated different results and the WHO does not advise restrictions	
Levonorgestrel butanoate	10–50 mg IM every 3 months	Prevents the need for daily dosing Thought to not affect BMD as Depo-Provera does	Reformulation underway; early trials planned
Mirena® Intra-Uterine System	Contains 52 mg LNG; releases 20 μg/day	Less pain and bleeding compared with copper IUDs Approved for only 5 years Can cause steroid-related side effects such as acne and ovarian cysts (Sivin et al. 1991; Chrisman et al. 2007; Mishell 2007)	In the clinic since 2000
Barrier methods			
SILCS™	Silicone cervical diaphragm	Available in one size believed to fit most women (Schwartz et al. 2008; PATH 2008)	Phase III contraceptive effectiveness trial completed and in analysis
FC-2®	Female condom made of synthetic latex	Should cost less than original female condom (Female Health Co 2008) Performance and acceptability of original female condom and FC2® were comparable in South African trial (Beksinska et al. 2006; Smit et al. 2006)	Has received CE mark for Product and Manufacturing but is not approved in USA
Reddy latex female condom	Soft polyurethane sponge aids insertion	Appeared more acceptable than original female condom in Indian trial (Smita et al. 2005)	Not approved in USA
PATH Woman's condom	Four small foam dots on outside of inner ring adhere to vagina to hold condom in place	Closed end of condom is gathered into gelatin-based capsule that facilitates insertion then dissolves after use	Phase I trial completed
UsherCell™	Cellulose 6% sulfate vaginal gel to be inserted before intercourse	Inhibits sperm function and egg binding (Anderson et al. 2002). Has shown promise in contraceptive efficacy trials (Mauck et al. 2008), but two HIV	No activity: development on hold

(*continued*)

Table 1 (continued)

Product	Method of use	Effect and/or benefit	Stage of development
		prevention trials were stopped for futility and possible increased risk, of infection, which in the final intent-to-treat analysis proved not significant (Van Damme et al. 2008)	
BufferGel®	Carbopol-based gel to be inserted before intercourse	Immobilizes spermatozoa by lowering pH (Olmsted et al. 2000) Has shown promise in contraceptive efficacy trials (Barnhart et al. 2007) but was ineffective for HIV prevention	Phase III contraceptive trial of BufferGel with diaphragm completed; Phase III HIV prevention trial failed
Nonsurgical female sterilization			
Ovabloc®	Liquid siloxane is inserted into the tubal orifice, then polymerizes into a plug		Not approved in US: available in Netherlands
Essure®	A small metal coil is placed into each Fallopian tube through the cervix using a catheter. Once in place, the device elicits tissue growth to form an occlusion		Marketed
Quinacrine	Chemical tubal occlusion; seven pellets are placed high in the uterus using a modified IUD inserter, then dissolve and cause scarring of the opening in of the Fallopian tube	10-year follow-up showed moderate effectiveness and minimal health risks (Sokal et al. 2008a, b)	Abandoned due to carcinogenicity concerns (Cancel et al. 2006; FHI 2007)

can be useful for treatment of gynecological conditions, such as PCOS or pelvic inflammatory disease, as well as for contraception.

The usual dosing regimen for oral contraceptives has been 21 days of active pills, followed by 7 days of placebo pills. New dosing regimens to reduce the number of menstrual periods in the year have also been introduced (Seasonale, Seasonique, and Lybrel). Provider-dependent methods, such as monthly injectables containing both a progestin and an estrogen, and implants and IUDs releasing progestational steroids over a long time provide alternate choices for the consumer. Consumer-controlled methods of delivery that are easily started and stopped are exemplified by vaginal rings and transdermal patches and creams. More recently, an existing oral contraceptive has been reformulated as a mint-flavored, iron-containing chewable tablet (see: http://fdb.rxlist.com/drugs/drug-145900-Femcon+Fe+Oral.aspx?drugid=145900&drugname=Femcon+Fe+Oral) to improve patient compliance, especially among teenagers, and another has an added androgen to increase sex-drive in women (see: http://www.medicalnewstoday.com/articles/120878.php). Thus, in the 50 years since the initial studies of Pincus and colleagues, a whole armamentarium of steroidal methods has been developed, but these methods are not radically new (Harper 2005, 2007). Much of the recent activity has focused on the different dosing regimens, especially the nonoral ones. However, users of patches and rings are thought to be at increased risk for venous thromboembolic events (Cole et al. 2007: Jick and Jick 2007; Jick et al. 2007). Implanon is a single rod releasing a progestin, etonorgestrel, which is effective for 5 years. Mirena is an IUD releasing LNG and is effective for more than 5 years, which not only provides very effective contraception but also other health benefits (Sivin et al. 1991; Chrisman et al. 2007; Mishell 2007).

Progestin-only pills have a limited following, since missing a pill is more likely to cause failure of the method than missing a combined pill. Furthermore, contraceptive efficacy is not as good as such progestin-only regimens do not inhibit ovulation, except at high doses, but work by altering cervical mucus consistency or sperm transport in the female genital tract. In addition, there is increased bleeding and spotting, which is not acceptable to many women.

In younger women, or those not having regular sexual intercourse, there is a risk of unprotected intercourse or even if a condom is used, a condom breakage, which puts them at risk for an unwanted pregnancy. For such situations, there is available a dedicated emergency contraceptive product (Plan B in the US: Postinor-1 or -2 elsewhere) consisting of two tablets, each containing 0.75 mg LNG to be taken 12 h apart, which must be taken as soon as possible after intercourse and no later than 72 h (WHO Task Force on Postovulatory Methods of Fertility Regulation 1998). There is evidence that taking both tablets at once may be just as efficacious. In this modality, LNG works by inhibiting ovulation, but it must be taken before follicular size is 18 mm or greater and this may account for the approximately 10–25% failure rate, even when taken properly (Novikova et al. 2007). Another steroid, CDB-2914 (also known as VA-2914 or ulipristal), a progesterone receptor modulator (PRM) with many of the same antagonistic activities as mifepristone has also been used for emergency contraception (Orihuela 2007). A Phase III trial in the United States and

other countries has just been completed (Creinin et al. 2006; Glasier et al. 2010). It was concluded that ulipristal can provide an effective alternative to LNG that can be used for up to 5 days after unprotected intercourse (Glasier et al. 2010). Ulipristal is thought to work not only by inhibiting ovulation but also by affecting endometrial function.

