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Mitochondrial DNA, Mitochondria, Disease and Stem Cells

Humana Press

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Preface

The transmission of mutated and deleted mitochondrial DNA (mtDNA) from one generation to another through the maternal lineage can result in a series of diseases that, if not severely debilitating, are lethal. These diseases primarily arise through the inability of the affected cells to generate sufficient cellular energy, ATP. The number of diseases described has been increasing steadily over the last 20 years. The first chapter in this book describes the various mitochondrial diseases, their aetiologies and prevalences and provides a clinical approach to their diagnosis.

We then concentrate on Complex I of the electron transfer chain and how deficiencies to this complex can lead to severe cases of disease. This chapter also describes how Complex I is assembled and how mutations to the assembly genes result in mitochondrial disease.

We progress to describe how embryonic stem cells function and how they can be mimicked by somatic cells that have been reprogrammed to behave like embryonic stem cells, namely induced pluripotent stem cells. This sets the scene for the following chapters, the first of which examines the role and need for mitochondria as they pass from the oocyte through to the pluripotent stem cells and into fully differentiated cells. This is followed by an account of how replication of the mitochondrial genome is strictly controlled from the oocyte and into undifferentiated pluripotent and differentiating embryonic stem cells and how this is a vital step during development. It also critically assesses whether induced pluripotent stem cells regulate their mtDNA copy number effectively during differentiation and whether they could thus have any therapeutic benefit. This is followed by a chapter discussing the role of mitochondria and mtDNA in tumor-initiating cells and during tumourigenesis and whether mtDNA defects can lead to cancer. Collectively, these chapters related to stem cell biology demonstrate that the processes of mitochondrial biogenesis, mtDNA replication, pluripotency and differentiation are tightly linked and interdependent.

Finally, we round off with an account of how mtDNA replication is regulated during development and how it is transmitted and segregated. We then discuss how certain assisted reproductive technologies can result in two populations of mtDNA being transmitted to the offspring. We discuss the pitfalls of some the

assisted reproductive technologies that have been proposed to prevent the transmission of mutant mtDNA from one generation to the next.

The contributing authors are experienced and accomplished scientists and clinicians. They have provided in depth accounts and state-of-the-art knowledge from their own specialized areas.

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Chapter 1
Clinical Approach to the Diagnosis of Mitochondrial Disease

Dominic Thyagarajan

Abstract Mitochondrial diseases are primary disorders of the mitochondrial respiratory chain. They are caused by known or presumed genetic mutations in mitochondrial or nuclear genes encoding subunits of the respiratory chain or a number of nuclear genes required for: import of respiratory chain subunits into mitochondria; assembly of functional respiratory chain complexes; replication, transcription and translation of mitochondrial DNA. This complexity is reflected in the different inheritance patterns of mitochondrial diseases and considerable variability in the clinical presentations throughout life. This chapter summarises the epidemiology and presentation of mitochondrial diseases, the major clinical features and classification schemes and presents a diagnostic approach to patient with a presumed mitochondrial disease.

1.1 Introduction

Mitochondria are key organelles in cellular physiology, involved in: ATP production; generation and detoxification of reactive oxygen species (ROS); apoptosis; cellular differentiation; intracellular Ca^{2+} regulation; the urea cycle; steroid hormone and porphyrin synthesis; lipid metabolism; and interconversion of amino acids amongst many other functions and processes. Only half of the $\sim$1,500 proteins estimated to contribute to mitochondrial structure and function are known [1]. Moreover, it is now clear that several human diseases, e.g. certain types of

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inherited neuropathy, are primarily caused by abnormal mitochondrial motility, fission and fusion and that a defect in autophagy of the mitochondrion, 'mitophagy', may be a key process in Parkinson's disease [2].

In common usage, 'mitochondrial disorders' are primary mitochondrial respiratory chain disorders, of presumed genetic origin, affecting oxidative phosphorylation (OXPHOS). OXPHOS, a series of linked biochemical reactions in which the transfer of electrons from the reducing agents NADH (from the metabolism of carbohydrates through the Kreb's cycle) and $FADH_2$ (from the beta oxidation of fatty acids), reduces molecular oxygen to water and generates a proton gradient across the inner mitochondrial membrane which drives the phosphorylation of ADP to ATP.

The clinical approach to mitochondrial disorders is informed by an understanding of the diversity of presentations at various ages and the molecular genetics of the electron transport chain (ETC) or the OXPHOS chain. The OXPHOS chain comprises five multi-subunit enzyme complexes (I–V) containing $\simeq 87$ proteins of which 13 are encoded by the mitochondrial DNA (mtDNA), a circular, double-stranded $\simeq 16{,}569$ bp molecule also encoding two ribosomal RNAs (rRNA) and a complete set of 22 transfer RNAs (tRNA). Nuclear DNA (nDNA) encodes the rest. In addition, a number of nuclear-encoded proteins are necessary for: the import of OXPHOS subunits into the mitochondrion; the assembly of functional multi-subunit OXPHOS complexes from imported to mtDNA-encoded proteins; and replication, transcription and translation of mtDNA. Thus, the OXPHOS chain is a hybrid product of two coordinated genetic systems: nDNA, with Mendelian and mtDNA with maternal transmission. Mitochondrial disease may therefore be (a) autosomal dominant or autosomal recessive, when an nDNA-encoded subunit of the ETC or other protein important in biogenesis of the ETC is affected or (b) maternally inherited, when mtDNA is mutated. Single large-scale rearrangements of mtDNA are usually sporadic, an unaffected mother being unlikely to have more than one affected child and affected mothers only having a small (1/24) chance of having an affected child [3]. The much larger number of nuclear genes involved in mitochondrial biogenesis, OXPHOS complex composition and assembly, mtDNA maintenance, transcription and translation and mitochondrial dynamics predicts that nDNA mutations directly or indirectly affecting OXPHOS function far outnumber mtDNA mutations of which well over 200 point mutations and a similar number of rearrangements have been described.

1.2 Historical Note

Techniques investigating normal mitochondrial anatomy and physiology have progressed through a series of major morphological, biochemical and molecular biological stages, laying the basis for the field of "mitochondrial medicine". Mitochondria were first recognised in the latter part of the nineteenth century, as improved lenses became available. Carl Benda named them in 1898 from the Greek 'mitos' (thread) and 'chondros' (granules) [4]. Pioneering experiments by

Wielend, Warburg and Kielin in the 1920s led to the concept of cellular respiration, dependent upon a highly organised system of bound electron carriers in the inner mitochondrial membrane. Supravital dyes developed in the early twentieth century, allowed mitochondria to be stained in fresh, unfixed preparations. Engel and Cunningham [5] modified one of these (the modified Gomori trichome stain) to examine frozen muscle sections. The application of this stain and later the Seligman cytochrome oxidase (COX) reaction [6] ultimately permitted the histological characterisation of mitochondrial disease.