2.1.2 Nonsteroidal

Table 1 also lists the much smaller range of nonsteroidal contraceptives. These are the copper releasing IUD and various barrier contraceptives, such as cervical caps, diaphragms, and female condoms.

Barrier methods that have been available for many years are the Ortho diaphragm and the FC-1 female condom. Recently, there has been renewed interest in improving user acceptability of such devices. The diaphragm was designed with feedback from volunteers with the hope that it should be very acceptable to women. It also has the advantage that one size may fit most women (Schwartz et al. 2008; PATH 2008). A Phase IIb efficacy trial with BufferGel as the spermicidal active has just been completed. The final results are in analysis. The female condom FC-1 has the disadvantage of high cost. This problem has been overcome with development of FC-2 made of latex. Its performance and acceptability were similar to FC-1 in South Africa (Beksinska et al. 2006; Smit et al. 2006). Other female condoms are under development. Nonoxynol-9 has been the most widely used ingredient of spermicidal vaginal gels used for contraception. It acts by immobilizing and killing spermatozoa in the vaginal tract. However, its failure to protect women from HIV, and indeed to increase risk in women with multiple uses per day (Van Damme et al. 2002) led to efforts to develop more benign alternatives. Cellulose sulfate, a polyanion, proved to be effective as a contraceptive (Anderson et al. 2002; Mauck et al. 2008), but did not prevent HIV transmission and may even have increased risk of infection (Van Damme et al. 2008). A different approach was development of BufferGel, a carbopol-based vaginal gel, which acts by killing sperm and potentially viruses by its pH lowering effect (Olmsted et al. 2000). It has already been shown to be as effective as nonoxynol-9 as a contraceptive when used with a diaphragm (Barnhart et al. 2007). Unfortunately, neither of the spermicidal vaginal gels – BufferGel and SAVVY – nor the contraceptive or noncontraceptive polyanion gels – cellulose sulfate, PRO2000, and Carraguard™ – were effective in preventing HIV infection (Morris and Lacey 2010).

Female sterilization has always been an option for those who have reached their desired family size, but involves surgery by laparotomy or laparoscopically. Reversibility is difficult or impossible depending on how much of the Fallopian tube has been removed. Recently, interest has been rekindled in transcervical methods of sterilization using either liquid siloxane (Ovabloc) which forms a plug in the tubal opening (Ligt-Veneman et al. 1999) or alternatively a metal coil (Essure) is inserted which causes an inflammatory reaction and tissue ingrowth (Chapman and Magos 2008). Essure is judged to provide significant cost savings

compared with laparoscopic sterilization (Thiel and Carson 2008). Since, however, both tubal blocking procedures require hysteroscopy to ensure correct placement, they would be less suitable for resource-poor settings. A cheap and simple method of sterilization is provided by insertion of quinacrine tablets into the uterus, but a single insertion does not achieve 100% tubal closure. A second insertion 1-month later does achieve 98% efficacy (Zipper and Kessel 2003). Its use fell out of mainstream favor because of toxicity concerns, especially cancer. However, in a 10-year follow-up of patients sterilized with quinacrine risks to health were felt to be minimal (Sokal et al. 2008a), but effectiveness was less than for other forms of sterilization (Sokal et al. 2008b). A further follow-up of Chilean women during 23,894 person-years revealed only 41 invasive cancers (including 16 new cases since the previous follow-up). It was concluded that rates of cancer among quinacrine treated women are similar to population-based rates (Sokal et al. 2010). Nevertheless, there are still concerns about the incidence of cancer in small animal toxicology studies (Cancel et al. 2006; Family Health International 2007).

It has been known for a long time that in animals, administration of a cyclooxygenase-2 (COX-2) inhibitor of prostaglandin biosynthesis prevents follicular rupture. That a similar effect occurs in women was deduced from a study by Killik and Elstein (1987) who showed using ultrasound that indomethacin could cause 100% luteinized unruptured follicles (i.e., causing entrapment of the oocyte). Indomethacin inhibits both COX-1 and -2 and has unacceptable side effects. With the advent of specific COX-2 inhibitors, this idea has been revisited, first as an adjunct to LNG treatment (Massai et al. 2007) and then as a stand-alone treatment (Bata et al. 2006). The drug chosen was meloxicam, which is available as a generic and over the counter in many countries, and has a long history of few side effects. A single dose of 15 mg given with LNG appeared to extend the window of efficacy for emergency contraception as judged by ultrasound (Massai et al. 2007) and meloxicem given alone for 5 days commencing at a follicular size of 18 mm, seemed to have a similar effect (Bata et al. 2006). A dose-finding study showed that 30 mg/day causes a greater degree of unruptured follicles than 15 mg/day, 91 vs. 50%, respectively (Jesam et al. 2010). The conclusion that lack of unruptured follicles in women seen with ultrasound was buttressed by a study in cynomolgus monkeys where a 5-day course of meloxicam (0.5 mg/kg/day p.o.) given around the time of ovulation significantly reduced the rate of oocyte release (using histological examination) without alteration of hormone levels or menstrual cycle length (Hester et al. 2010). However, the suggested recommended dose of meloxicam for osteoarthritis is 15 mg/day and other COX-2 inhibitors have caused severe cardiovascular adverse effects. Less selective NSAIDs, e.g., meloxicam, appear to have less risk for thromboembolic events than more selective ones, e.g., celecoxib and rofecoxib (Layton et al. 2003a, b). It should be noted that the reported cardiovascular and thromboembolic side effects of meloxicam are no greater than those seen with steroidal oral contraceptives (Layton et al. 2003a, b; Helin-Salmivaara et al. 2006; Lidegaard et al. 2009; van Hylckama Vlieg et al. 2009). There is, however, still a risk of GI bleeding with meloxicam, although this should be ameliorated by pill-free days. Five days treatment for emergency contraception may also not be as

acceptable to women when one or two pills of LNG will suffice. At the very least, meloxicam could be a useful adjunct to LNG for emergency contraception.

In addition, consideration is now being given to use of meloxicam on a monthly basis in a study in Chile. Placebo pills will be given on cycle days 1–4, meloxicam (15 or 30 mg/day p.o.) on days 5–22, and placebo again for the remainder of the cycle. On onset of bleeding a new pack of pills would be started. Concurrently, a pregnancy inhibition study in monkeys is proposed using the same dosing protocol as in the human study.

2.2 Male Methods

Historically, there have been fewer contraceptive options for men – condoms, vasectomy, and withdrawal. This area did not attract the same degree of research as that for female contraceptives. This may have been because of a mistaken perception that since women get pregnant, they are the ones motivated to use contraceptives. However, as we have seen in Sect. 1.2 above, men are willing to share the burden of family planning and would use even the inconvenient methods now under development. The new options are shown in Table 2.

WHO has conducted two major studies using large doses of testosterone enanthate (200 mg i.m. weekly) as a male contraceptive (WHO 1990, 1996). In the first trial, only about 65% of men reached azoospermia and in such men there was only one pregnancy in 1,486 months of use. Recovery of spermatogenesis took about 4 months (WHO 1990). The second study showed that even men not azoospermic, but with sperm counts of less than 3 million/ml were effectively infertile; only four pregnancies occurred in 49.5 person-years of use (WHO 1996). Although these pioneering studies proved the concept, the frequent injections were not well accepted.

From the various, generally small trials that have been conducted, it is known that a regimen combining an androgen and a progestin can provide a reliable way to inhibit spermatogenesis by suppressing gonadotropin secretion from the pituitary. New formulations of testosterone undecanoate and norethisterone enanthate permit longer dosing intervals, although this particular combination has not been tested heretofore for efficacy, only for degree of azoospermia and blood levels (Meriggiola et al. 2005; Qoubaitary et al. 2006). Doses of 1,000 mg TU and 200 mg NET-EN given bi-monthly were selected as the best choice and they are being used in the present trial, which also differs from the pilot study in that when men reach azoospermia or less than 1 million sperm/ml in the ejaculate, they will be allowed to have intercourse without need for a condom. There will be a 6-month suppression phase, a 1-year efficacy phase and 1-year for recovery. Much hope is pinned on the trial which is in progress. As of March 2010, about 230 men have been enrolled and of those 151 had entered the efficacy phase. A small number are in the recovery phase. Recruitment will end at the end of August 2010 and results should be

Table 2 Examples of products for men recently introduced in the clinic and in clinical trials

Male steroidal methods			
Testosterone enanthate (TE)	200 mg TE injected intramuscularly weekly for 6 months	Azoospermia in 65% of men within 6 months, only 1 pregnancy in 1,486 months of use (WHO 1990) Recovery of sperm counts within about 4 months of stopping treatment (WHO 1990) Second study determined that among men who achieved oligospermia only four pregnancies occurred in 49.5 person-years of use and in azoospermic men there were no pregnancies in 230.4 person-years of use (WHO 1996) Frequent injection schedule caused 5% to discontinue (WHO 1996)	
Testosterone undecanoate (TU) plus etonorgestrel implant	750 mg TU every 10–12 weeks or 1,000 mg every 12 weeks plus a low or high release etonorgestrel implant	Spermatogenesis suppressed to less than 1 million/ml in 89% of men by week 16; 3% never achieved this level of sperm inhibition. Regimen well tolerated but not ideal (Mommers et al. 2008)	Contraceptive study in 354 men completed corporate decision not to continue development
TU plus norethisterone enanthate (NET-EN)	750 or 1,000 mg TU and 200 mg NET-EN given in two separate intramuscular injections every 8 or 12 weeks	PK study determined doses for larger efficacy study (Qoubaitary et al. 2006) With 8-week interval 90% reached azoospermia compared with 38% in 12-week group (Meriggiola et al. 2005)	8-week interval and 1,000 mg TU plus 200 mg NET-EN chosen for contraceptive study
TU plus NET-EN	1,000 mg TU plus 200 mg NET-EN every 8 weeks in 2 oil i.m. injections	When sperm concentrations reach azoospermia or less than 1 million/ml couples will cease to use other contraceptives for 1-year efficacy period, followed by a 1-year recovery	Phase IIb trial started in 8 centers in 7 countries. Enrolment is in progress

(*continued*)

Table 2 (continued)

Male non-steroidal methods			
Vasal occlusion			
Intra Vas Device ("Shug")	Two types: a pair of silicone plugs and urethane tube lined with nylon sieve	Trap sperm but allow fluid in the vas to pass through, reducing likelihood of congestive epididymitis Double plug design did not completely block sperm transport through the epididymis, failing in 3 out of 30 men (Zaneveld et al. 1998)	Project being continued by Shepherd Medical. Effectiveness trial in 90 men planned to start in 2006 and end 2009. No active recruitment (Shepherd Medical Web site 2008)
Reversible Inhibition of Sperm Under Guidance (RISUG)	Styrene maleic anhydride in a solvent vehicle of dimethyl sulfoxide gel injected into the vas	Partially occlude vas and have direct toxic effect on sperm for at least 1 year (Guha et al. 1997) Reversible using a complicated noninvasive technique which can be repeated (Lohiya et al. 1998, 2000)	In Phase III clinical trials in India

available by 2012. If a hormonal method of male contraception is going to be available in 5 or do years, this is the only possible candidate.