In 1962, Luft and colleagues established that a hypermetabolic state of non-thyroidal origin was related to loose mitochondrial coupling between respiration and phosphorylation of ADP [7]. Only one definite case with Luft syndrome has been identified since [8], but the discovery was seminal because for the first time, both ultrastructure and Warburg manometry work were applied to the study of human material. Shy and Gonatas [9], using the new ultrastructural techniques divided certain childhood myopathies into one group with proliferated mitochondria and normal appearance (pleoclonial myopathy), and another with enlarged, abnormal mitochondria and disoriented cristae (megaconial myopathy). Drachman [10], Kearns, and Sayre [11] described chronic progressive external ophthalmoplegia (CPEO) with other features and, in such patients, Olsen et al. [12] coined the phrase 'ragged-red' fibres to describe distinctive subsarcolemmal clustering of skeletal muscle mitochondria on the Gomori modified trichrome stain. Ultrastructurally, the mitochondria were enlarged, had abnormal cristae, and sometimes contained para-crystalline inclusions. It became evident that collection of such 'mitochondrial encephalomyopathies' [13] or 'mitochondrial cytopathies' [14] were not necessarily associated with CPEO, and were diverse in their manifestations, including disorders of vision (retinal degeneration, optic atrophy, cataract and glaucoma), deafness, proximal myopathy, neuropathy, encephalopathy, short stature, renal tubular disorders, endocrinopathies and lactic acidosis. In some of these cases, OXPHOS defects were identified biochemically in cytochrome *b*, ATPase, NADH-Coenzyme Q10 (CoQ) reductase, and cytochrome *c* oxidoreductase (COX). Immunohistochemistry for respiratory chain complexes and their individual components (nuclear and mtDNA encoded) identified that in many encephalomyopathies there was a general depression of all subunits, whereas in others, specific subunit deficiencies were found, and also considerable variation existed between adjacent cells, particularly in skeletal muscle.

In the 1980s, with complete sequencing of human mtDNA [15] and the discovery of the molecular genetic basis of mitochondrial disorders came a watershed. Egger and Wilson [16] noted the excess of maternal inheritance in pedigrees with mitochondrial cytopathy and leber's hereditary optic atrophy (LHON), and proposed mitochondrial genetic inheritance, since it was known that mammalian mtDNA was maternally inherited [17]. Then, a specific point mutation in a structural gene of mtDNA in LHON [18], and large-scale deletions in muscle mtDNA from patients with sporadic mitochondrial encephalomyopathies were found in the same year [19]. An explosion of genotype-phenotype correlation followed, revealing certain classes of mutations such as mutations of tRNA first

found in the characteristic syndromes of Mitochondrial encephalomyopathy with lactic acidosis and stroke (MELAS) [20] and Myoclonus Epilepsy with Ragged-Red Fibres (MERRF) [21]. In most, but not all instances (LHON a notable counter example), the mutated mtDNA was found to coexist with the normal "wild type" (heteroplasmy). In general, mtDNA mutations impairing mitochondrial protein synthesis (tRNA mutations and deletions) were noted to be associated with the ragged-red fibres on muscle biopsy, while a morphological clue was absent in mutations of the mitochondrial structural genes.

The more difficult task of identifying mitochondrial disease of nuclear genetic origin began with the finding of dominant inheritance of multiple mtDNA deletions [22]. Recessive and dominantly inherited genes affecting replication or maintenance of mtDNA have now been found to be mutated in a variety of mitochondrial syndromes with considerable phenotypic expression associated with multiple mitochondrial deletions [23–26] or depleted copy number [25, 27, 28]. Of these, mutations in the catalytic subunit gene of mtDNA polymerase, called polymerase gamma gene (POLG), were found to be a relatively common cause of a wide range of dominantly or recessively inherited neurological phenotypes in adults and children including Alper's poliodystrophy, CPEO, ataxia, epilepsy and neuropathy syndromes. This gene has, in addition to a DNA polymerase activity, a 3′–5′ proofreading exonuclease activity. The mutations are associated with multiple mtDNA deletions or depletion. These were disorders of 'intergenomic signalling'. A separate category of gene mutations in nuclear-encoded subunits of OXPHOS emerged, particularly of Complex I, usually presenting with Leigh syndrome (LS) [29, 30]. Another category consisted of defects in nuclear-encoded ancillary OXPHOS proteins, for example Complex IV assembly proteins [31, 32], and an ancillary protein of Complex III [33]. Finally, defects of mitochondrial translation were demonstrated in rare cases, including mutation in a gene encoding mitochondrial ribosomal protein subunit 16 (*MPRS*16) [34] and in another situation of mitochondrial myopathy with sideroblastic anæmia (MLASA)-defective pseudouridylation of mitochondrial tRNA gene due to a homozygous missense mutation in *PUS*1 gene which encoded the mitochondrial enzyme pseudouridine synthase I [35].

1.3 Epidemiology and Presentation of Mitochondrial Disorders

Results of several epidemiological studies of mitochondrial disorders vary in accordance with the methodology, the diagnostic criteria and the population studied. Indeed, such studies are very difficult to carry out and interpret because inaccurate case ascertainment, imprecise diagnosis, population genetic bottlenecks, founder effects and incomplete knowledge of clinical phenotypic spectrum may introduce bias [36]. Because of the high mortality in early childhood, incidence rather than prevalence is a better guide to the frequency of these disorders in

the population. Cases ascertained from several registers in Western Swedish healthcare region over a 15 year period identified 32 children under the age of 16, yielding an incidence of about nine in 100,000 in preschoolers and a minimum point prevalence of 4.7 in 100,000 [37]. A retrospective south eastern Australian study of material referred to a diagnostic referral Centre in a similar 10 year period, gave a very similar minimum birth prevalence for OXPHOS disorders of five in 100,000 [38]. In both studies, only $\simeq 15$ % of children had an identified mtDNA mutation, which is different from mitochondrial disorders diagnosed in adulthood, where mtDNA mutations are more likely to be found [36].

Especially in children, the presentation of mitochondrial disorders is highly variable and may be quite non-specific. A recent British population-based study of progressive intellectual and neurological deterioration in children [39], found that 112 of 1,047 ($\simeq 11$ %) cases with this phenotype in whom a diagnosis had been made had a mitochondrial disorder, the second largest diagnostic group [39], with a 2006 point prevalence of this presentation of 0.62 in 100,000. Of these, the largest group had no characteristic syndrome or specific molecular diagnosis and the presentation was rather non-specific with hypotonia, developmental delay, failure to thrive, gait disturbance/ataxia or seizures. Children with LS and mutations in the mitochondrial ATPase 6 gene comprised the next largest groups, confirming another study revealing LS as the most commonly recognisable syndrome in childhood mitochondrial disease [40]. The best estimate for the lifetime prevalence of mitochondrial disease is one in 5,000 [36]. Due to reduced penetrance, the prevalence of mtDNA mutations in adults may be much higher, one estimate from analysis of neonatal cord blood is that at least one in 200 adults harbour an mtDNA mutation capable of transmission and the de novo mutation rate is $\simeq 0.001$ % [41].

Practically any organ or tissue may be involved, and involvement of three or more organ systems without any better explanation should raise the suspicion of a mitochondrial disease. A progressive, often fluctuating course is typical. Generally speaking, involvement of tissues with higher energy demands such as brain, skeletal and cardiac muscle, the retina, the kidney and endocrine organs predominates.