Two pharmaceutical companies, Schering AG (now Bayer Schering Pharma AG) and Organon (now part of Merck & Co. Inc.) had joined forces to test the combination of the etonorgestrel implant (good for a year) and TU given i.m. bimonthly as a possible method of male contraception. It was concluded that this was a well-tolerated method for inducing reversible suppression of spermatogenesis. Approximately, 90% of men reached and maintained azoospermia from week 16 to the end of the study at 42 or 44 weeks (Mommers et al. 2008). It was also concluded that having two methods of delivery – implant and injection – was not ideal. For corporate reasons, it was decided not to pursue this combination further.

The only other male methods near to fruition involve vassal occlusion. Lohiya et al. (2001) conclude that the vas deferens is an organ that can be manipulated for male contraception without undue side effects. Vasectomy is the most widely used approach for sterilization and can be done surgically or with the newer no scalpel technique. Other ways of blocking the vas have also been explored (see full discussion: http://www.newmalecontraception.org/vas.htm accessed February 17, 2010).

One intravas device known as the "SHUG" has been in development for many years (Zaneveld et al. 1998). It is now known as the Intra Vas Device (IVD). This consists of two hollow silicone plugs joined by a thread. The plugs are placed in the vas and the thread left outside the vas to facilitate removal. Unfortunately, in the only clinical trial reported, 3 out of 30 men did not have complete blockade of sperm passage (Zaneveld et al. 1998). Further modifications have been made to improve efficacy and the project has been taken over by Shepherd Medical Company who planned to start a clinical trial in 90 men beginning in 2006. This was to be funded by NIH Office of Extramural Research starting in 2004 and ending in 2007 (Office of Extramural Research 2004). However, according to the ClinicalTrials.gov Web site, there is no active recruitment of participants at last reported update in 2007 (see: http://clinicaltrials.gov/ct/show/NCT00335361?order=2). No results from this trial have been published.

A more promising approach is provided by RISUG (an acronym for Reversible Inhibition of Sperm under Guidance). This method consists of styrene maleic anhydride powder dissolved in dimethyl sulfoxide (DMSO), which is injected into the vas in sufficient quantity to coat the inner lining of the vas and to partially block it. Not only does the SMA block the vas but it also actively kills sperm (Guha et al. 1997). In a small clinical trial, 12 men whose wives were not using other methods of contraception had their vas injected with RISUG and followed up for at least 12 months. Azoospermia was maintained for this period and no pregnancies were reported (Guha et al. 1997). Experiments conducted in langurs showed that the blockade can be reversed by a noninvasive, but complicated, procedure which involves palpation, percutaneous electrical stimulation, forced vibratory movement, suprapubic percussion, and rectal massage of the vas (Lohiya et al. 1998). Whether all these maneuvers are necessary is unknown since each was not tested individually. In a follow-up study also in langurs, it was shown that blockade lasting

150 days could be reversed and then the vas reoccluded and reversed successfully again (Lohiya et al. 2000). According to Wikipedia, Dr. Guha claims that there has been only one pregnancy among partners of 250 men treated and that the effect lasts 10 years. The Indian Council of Medical Research has sanctioned a Phase III trial in India despite some lingering doubts about the toxicity of DMSO. About 60 of a planned 500 subjects have been enrolled, and an interim analysis was expected a year from now (Badri Saxena, personal communication October 2008), but has been further delayed.

2.3 Need for Improved Methods

As has been discussed in Sect. 2.1, although there are a large number of different modalities of contraception for women, mostly they involve delivery of steroids (with admittedly somewhat different activities on the progesterone receptor) packaged in various ways – oral, transdermal, implant, vaginal ring, and IUD. If a woman has unacceptable side effects with one preparation, then by trial and error, she probably can find one that suits her needs and lifestyle (spacing or limiting). Nevertheless, it is clear that despite availability, many women do not use contraceptives. Nonuse is responsible for 90% of unwanted births (Collumbien et al. 2004) and this unmet need ranges from 10 to 12% in developing countries (Sedgh et al. 2007). Even in the United States, women who feel dissatisfied or neutral about their contraceptive method may not use contraception for at least 1 month every year (Frost et al. 2007).

For men, the situation is worse, as choice is so limited. The only new method that may appear in the next 5 years is a steroidal one, and delivery of which, although men claim to find oily injections in the buttocks acceptable in the trials, is less than optimal. It is plausible that men who are in the ongoing trials are early adopters and more motivated. Whether a method involving a clinic visit every 2 months will be acceptable to men as a routine method is uncertain. Different delivery methods such as transdermal ones could prove more acceptable, but here there is the drawback of daily application and even potentially spread to the partner.

Clearly, no one method will be suitable for all couples, especially given the different needs at different times in the reproductive life-cycle – career delay, birth spacing, desired family size achieved, and degree of sharing responsibility between a man and his partner. Long-acting methods such as IUDs or implants are more suitable for those women not desiring further children for at least for 5 years or so and have the advantage of decreasing unintended pregnancy (Speidel et al. 2008). Taking into account the pandemic of HIV/AIDS in many sub-Saharan countries, methods that provide dual protection – contraception and inhibition of HIV and other sexually transmitted diseases (a.k.a. microbicides) – are urgently needed.