Certain clinical features are suggestive of mitochondrial disease, particularly when they cluster in recognisable syndromes. In the brain, such features include: 'stroke-like' episodes affecting mainly grey matter in distribution unconforming to vascular territories, usually in the occipital or parietal lobes (typical of MELAS syndrome) [42, 43]; recurrent encephalopathy especially if aggravated or triggered by sodium valproate; and myoclonus with ataxia. Suggestive brain imaging features are symmetrical high T2-weighted and fluid attenuation recovery sequence (FLAIR) signal and low T1-weighted signal in the deep grey matter on magnetic resonance imaging (MRI) in LS and familial bilateral striatal necrosis (FBSN) [43, 44]. Topography aside, the MRI appearance of stroke-like episodes in MELAS in the acute phase is different from acute ischæmic stroke where there is a decline in apparent diffusion coefficient (ADC); in MELAS the ADC is normal or increased, suggesting that the extracellular œdema is a significant component of the lesion [45, 46]. CPEO with ptosis is a characteristic ocular manifestation of some

syndromes. Another characteristic ophthalmologic presentation is the subacute, often bilateral optic neuropathy occurring in LHON. 'Red-flag' features of mitochondrial disease have been suggested in other organ systems [47], but it is important to appreciate that clinical features overlap with many other pædiatric and adult neurogenetic and neurometabolic disorders; hence many findings are relatively non-specific, for example: constitutional features such as short stature and asthenia; neurological features such as bilateral sensorineural hearing loss, intractable epilepsy; basal ganglia calcification or an unexplained leukodystrophy on cerebral imaging; ophthalmological features such as pigmentary retinopathy etc. When these features occur in combination, however, the likelihood of a mitochondrial disorder increases. The confident diagnosis of a mitochondrial disorder can therefore require synthesis of many clinical and relatively detailed diagnostic tests: a complex and demanding task.

Generally speaking, the later mitochondrial disease presents in life, the less florid the course and progression. Individuals are often encountered in adulthood who have enjoyed apparently normal health for a long period. A chronic, insidious course is not invariable; another typical presentation, particularly in young children is that of an episodic illness with rapid progression followed by regression, after triggered by some intercurrent physiological stressor such as infection or surgery.

1.4 Diagnostic Classifications

To aid the diagnosis of mitochondrial disorders, several diagnostic criteria have been proposed. The validity and utility of these schemes has been debated. The first of these [48] was derived from adults and used clinical features and ancillary investigations to classify mitochondrial disorders into possible, probable, and definite categories. However, when an attempt was made to apply these diagnostic criteria to subsequently evaluated groups comprising children with OXPHOS disorders, several difficulties were encountered [49, 50]. The presentation in infants and children is less specific than in adults; most pædiatric patients lack histopathological hallmarks like "ragged-red" or COX-negative fibres and mtDNA mutations [51]. The continuous data derived from biochemical assays of OXPHOS complexes could not be easily fitted to the categorical divisions and there was a lack of additivity of the clinical features and the biochemical assays. Moreover, the biochemical criteria in this scheme did not include studies of the whole OXPHOS chain such as substrate oxidation rates or ATP production even though 30–40 % of all defects in energy metabolism cannot be assigned to a single or combined deficiency of OXPHOS complexes [49]. A modification was subsequently proposed to accommodate children [50].

This modified scheme was further adapted and applied to a group of 30 pædiatric cases. This mitochondrial disease criteria (MDC) scheme [49] uses clinical, laboratory, pathological and biochemical items, and rather than scoring

complete clinical syndromes, scores single clinical symptoms which adapt the scheme to both classic presentations and diffuse paediatric clinical patterns. The scheme design also enables some clinical decision making in investigation because patients are pre-classified on general clinical and metabolic criteria before a muscle biopsy is performed; a biopsy would be recommended if an undiagnosed disorder reaches the probable or definite general classification.

When measurement of ATP synthesis was added to the original classification scheme proposed for adults, the modified scheme proposed by Bernier et al. [50] and the MDC performed very similarly, with some differences in the level of certainty.

At a more conceptual rather than clinical level, the genetic classification of mitochondrial disorders provides a useful framework for the clinician and geneticist. Mitochondrial disorders may be thus classified as in Table 1.1. There is not a one-to-one, but rather a many-to-many relationship between the genetic classification and recognisable syndromes (Table 1.2).

1.5 Classic Mitochondrial Syndromes

Whilst in many cases the presentation of a mitochondrial disease is non-specific, well-characterised syndromes are recognised (Table 1.2). These fall into three broad overlapping groups:

1. mainly skeletal muscle involvement centred on CPEO and limb muscle fatigability
2. multisystem manifestations with an emphasis of central nervous system (CNS) involvement
3. oligosymptomatic syndromes with an emphasis on a tissue other than skeletal muscle, examples being LHON or antibiotic-induced non-syndromic deafness

Table 1.2 summarises the key features of some of the more common and better known syndromes.

1.6 Diagnostic Approach

This begins, as usual, with a thorough history and examination and basic laboratory and radiological investigations, from which it may be possible to fit the case to an easily recognisable syndrome (see Table 1.2). In considering laboratory testing, the main principles of mitochondrial medicine should be borne in mind.

1. Mendelian transmission, maternal inheritance and sporadic conditions are all possible. There is considerable phenotypic overlap and some Mendelian genes, POLG being the most important example, may result in autosomal dominant and recessive disease.

Table 1.1 Genetic Classification of Mitochondrial Disorders

Disorder of:	General Class of defect	Genes Involved	Main syndromes
mtDNA	Protein synthesis	point mutations in and deletions involving tRNA and rRNA genes	KSS;PS;CPEO;MELAS;MERRF
		ND genes and ATPase6	LHON;NARP/LS/FBSN;MELAS overlaps
	Protein coding genes	ND genes;Cytb;COX	Myopathy
Intergenomic signalling (nDNA)	Multiple mtDNA deletions	ANT1;POLG;TWINKLE;TP	Dominant and recessive PEO; MNGIE
	mtDNA depletion	dGK;POLG;TK2	hepatocerebral; Alpers poliodystrophy; myopathy
	Defect of mtDNA translation	EFG1;MRPS16;PUS1	hepatocerebral;generalised
Other nDNA mutations	ETC subunits	NDUFS;NDUFV;SDHA	LS
	Assembly proteins	BCS1L;SURF1;SCO2; SCO1; COX10; COX15; LRPPRC; ETHE1	LS; Leigh syndrome French Canadian Type (LSFC); ethylmalonic encephalomyopathy (EE); growth retardation, aminoaciduria, iron overload, lactic acidosis, early death (GRACILE)
	Fusion/fission/motility	OPA1;MNF2;KIF5A	Autosomal dominant optic atrophy; CMT2A;HSP
	Lipid milieu	G4.5	Barth syndrome

Table 1.2 Some Mitochondrial Syndromes

MELAS	Short stature; migraine; dementia; senosorineural deafness; stroke-like episodes (often occipital and not conforming to metabolic territories), seizures, exercise intolerance, asthenic build and muscle weakness; diabetes mellitus and various other endocrinopathies; intracerebral calcification, cerebral atrophy
MERRF	Myoclonus epilepsy; limb muscle weakness and wasting, particularly respiratory muscle weakness in older patients, ataxia, deafness, retinal pigmentary degeneration
CPEO	Ptosis and progressive complex external ophthalmoplegia; limb muscle weakness and wasting; exercise intolerance; intracerebral calcification, white matter abnormalities on MRI.
KSS	CPEO with onset before age 20, retinal pigmentary degeneration, high CSF protein, heart block (almost invariable before age 50) white matter abnormalities on MRI
LS/FBSN	Psychomotor retardation, poor suck/swallow in infancy and failure to thrive, signs of brainstem dysfunction (respiratory abnormalities, sudden death in infancy, eye movement disturbance, nystagmus); peripheral neuropathy; dystonia and other movement disorders, characteristic bilateral, symmetrical periventricular T2 signal hyperintensities on MRI in the deep gray matter; spongiform change, gliosis and microangiopathic necrosis in the deep gray matter
LHON	Subacute visual failure, particularly in males (M:F ratio 9:1). Dystonia in some patients with the T14484C mtDNA mutation
MNGIE	Gastric hypomotility, CPEO, wasting and weakness, deafness
PS	Infantile sideroblastic anemia
Aminoglycoside-induced deafness	Non-syndromic sensorineural deafness following exposure to aminoglycoside antibiotics

2. Point mutations in mtDNA will be maternally inherited but this may be difficult to discern in the pedigree, particularly a small one.
3. mtDNA molecules are present in many copies and pathogenic mutations generally, with some important exceptions, affect a proportion of mtDNA molecules (heteroplasmy).
4. A certain percentage of mutant mtDNA molecules must be reached for OXPHOS to be impaired. Tissues with different dependence on OXPHOS may affect differently (threshold effect).
5. During mitosis, random segregation of mutant mtDNA molecules occurs, leading to a genetic and functional OXPHOS mosaicism of OXPHOS within and amongst tissues. In some syndromes, for example recurrent myoglobinuria with Cyt *b* mutations in mtDNA or in single sporadic mtDNA deletions associated with CPEO, the genetic abnormality is most abundant or only detectable in muscle.

Careful family history and thoughtful analysis of the pedigree are very important. Father to offspring transmission excludes an mtDNA mutation except in a single recorded circumstance [52, 53] and a pedigree consistent with maternal

inheritance should therefore prompt a search for an mtDNA mutation. In practise, the number of affected individuals is often low, and complete ascertainment is not possible. On the other hand, if extensive pedigree records are kept and new mutations are low, for example in the case of LHON in Australia, a diagnosis may be confidently reached based on the clinical presentation and the family history, using molecular genetic testing only for confirmation.

1.7 Biomarkers and Metabolic Screening

1.7.1 Lactate, Pyruvate and Creatine

There is no satisfactory biomarker for defects of OXPHOS[1]. If aerobic metabolism is impaired, lactate and pyruvate levels accumulate with a decrease in the NAD/NADH ratio. However, the rise in lactate (>2.1 mM) and pyruvate is neither sensitive nor specific enough and the accurate estimation of blood lactate is dependent on proper collection and treatment of the specimen. False positive elevations in lactate may occur with the use of a tourniquet in venepuncture or after exercise/physical agitation as part of what is really a physiological rise. Other causes of lactic acidæmia such as hypoxia, sepsis, shock, renal failure, etc. and other metabolic disorders such as amino acid and organic acidæmias, urea cycle defects, Krebs cycle, fatty acid oxidation disorders and pyruvate metabolism defects, disorders of liver glycogen metabolism and liver gluconeogenesis should be considered [47]. In the brain, prolonged seizures, stroke, malignancy and meningitis may lead to raised CSF lactate levels, but the levels are not influenced by collection technique [54]. Handling of the sample is important in the pyruvate estimation. It should be collected in 8 % perchlorate, immediately placed on ice, and rapidly analysed. The timing of the specimen collection in relation to meal-time is also important; plasma pyruvate is raised in normal individuals for a few hours following a meal. Concurrent measurement of alanine levels may be a useful indicator of long-standing pyruvate accumulation in this situation.

The resting lactate has quite a low sensitivity of only around 26 % and a specificity of 84 % [55] in mitochondrial myopathies but after a 15 min, constant 30 W workload on a bicycle ergometer, the sensitivity rises to 69 % with the same specificity. This is a simple and inexpensive investigation in the outpatient setting. In patients with predominantly encephalopathic symptoms, the CSF pyruvate and lactate levels may be raised even when the plasma levels are normal and sometimes only during periods of acute metabolic decompensation. Thus, lactate and pyruvate estimation should be informed by the clinical picture.

[1] Recently, a raised serum level of FGF-21 has been reported to be a very sensitive and specific biomarker for muscle-manifesting mitochondrial disease, the area under the receiver-operating curve being 0.95 [62], but this awaits further evaluation.

Recently, liquid chromatography coupled with mass spectrometry, applied to derive a metabolic profile from spent media in myotube cultures and then validated in a cohort of mitochondrial disease patients with muscle involvement, has shown a robust elevation of creatine compared with controls [56]. Further independent clinical validation in a range of patients with different presentations will be required before the value of creatine as a reliable marker can be assessed further.

1.7.2 Amino Acid, Organic Acid and Carnitine Profile

Absolute hyperalaninemia (>450 μM) or a relative increase assessed by a raised alanine: lysine (n: < 3) or alanine: (phenylalanine + tyrosine) (n < 4) [49] is suggestive of an OXPHOS defect, but like lactate estimation, the sensitivity is low, the main benefit being the resistance of alanine to artefacts of specimen collection. Urinary organic acids show abnormal profiles in mitochondrial disease. These are routinely measured in the differential diagnosis of metabolic encephalopathies in childhood but do not provide sensitive or specific diagnostic information in mitochondrial disease [57].

Carnitine shuttles long-chain acyl groups from fatty acids into the mitochondrial matrix, so they can be broken down through β-oxidation to acetyl-CoA. Acyl-carnitine profiling and measurement of total and free carnitine levels, along with acyl-carnitine profiling is used to identify defects of fatty acid oxidation which includes secondary fatty acid oxidation defects and carnitine deficiency which may occur in primary OXPHOS disorders.

1.8 Cerebral Imaging

MRI and proton (H) magnetic resonance spectroscopy (MRS) have greatly aided the diagnosis of mitochondrial disorders with an encephalopathic presentation. However, it is important to realise that both investigations may be completely normal, especially if the CNS is not clinically involved and that a number of the findings such as a diffuse leucoencephalopathy, which may be seen in CPEO or MNGIE, or cerebral and cerebellar atrophy are quite non-specific changes [43]. The same applies to non-specific changes such as basal ganglia calcification, most easily appreciated on the computerised tomography. The most striking MRI abnormalities and ones characteristic of a mitochondrial syndrome are the stroke-like lesions in MELAS (Fig. 1.1) and focal bilateral symmetrical signal changes in the deep grey matter in LS and related conditions such as FBSN [43, 45, 46, 57]. Figure 1.2 shows typical changes in LS in 14 year old boy presenting with developmental regression, poor suck and swallow, and hypotonia. LS is the commonest mitochondrial diagnosis in non-specific presentations in children and so MRI can be a very helpful diagnostic aid in this setting.