At present, research on HIV prevention and contraception is mostly being conducted separately and is not well coordinated. Although there is adequate funding for microbicides, funding for contraception is constrained. The initial

inventors in academia or biotech cannot fund the translational research themselves. Absent adequate funding at worst new developments may not reach fruition or at least will be seriously delayed. (This theme is discussed further in Sect. 5.) As Potts and Fotso (2007) urge, it is time to once again to place population and family planning center stage in global efforts to improve reproductive health and fight poverty. In a recent review, we made the case, which we reiterate here, that knowing what we know, what is possible, what we need to do, and how the OMIC revolution permits us to focus our efforts to provide new methods for both men and women (Aitken et al. 2008), the funding of new initiatives will not go in vain.

3 New Leads in the Preclinical Discovery Phase

When considering developing a new lead, certain considerations need to be taken into account. First is validation of the target, which can be achieved by interfering with the biological activity of the target. Inhibition can be accomplished by means of knocking out or silencing the relevant gene in mice (provided there is a human homolog), antibodies to the target (preferably in a primate model) or small molecular weight inhibitors (if reasonably specific such be known). The target should preferably be specific for the reproductive tract with minimal cross-reactivity in liver and kidney and other organs and not be able to be substituted for by another member of the gene or protein family. Ideally, the target needs to be druggable. Good targets are ion channels, receptors, and enzymes which are more readily susceptible to inhibition by small molecules. Some targets may be readily inhibited by small peptides, i.e., by mutated ligands that bind to a receptor but do not cause an active read-out. The problem with peptides is that they are not orally active and must be delivered by another route. Since the half-life of peptides, even if extended by conjugation with polyethylene glycol (PEGylated), will be measured in days not weeks or months, frequent administration will be needed. Injections, more frequent than once-a-month, are not acceptable for contraception. This is not true for other modes of delivery. It has been known for a long time that vaginal absorption is an effective means to deliver systemically a wide range of substances, including proteins and antigens (reviewed by Benziger and Edelson 1983). In a more recent review, it is reemphasized that vaginal absorption of peptides and therapeutically important macromolecules is feasible (Hussain and Ahsan 2005). Antibodies, including monoclonal IgG and IgM, have been released from a vaginal ring over a 30-day period, but concentrations were about 100-fold lower in blood and other tissues than in vaginal secretions (Saltzman et al. 2000). Insulin uptake was minimal from the rat vagina without use of enhancers like the surfactant polyoxyethylene-9-lauryl ester (Richardson et al. 1992). More relevant to contraception, it has been shown that delivery of magainin II amide (23 amino acids) from a vaginal tampon was absorbed sufficiently to interfere with implantation in the rhesus monkey (Dhawan et al. 2000). Other dosage forms can be considered, such as

nasal sprays, but for practicality vaginal gels to test the concept in vivo, followed by development of a vaginal release device seem to be the easiest path to delivery of peptides.

3.1 Potential New Female Methods

A selection of new leads has been recently discussed (Aitken et al. 2008). These included both steroidal and nonsteroidal moieties. Conventional wisdom predicates that ten targets are needed to get one lead all the way from discovery to clinical testing. As will become clear from the following vignettes, the number of exciting leads is low.

Progesterone receptor modulators (PRMs), such as mifepristone and ulipristal, have been shown to be effective for early abortion (Kovacs et al. 1984; Swahn et al. 1994) and for emergency contraception and as a daily contraceptive (Baird et al. 2003; Chabbert-Buffet et al. 2007; Orihuela 2007). Other PRMs have been tested in animals, including monkeys (Brenner and Slayden 2005; Slayden et al. 2006), but there has been reluctance to develop such agents for contraception due to the possibility of their use for medical abortion, and the fact that mifepristone is widely used and well tolerated. Other agents further back in the pipeline include inhibitors of leukemia inhibitory factor (LIF) receptor and its close relative IL-11 receptor α, inhibitors of leptin receptor and proprotein convertase 6 (PC6). All of these approaches would work postfertilization by interfering with endometrial receptivity for blastocyst implantation.

In mice, LIF is obligatory for implantation (Bhatt et al. 1991: Stewart et al. 1992) and appears also to play a key role in monkeys (Yue et al. 2000; Sengupta et al. 2006) and in women (Kojima et al. 1994; Charnock-Jones et al. 1994). Peptides derived from mutated human LIF have proved to be good inhibitors of LIFR activation being 1,000-fold more potent binding to LIFR in vitro than the native molecule (Fairlie et al. 2004). Despite its short half-life in vivo, multiple i.p. injections and delivery from an osmotic minipump of the antagonist between days 2.5 and 4.5 of pregnancy prevented implantation in mice (White et al. 2007). Conjugation of the antagonist with polyethylene glycol (PEGLA) increased the half-life considerably. In vitro the PEGylated form could still block LIFR signaling, and in vivo only three i.p. injections between days 2.5 and 3.5 were needed to inhibit implantation (White et al. 2007).

Application per vaginam in mice, however, proved problematic, as there was leakage and grooming, and this combined with the small volume of the vagina, made delivery of adequate amounts unreliable and difficult. This problem was a factor for all the peptides delivered vaginally in mice – LIFR, IL-11Rα, Leptin, and PC6 antagonists. Alternative animal models are being sought. A dose-finding exercise in cynomolgus monkeys is now in progress. Subcutaneous administration will be tested first to determine what dose should be used to achieve measurable

uterine levels of PEGLA, and then vaginal application tried to achieve the same uterine level.

A similar strategy has been pursued with IL-11 receptor α subunit. Mice null for this receptor are infertile due to defective decidualization (Robb et al. 1998). Both the mRNA and protein of IL-11R are highly expressed in primate endometrial luminal and glandular epithelium at the time of uterine receptivity (Dimitriadis et al. 2003, 2006a). Inhibitory peptides have been made which block IL-11R signaling via the JAK/STAT pathway in vitro and are therefore likely to be active in vivo also. The expectation is that the antagonist will act on the epithelium prior to blastocyst attachment. In contrast in the mouse, IL-11 and its receptor are completely decidual specific (Robb et al. 1998) and thus the antagonist will work only after blastocyst attachment. Like the LIFR antagonist, the IL-11R antagonist had to be PEGylated also. These antagonists have not yet been tested in vivo. Work has concentrated on the PEGLA. If the initial promise holds up, one can imagine a dual acting mixture of the LIFR and IL-11R antagonists having a synergistic effect, they both activate the JAK/STAT pathway (Bao et al. 2006; Dimitriadis et al. 2006b). The IL-11R antagonist development is clearly much further back in the pipeline than the LIFR one.