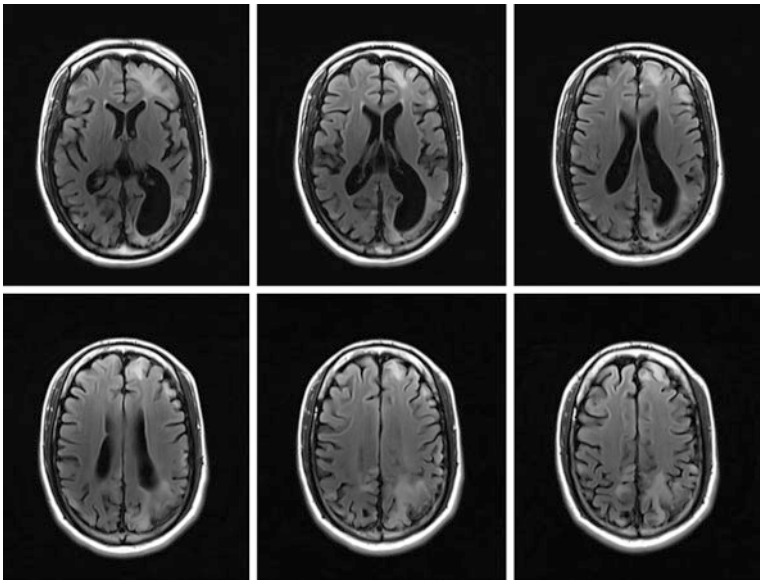

Fig. 1.1 T2 weighted brain MRI in MELAS

The chemical shift peaks expressed in parts per million (ppm), caused by the unique resonance frequency emitted by the most commonly studied brain compounds with MRS are lactate (1.33 ppm), N-acetyl-L-aspartate (2.02 ppm), succinate (2.39/2.40 ppm), total creatine (3.03 ppm), choline (3.22 ppm) and myo-inositol (3.55 pm). Peak area is a rough estimate of metabolite concentration. An elevated lactate peak may be seen even in absence of lactic acidæmia. Elevated CNS lactate is a relatively constant feature of LS, in which lactic acidæmia is often not found and so MRS can help greatly in making the diagnosis figure is the MRS study of the same patient with LS depicted in Fig. 1.3. It is important to realise that the field strength of the magnet and different acquisition parameters affect the spectroscopic pattern and comparison across sites may be difficult. For example, the lipid resonance at 1.0–1.7 ppm in a 1.5 Tesla scanner may mask the lactate peak resonance at 1.33 ppm at short TE times (i.e., 35 ms). At intermediate TE times (i.e., 135 ms), the lactate peak inverts to become distinct, an inversion not so evident on a 3 Tesla scanner; therefore a longer TE time (i.e., 288 ms) may be required. Moreover, many centres report peak area ratios because the MRS signals they acquire are not calibrated with pure compounds of known concentrations before clinical use and so comparison across machines may be difficult [43, 57].

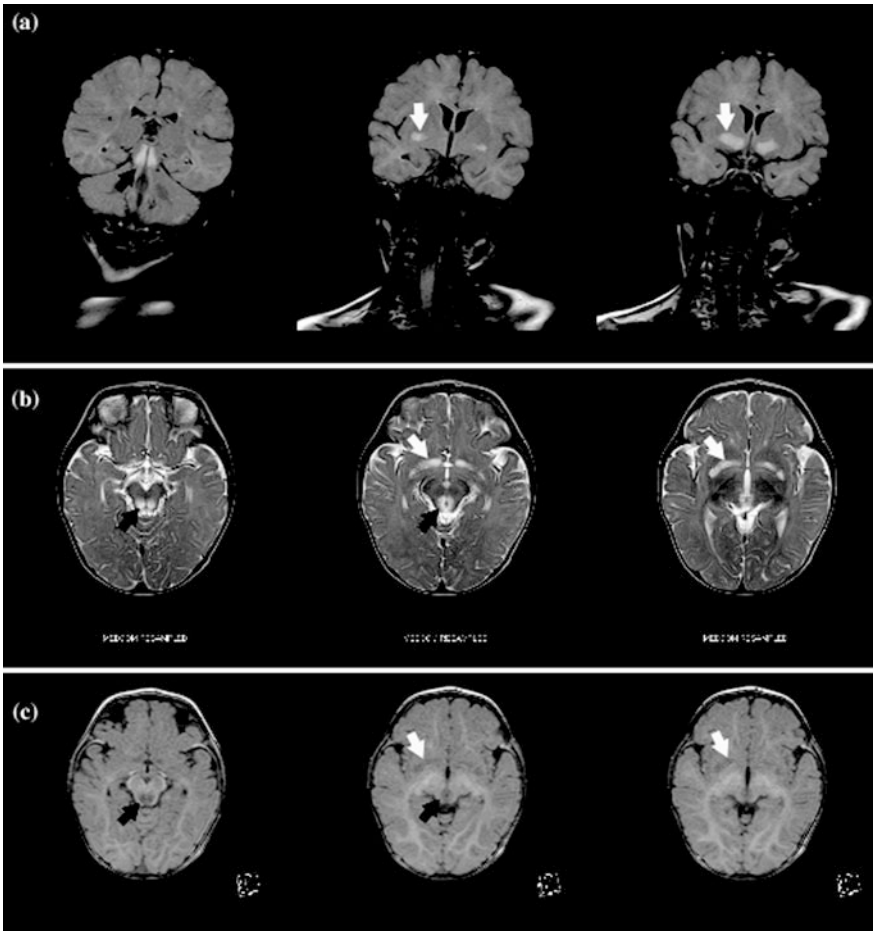

Fig. 1.2 MRI in LS. **a**. Coronal FLAIR; **b**. Axial T2; C. Axial T1. *Dark arrows*: brainstem. *White arrows*: globus pallidus and ventrolateral putamen

1.9 Invasive Testing

In primary mitochondrial disease, the skeletal muscle is the main accessible tissue affected, and in general is the preferred tissue for study if it is available. There are occasional situations in which other tissues are predominantly affected clinically, for example liver or cardiac muscle, and skeletal muscle may show no detectable OXPHOS defect [57]. In such situations, the affected tissue should be biopsied. However, mtDNA point mutations may be detected in other tissues such as blood, hair follicles and urinary sediment, so careful thought should be given to whether a non-invasive molecular genetic screen can be performed first on blood or another easily available tissue, based on the clinical picture, family history and information

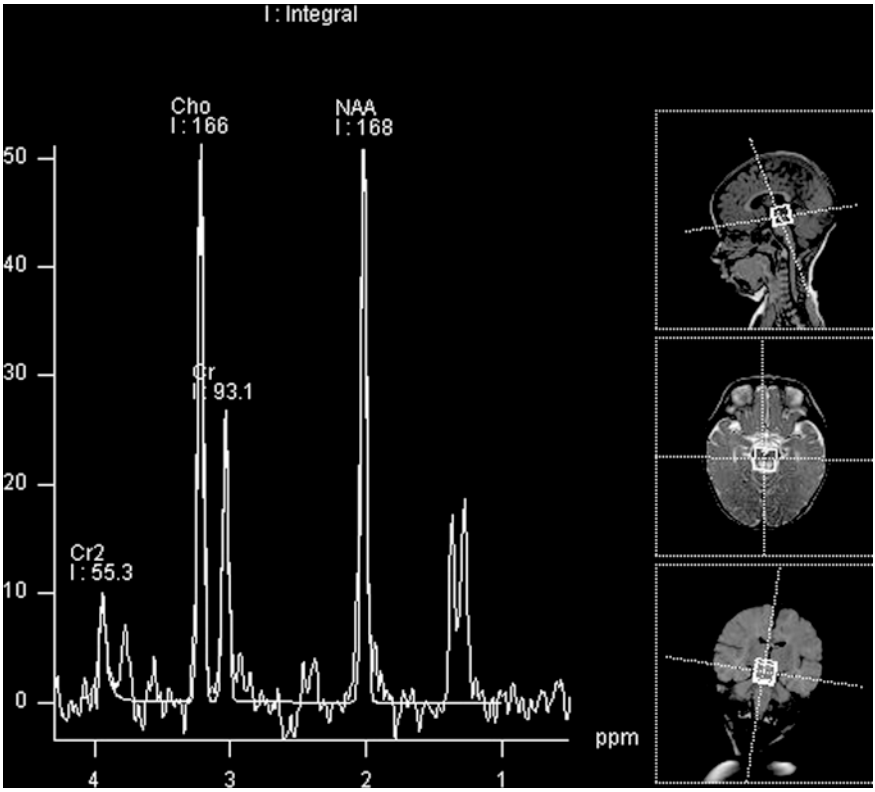

Fig. 1.3 Proton MRS spectroscopy in LS. The characteristic lactate elevation is seen as the double peak at 1.3 ppm

from cerebral imaging. Generally speaking, in children, where non-specific presentations of mitochondrial disease are more likely, this is difficult to achieve.

1.9.1 Muscle Biopsy

Either from an open biopsy or a core biopsy using a Bergstrom needle from which 200 mg of muscle can be obtained in 3 passages in appropriate patients, portions of the biopsy should be kept for morphological studies, biochemical evaluation of OXPHOS activity and DNA extraction. Standard testing requested should include:

1. Histochemistry. A fresh core of about 80 mg in size is placed in a sealed jar and transferred immediately to the laboratory on ice (not dry ice). At the laboratory, the core is frozen in liquid nitrogen pre-cooled isopentane and transferred to a −70° freezer prior to section in a cryostat.

2. Electron Microscopy (EM). Half a needle biopsy core (approximately 35 mg of tissue) is placed in gluteraldehyde for EM. With open biopsy, the EM specimen should be fixed in clamps prior to section and transferred to gluteraldehyde.
3. Biochemistry. A fresh specimen is taken for assays of OXPHOS complex activities (one core/70 mg). Again, this is placed in a sealed jar and transferred on ice to the laboratory. In some instances, muscle is also kept for high resolution respirometry studies of integrated OXPHOS capacity.
4. Molecular Genetics. DNA is extracted from half a needle biopsy core (35 mg).

These investigations may be carried out at different laboratories, though the problem of lost specimens will be avoided if one laboratory, usually the histochemistry laboratory, is responsible for further distribution and optimal use of specimens. If, for unavoidable technical reasons, the amount of material available is less than ideal, it may be necessary for the laboratory to confer with the clinician so that priority may be attached to the testing.

Historically, since the introduction of the Gomori trichome stain, histochemistry has been one of the mainstays of diagnosis in mitochondrial medicine. Reliable enzyme histochemistry, especially the Seligman COX method, further refined diagnosis. Other useful histochemical stains for analysis of mitochondrial enzyme activity are NADH dehydrogenase, and succinate dehydrogenase (SDH). The SDH is for complex II; a complex encoded entirely by nuclear genes, and may also identify subsarcolemmal mitochondrial accumulation. The COX stain evaluates complex IV, which is encoded by both mitochondrial and nuclear genomes. Upon sequential application of these two histochemical stains to a single muscle section, abnormal COX-deficient fibres will appear blue among normal COX activity fibres, increasing the sensitivity of detection of abnormal fibres which might otherwise go undetected against the normal COX positive fibre background, so-called 'ragged-blue' fibres.

All muscle biopsies will be subjected to a range of histochemical analyses including Sudan black or other lipid stain, and ATPase reactions. There may be a mild excessive lipid in some cases as the only abnormality—pyruvate dehydrogenase deficiency, for example.

Typically ragged-red fibres are often identified in mtDNA mutations affecting protein synthesis but are likely to be absent in point mutations of mtDNA structural genes and nDNA mutations affecting OXPHOS subunits, assembly proteins or mitochondrial fusion/fission/motility proteins. They are uncommon in childhood; here, the subtler finding of sub-sarcolemmal accumulations of mitochondria is more common, representing a milder or earlier manifestation of mitochondrial proliferation, still absent in 35 % of 113 paediatric patients with proven mitochondrial dysfunction [58]. The number of ragged-red fibres varies considerably, ranging from only 4 to 5 fibres in a biopsy to 30–40 % of all fibres. A small number of ragged-red fibres lack specificity as they may accumulate with ageing and as a secondary phenomenon in other disorders such as muscular dystrophies, myotonic dystrophy, inflammatory myopathies, glycogenoses, and congenital myopathies.

COX-negative fibres accumulate from the age of about 40, and very small numbers of COX-negative fibres require cautious interpretation; the reasonable