Although leptin is a key regulator of food intake and energy balance, it also regulates implantation in mice. Mice null for the leptin receptor are infertile due to a failure of implantation (Malik et al. 2001). Leptin signals through the JAK/STAT pathway, like LIF, and this can be blocked by inhibitory peptides or antibodies to the leptin receptor (Gonzalez et al. 2004). Leptin inhibits decidualization in vitro (Tanaka et al. 2003). Peptide antagonists of the leptin receptor inhibit implantation in mice after intrauterine administration on day 3 of pregnancy, while scrambled peptides have no effect (Ramos et al. 2005). As with the LIFR and IL-11Rα antagonists, PEGylation of the leptin receptor antagonist increased its half-life to 19–68 h. Formulation of this PEGylated antagonist in a gel which was then administered intravaginally on days 1–6 of pregnancy prevented implantation in mice (Gonzalez et al. 2007). Unfortunately, a fresh supplier of PEG had to be found. Replication of the original results with the new material proved difficult. Although radiolabeled peptide was found in the uterus, ovaries and mammary glands following vaginal administration, insignificant amounts were found in the central nervous system (suggesting no crossing the blood–brain barrier) and there was no effect on energy balance (Gonzalez 2006; Gonzalez et al. 2007). However, the fact that leptin has so many other functions encourages caution with its development for contraception.

Both mRNA and protein of a serine protease, proprotein convertase 6 (PC6), were found to be present in mouse and human endometrium and associated with the window of endometrial receptivity for implantation (Nie et al. 2005a). Intrauterine administration of morpholino antisense oligonucleotides blocked PC6 protein production and inhibited implantation (Nie et al. 2005b). Although there are other members of the PC family found in the endometrium, only PC6 was specifically upregulated during decidualization; all the others were just constitutively expressed (Freyer et al. 2007). This suggests that it may be a good target. Experiments have

examined the effect of a known inhibitor of PCs, D-type 9-mer-Polyarginine (Poly-R) (Fugere et al. 2007) as an antifertility agent in mice. Although positive results were achieved with vaginal application, replicability is a problem. The rabbit is being used as an alternative model.

It should be noted that except for LIF no primate studies have been undertaken with any of these antagonistic peptides. Whether a successful result in a monkey species is the gatekeeper to going forward with development and into women is uncertain. A positive result would certainly be encouraging. However, monkey studies are expensive, difficult to conduct and pregnancy rates in untreated monkeys are only about 65% depending on the caging available for mating. The number of centers able to do such studies is also a limiting factor. Other smaller primates, such as marmosets, might provide a solution.

Other possible leads are genes upregulated at the time of the window of endometrial receptivity, such as laminin β3, microfibril-associated protein 5, angiopoietin-like 1, endocrine gland-derived vascular endothelial growth factor, and nuclear localized factor 2, but these are at a much earlier stage and their ultimate success or failure is unknown (Haouzi et al. 2009).

3.2 Potential New Female or Male Methods

Here again, a selection of leads which work during different reproductive stages has been discussed by Aitken et al. (2008). Male germ cells develop an intracellular bridge during spermatogonial cell differentiation (Greenbaum et al. 2006, 2007). These bridges link all male germ cells during subsequent meiosis and mitosis, which results in a syncytium of more than 1,000 cells. An essential protein in the formation of these bridges appears to be TEX14 which localizes to the intercellular bridge during spermatogenesis. Mice null for TEX14 show disrupted intercellular bridges and are infertile (Greenbaum et al. 2006). Recently, it has been found that intercellular bridges between the gonocytes and oogonia are absent in TEX14 null female mice, and yet they remain fully fertile (Greenbaum et al. 2009). No inhibitors of TEX14 have so far been reported.

A small molecule, Adjudin (AF2364) related to Lonidamine, an anticancer drug, targets protein complexes restricted to the interface between Sertoli cells and elongating spermatids in the testis. This disrupts the anchoring junction which causes premature exfoliation of spermatozoa. These immature spermatozoa cannot fertilize oocytes (Cheng et al. 2005; Mruk et al. 2006). Owing to poor oral bioavailability large doses had to be administered systemically to achieve the desired antifertility effect. This resulted in liver toxicity (Cheng et al. 2005). A pharmacokinetic study in rabbits showed that the AUC for i.v. and oral administration of Adjudin were 20.1 and 2.2(mg h)/L, respectively, thus confirming the lack of oral bioavailability (Hu et al. 2009). One way of overcoming this problem was the conjugation of Adjudin to a mutant FSH molecule that bound the FSH receptor but

did not activate it. Since FSH receptors are only found on Sertoli cells, this conjugate targeted Adjudin to the testis where after i.p. administration it was 100,000 times more potent and not toxic than the unconjugated Adjudin (Mruk et al. 2006). However, to administer the conjugate practically is still difficult.

A different approach was taken by others (Tash et al. 2008a, b). Chemical modifications of the parent compound, Lonidamine, produced indazole carboxylic acids that were much more bioavailable and potent. One of which named gamendazole was selected for further study (Tash et al. 2008a). In seven fertile male rats, it was found that an infertility rate of 100% was achieved 3 weeks after a single oral dose of 6 mg/kg. Reversibility occurred by 9 weeks in four out of the seven (Tash et al. 2008a). It appears that gamendazole targets the Sertoli cells in a similar way to Lonidamine and Adjudin, but is more effective because of the good bioavailability. Whether this compound is toxic to the liver at these low doses is unknown. This could be a very attractive method of male contraception because, once infertility is established, a monthly pill should be effective to maintain it as the next treatment would occur before mature sperm could again be exfoliated from the seminiferous epithelium.