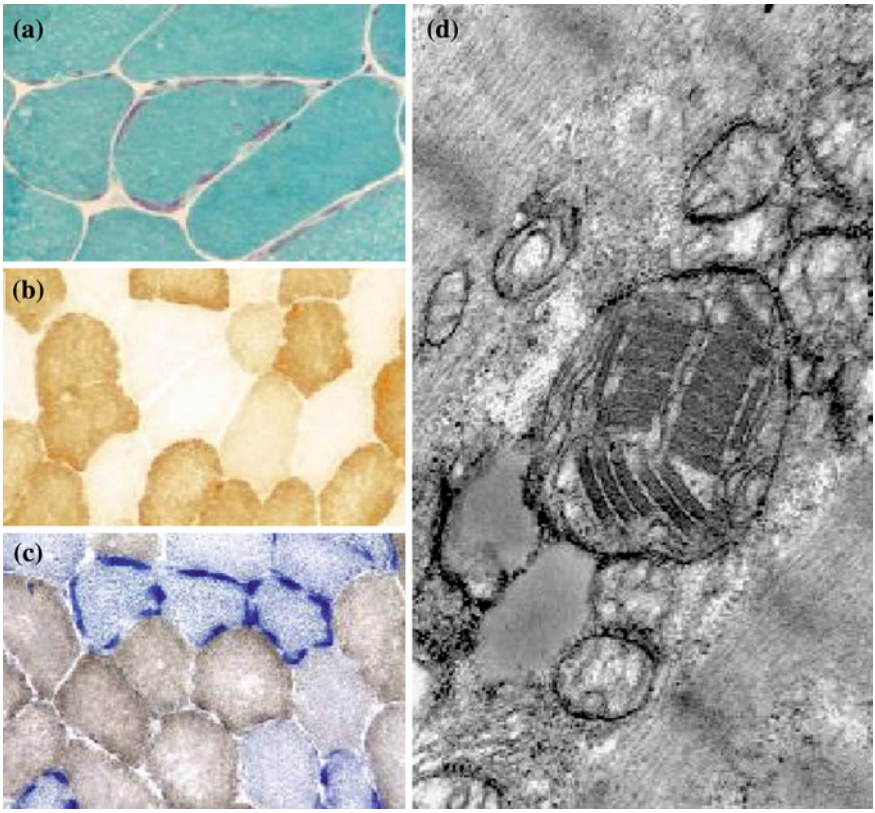

Fig. 1.4 Muscle pathology in mitochondrial disease. **a**. *Ragged-red* fibres on the Gomori trichrome stain. **b**. COX stain showing a mosaic of COX-negative, COX-deficient and COX-positive fibres **c**. COX/SDH stain showing '*ragged-blue*' fibres and **d**. EM showing distorted, simplified cristae, abnormally shaped and sized mitochondria with paracrystalline inclusions

suspicion of an mtDNA-related myopathy requires more prevalent COX-negative fibres than expected in a patient of that age. The number of COX-negative fibres usually greatly exceeds the number of ragged-red fibres. Less commonly, as in some limb myopathies with cytochrome *b* deficiency, or mutations in complex I subunits, COX positive ragged-red fibres may be found. Some infantile myopathies with selective COX deficiency are characterised by a total lack of activity histochemically in all fibres in the biopsy, indicating a nuclear rather than a mitochondrial genetic defect, for example in one of the COX assembly genes (Fig. 1.4).

EM study reveals characteristic ultrastructural abnormalities such as increased mitochondrial number and size, distorted or absent cristae, and osmophilic or paracrystalline inclusions, and they may be the only morphological changes evident, but the changes are non-specific and may be seen in other myopathic and neuropathic diseases [59].

Biochemical investigations include spectrophotometric assays of enzyme activity in isolated mitochondria from fresh tissues, in tissue homogenate or whole cells, and depending on the availability, functional studies of intact mitochondria. Unfortunately, no universally agreed standardisation for these spectrophotometric assays or assay conditions exists, and interlaboratory variability in test results is common. The usual studies are of complex I (NADH-ubiquinone oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), complex III (decylubiquinone-cytochrome c reductase) or complex IV (cytochrome c oxidase), or they can be studied together as complex I + III (NADH-cytochrome c reductase) or complex II + III (succinate-cytochrome c reductase). Robust luciferase based assays of ATP synthase (complex V) have been developed for living cells but are not routine in most laboratories. ATPase hydrolytic activity can be directly assayed by spectrophotometric technique. Activity measurements are reported normalised relative to a marker enzyme, such as citrate synthase, or as internal ratios rather than relative to protein concentration. Despite considerable variability inherent in these investigations, an isolated defect in one complex indicates a mutation of either an mtDNA-encoded or nDNA-encoded subunit or assembly factor of that particular complex (e.g. SURF1 mutation in COX deficiency). Partial defects involving complex I, III and IV are typical of patients with a mitochondrial protein synthetic defect due to either mtDNA deletions or tRNA mutations. Defects in several complexes are also seen in mitochondrial polymerase defects. Combined measurements of I + III and II + III provide useful information in the diagnosis of the potentially treatable coenzyme Q10 deficiency disorders which can subsequently be confirmed with coenzyme Q10 quantification in muscle.

The Clark oxygen electrode used in polarographic studies measures oxygen consumption by isolated muscle mitochondria in the presence of various substrates (e.g., malate + pyruvate or malate + glutamate to donate NADH, succinate to donate FADH, or TMPD + ascorbate to donate electrons directly to cytochrome C). It is not available in all centres and requires the used of fresh tissue. The function of the entire respiratory chain may also be studied using radioactively labelled substrates (e.g., [1–C]pyruvate, [U–C]malate and [1,4–C]succinate) in the absence or presence of various inhibitors to measure production of CO and ATP in relation to citrate synthase activity as a marker of mitochondrial content.

Primary coenzyme Q10 deficiency is quite rare but important to recognise because of the striking benefit of coenzyme Q10 supplementation. It results from deficiencies in enzymes needed for its synthesis and establishing this diagnosis requires detection of impaired coenzyme Q10-dependent respiratory chain activity and a tissue-specific reduction of coenzyme Q10 levels. Plasma levels of coenzyme Q10 are usually normal in primary muscle coenzyme Q10 deficiency.

There are currently four broadly recognised phenotypes for which some patients overlap:

1. an encephalomyopathy with exercise intolerance, myopathy, myoglobinuria, seizures, and ataxia,
2. severe infantile encephalopathy with renal tubulopathy

3. myopathic form with exercise intolerance, myopathy and rhabdomyolysis
4. encephalopathy with ataxia, seizures and basal ganglia disease

1.9.2 Fibroblast Culture

Fibroblasts can be easily cultured from a punch biopsy of skin, stored indefinitely and used as a renewable source of DNA, and re-cultured for testing as new tests become available. The major drawback is that fibroblasts are not always involved in mitochondrial disease and OXPHOS defects expressed in muscle tissue may not be expressed in fibroblasts partly due to altered heteroplasmy and a high tissue regeneration rate of fibroblasts. Therefore, the absence of a biochemical defect in fibroblasts cannot be taken as exclusion of a mitochondrial disorder. Fibroblasts should be cultured with uridine and pyruvate in the media to avoid the potential loss of mtDNA mutant-harbouring cells.