In mice, the retinoic acid receptor α (RARα) is essential for transduction of retinoid-mediated signaling and normal fertility. Its lack in mice homozygous for a null mutation causes multiple sperm defects, including failure of spermatids to align properly with the Sertoli cells (Chung and Wolgemuth 2004, Chung et al. 2005, 2009; Wolgemuth and Chung 2007). In a program to develop antagonists of RARα for therapeutic purposes, it was discovered that an antagonist, BMS-189453 (now known as compound 9), that blocked all three RARs – RARα, β, and γ – caused testicular degeneration and infertility in rats. Toxicity was only seen at 60 mg/kg/day (Schulze et al. 2001). Even a dose as low as 2 mg/kg orally for 1, 3, or 7 days caused testicular degeneration 1 month after dosing. The degree of atrophy was time and dose-dependent and was at these doses irreversible. A dose of 50 mg/kg for 1 week produced similar effects in rabbits (Schulze et al. 2001; Wolgemuth and Chung 2007). More recent testing of lower doses suggests that it is possible to reverse the testicular damage. Oral administration of 2.5 mg/kg/day for 4 weeks to mice caused infertility for 4 weeks following treatment: reversal occurred in 14 weeks. Whether lower doses and longer times of treatment will still prove effective and reversible remain to be determined. No toxicology was noted at this dose in the mice. Compound 9 has an advantage over new analogs in that a complete toxicology study was done in rats as part of its initial development during which the testicular effect was seen.

GAPDHS (known as GAPDH-2 in the human) is a glycolytic enzyme encoded by genes expressed only during spermatogenesis (Welch et al. 1992; Krisfalusi et al. 2006). GAPDHS is tightly bound to the fibrous sheath of the principal piece of the sperm flagellum (Welch et al. 1992), and mice that are homozygous null for this enzyme have immotile sperm and are infertile (Miki et al. 2004). GAPDHS is also found in the rat about 29 days postnatally and is localized to round and condensing spermatids (Welch et al. 2006). This suggests that GAPDHS may be a useful target for contraception in men and vaginally in women, but this depends on identification

of inhibitory small molecules. Structure activity relationship analyses are being done on in silico screening hits and high throughput screening is being done on various libraries of compounds. Hits to date have a low level of inhibitory activity. The same investigators have also shown that phosphoglycerate kinase-2 is essential for sperm function and male fertility in mice. This isoenzyme catalyzes the step following the action of GAPHS in the sperm glycolytic pathway, but the defect in fertility is less than that of GAPDHS (Danshina et al. 2010).

Sperm depend on motility to traverse the reproductive tract and hyperactivation to achieve penetration of the oocyte. Calcium is essential for motility and enters the sperm by a calcium ion channel which is comprised of four subunits of CatSper proteins 1–4 (Ren et al. 2001; Jin et al. 2005; Qi et al. 2007). All four subunits appear necessary for calcium influx. Their absence does not affect normal sperm motility but does abolish hyperactivated motility (Carlson et al. 2003; Quill et al. 2003; Jin et al. 2007). Patients with subfertility due to lack of sperm motility have a 3.5-fold difference in *CatSper* gene expression from infertile men with normal sperm motility (Nikpoor et al. 2004). High throughput screening and secondary confirmation by CASA have identified some leads but activity is in the high nM range (Carlson et al. 2009). This appears an attractive target, and inhibitors could be used by either men or women.

Odorant receptors (OR) have been found on human sperm (Spehr et al. 2003). A human testicular hOR17-4 has been identified which mediates sperm chemotaxis in various bioassays (Spehr et al. 2003; Spehr and Hatt 2005). It is thought that chemical signals may be involved in attraction of sperm to the ovulated oocyte to improve the chances of fertilization. Activation of the hOR17-4 receptor mediates distinct flagellar wave patterns and chemotactic behavior (Spehr et al. 2006), which suggests that this system may be a key player in the process of fertilization, even though the endogenous ligand released from the oocyte is unknown. Undecanol, an antagonist that inhibits signaling through the OR17-4, has been described (Spehr et al. 2003), but in the intervening period little progress seems to have been made in identifying more active compounds despite the attractiveness of the target. Specificity may be a problem as the family of olfactory receptors is diverse and is involved in the activity of enzymes, ligand-gated ion channels, and G protein-coupled receptors (Spehr and Munger 2009).

Most of the leads discussed so far have been validated by use of mice with the gene of interest deleted by targeted mutagenesis. However, use of passive administration of antibodies can also provide useful information. Eppin is a protein found in Sertoli cells in the testis and epididymal cells. It binds semenogelin and inhibits the activity of prostate-specific antigen, which normally causes the hydrolysis of semenogelin (Wang et al. 2007a, b). Antibodies specific for Eppin caused infertility in male monkeys, which eventually was reversed in the majority of monkeys (O'Rand et al. 2004). The antibodies were thought to bind to eppin on the sperm surface and the complex thus blocking the binding site for semenogelin (O'Rand et al. 2006). Furthermore, the anti-eppin antibodies from the monkeys reduced progressive motility of human sperm (O'Rand et al. 2009). It was concluded that eppin-seminogelin binding site is a key factor in removal of semenogelin during

semen liquefaction in vivo (O'Rand et al. 2009). Using recombinant semenogelin and fragments, it was determined Cys is the critical amino acid for biding to Eppin and inhibiting sperm motility (Mitra et al. 2010). Small molecule inhibitors of this binding site are being sought, which these investigators feel is an excellent target for a male contraceptive (O'Rand et al. 2009).

Another interesting approach to male contraception depends upon inhibition of sperm emission. Drug-induced inhibition of ejaculation, associated with otherwise normal orgasm, was observed as a side effect of treatment with therapeutic agents used for other purposes, e.g., guanethidine, thioridazine, and phenoxybenzamine (Green and Berman 1954; Singh 1963: Bauer et al. 1973). Studies using rat and human vas deferens defined the α1-adrenoceptor subtypes mediating noradrenaline contractions (Amobi and Smith 1992; Amobi et al. 1999). Subsequent studies with α1-adrenoceptor agonists and antagonists on human vas deferens obtained from vasectomy operations confirmed the rat findings (Amobi et al. 2003). The discovery of the mode of drug-action underlying the contraceptive side-effect of thioridazine and phenoxybenzamine led to the identification of a novel subset of drugs related to α-adrenoreceptor antagonists that replicate this unique mode of action (Amobi et al. 2010a, b). Further studies will use the ram as a suitable animal model for in vitro vas deferens studies and then for in vivo efficacy experiments. The attraction of this approach is that treatment would only be on a "as needed" basis and the effect could be over within 24 h, thus reducing the drug burden that could cause side effects.

The targets discussed briefly above are not an exhaustive list and only some of those farthest along in the development pathway are mentioned to give a snap shot of where things stand now.

4 Factors Influencing a Successful Outcome

All the leads for a novel nonhormonal method of contraception are at an early stage and even where inhibitors with adequate potency and specificity have already been identified the time that will be required to reach the clinic is at least 5 years, and probably longer. When one considers the length of time (10+ years) necessary to progress from Phase I to Phase III trials, it can be seen that if all goes according to plan it will be 15–20 years to reach fruition and cost upwards of US $100 million. And this does not take into account the fact that nine out of ten leads will fail for one reason or another. This is one essential reason why it is necessary to have a pipeline full of leads.

It is important that efficacy be as close to 100%, as possible because existing methods, such as oral contraceptive pills, implants, vaginal rings, or IUDs (copper or LNG releasing), achieve that level. Furthermore, the need for contraception is to avoid unwanted pregnancy, and if contraception fails that may result in the need to have an abortion, if that option exists. Better contraceptives should mean less need for abortion, legal and illegal. Lack of side effects, both specific and nonspecific ones, is an important element. Contraceptives are used by healthy individuals

usually for long periods of time, or in contrast very infrequently. Side effects occurring for a few days following use of an emergency contraception method may be tolerable, but not if they occur at the same level week after week or month after month. This, along with efficacy, is one of the factors in determining acceptability of a method. The method of delivery – oral, subcutaneous, intramuscular, transdermal, vaginal ring, film or gel, intrauterine, and sterilization – all play a role in the decision a person (up to now usually a woman) makes when selecting a method. In addition, the method must meet cultural norms and life stage needs – emergency, spacing, or limiting. Cost of a method is also a major factor unless it is subsidized through government programs.

Finally and outside the scope of this article, there is the whole issue of provider-dependent vs. provider-independent methods and what methods are available when a selection is made initially and whether the method will still be available at repeat visits to the supplier. This latter consideration is much more a problem in less developed, resource-poor countries where supply lines are often unreliable. However, such individuals deserve the best and most suitable contraceptives just as their counterparts in developed countries.

5 Resources to Complete the Translational Process

The time to market and the cost noted above provide a significant disincentive to potential developers of new contraceptives. I have recently addressed this issue (Harper 2008). Large pharmaceutical companies by-and-large have reduced their efforts in this arena and are only willing to commit their resources again after successful Phase II or more likely Phase III trials have been completed. Smaller companies generally do not have the funds to support all the activities necessary to reach these milestones. Part of the needed funds can be provided by organizations such as the US NICHD and Medical Research Councils of other countries. Philanthropic foundations have, in the past, been significant supporters of reproductive biology, with Ford and Rockefeller Foundations supporting training programs for foreign scientists from the 1970s, and Andrew W. Mellon Foundation with its support of selected US reproductive biology centers and a twinning program between the US centers and scientists in developing countries. From 1995, support for the field, and contraceptive development in particular, was provided by Rockefeller, Andrew W. Mellon, William and Flora Hewlett, David and Lucile Packard, Bill & Melinda Gates foundations, and also one that chooses to remain anonymous. Today this list has dwindled and only the anonymous Foundation is still active in this area. The drop out was largely due to donor fatigue over the long time and the large sums needed for a successful outcome.

Much has been made of the need to harness the knowhow and deep pockets of big Pharma, and the concept of public/private partnerships has been promoted vigorously since 1995. In some fields, such as development of treatments for TB and prevention of malaria and HIV, there has been substantial industry

involvement. In 2007, there were 23 Global Health Partnerships (Buse and Hammer 2007). With some notable exceptions this has not been the case for contraceptives. Schering AG (now Bayer Schering Pharma AG) was particularly active jointly supporting research with the Consortium for Industrial Collaboration in Contraceptive Research program of CONRAD into new leads for female and male contraception. These projects have now ended due to the corporate reorganization, but provide an excellent model provided willing and able partners can be found.

6 Conclusions

There is some good news and some bad news. The good news is that improvements in existing contraceptives will be forthcoming. There will likely be a positive outcome of the male steroidal approach now in Phase IIb trial. Maybe there will be an improvement in emergency contraception although this is mainly a niche area. Further development of different modes of delivery will continue and will provide options for those who do not like daily pill taking. The bad news is that radically different, nonsteroidal methods, with the potential exception of meloxicam, will not be available for 15 plus years. Hopefully, we can expect one new male and one new female lead to make it to the clinic by 2027. Which of the candidates in the pipeline, or even ones not yet identified, will make it is uncertain?

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Index