1.10 Molecular Genetic Analysis

If, based on the clinical, imaging and metabolic screening evaluations, a mitochondrial disease is likely, a staged approach to molecular diagnosis is most sensible, to contain costs and optimally use whatever diagnostic resources are available. This will undoubtedly change as sequence capture technology coupled with bi-genomic next-generation sequencing is employed for sequencing previously implicated and candidate mitochondrial and nuclear genes. This has been described in a dataset consisting of the entire mtDNA genome and coding sequences within >3,500 exons of 362 nuclear genes for proteins involved in mitochondrial function, for an aggregate target size of approximately 0.6 Mb excluding repetitive regions [60]. At present, the usual first step in most centres, particularly if a classic mitochondrial syndrome associated with mtDNA mutations (see Tables 1.1, 1.2) is suspected from the preliminary clinical evaluation, is a search for an mtDNA mutation using the traditional Sanger dideoxy-sequencing method, bearing in mind the preferred tissue is muscle. The limitations of the method are that low levels of heteroplasmy (especially when the test tissue is blood), are below the detection limits of the technique [61]. If this is negative, sequencing of the POLG gene should be considered, because it is the commonest nuclear gene mutated with highly variable phenotypes. An important rule of thumb is that 75–90 % of primary mitochondrial disease in childhood results from nDNA mutations whilst an mtDNA mutation is more likely to be found with adult onset presentations [36, 57]. If the mtDNA screen is unrewarding and muscle is not available, a skin biopsy should be sought for OXPHOS studies in fibroblast that may rationally guide further nDNA sequencing. If other tissues such as liver or heart are predominantly affected, then biopsy of these tissues should be considered. A number of

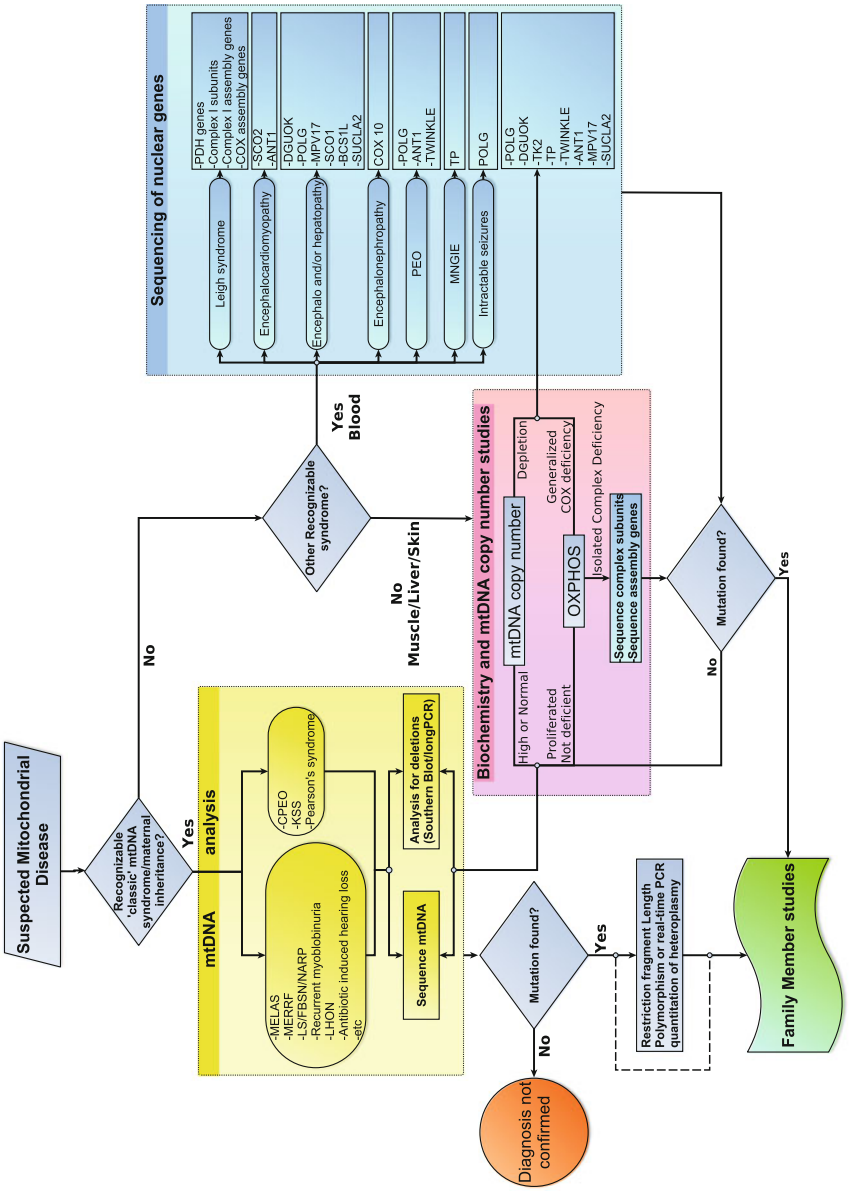

Fig. 1.5 Molecular diagnostic flowchart for suspected mitochondrial disease

approaches are employed in different centres. Figure 1.5 is a diagnostic flowchart modified from the scheme employed at the Baylor College of Medicine, Mitochondrial Diagnostic Laboratory [47]. It can be adapted to suit local centres.

1.11 Summary

In typical cases, mitochondrial disease diagnosis can be relatively straightforward especially if conducted by an informed and knowledgeable clinician. It may simply involve mtDNA testing on a blood sample. In complex or unusual cases, and in children, diagnosis is probably one of the most demanding areas in modern medical practise and requires a close collaboration between the clinical and laboratory team, interpreting results from different diagnostic areas.

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Chapter 2
Mitochondrial DNA Mutations and Their Effects on Complex I Biogenesis: Implications for Metabolic Disease

Matthew McKenzie

Abstract NADH-ubiquinone oxidoreductase (complex I) is a large, multimeric enzyme complex involved in the generation of ATP by oxidative phosphorylation (OXPHOS). It is comprised of 45 different polypeptide subunits, seven of which are encoded by the mitochondrial genome. For complex I to function efficiently it must be assembled correctly from these subunits in a coordinated manner. Disruption of this assembly process can result in complex I deficiency and a wide range of different mitochondrial disorders, including ophthalmological syndromes and fatal childhood encephalomyopathies. This chapter will describe our current understanding of complex I structure, function, and assembly. In particular, how mutations in mtDNA-encoded subunits disrupt complex I assembly and contribute to human disease pathogenesis will be discussed.

2.1 Introduction

Much of a cell's energy requirements are met by the mitochondria, organelles which generate ATP via a process known as oxidative phosphorylation (OXPHOS). The OXPHOS machinery is comprised of five enzyme complexes which are embedded within the mitochondrial inner membrane; NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (complex II), ubiquinol-cytochrome c oxidoreductase (complex III), cytochrome c oxidase (complex IV) and F_1F_o H^+-ATP synthase (complex V). Electrons derived from the

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Stem Cell Biology and Regenerative Medicine, DOI: 10.1007/978-1-62703-101-1_2,

oxidation of carbohydrates are transferred via NADH or $FADH_2$ to complex I or complex II. From here they reduce ubiquinone to ubisemiquinone (CoQH), then ubiquinol ($CoQH_2$), before being transferred to complex III. The electrons are passed to cytochrome c, then complex IV, and finally to $½O_2$ to give H_2O. The energy released by the electron transfer along the respiratory complexes is used to drive H^+ ions out of the inner mitochondrial matrix at complexes I, III, and IV. This creates a transmembrane electrochemical gradient $\Delta\psi_m$ that drives complex V to condense ADP and inorganic phosphate (P_i) to ATP.

Disorders of the OXHPOS system are the most common cause of inborn metabolic disease, affecting approximately one in 5,000 live births [1, 2]. They encompass a wide variety of multisystemic degenerative diseases, commonly referred to as mitochondrial encephalomyopathies, which can exhibit various combinations of clinical features. Brain and muscle are usually affected in these disorders, although other tissues that have high energy requirements may also be involved.

We now know that the function of the OXPHOS complexes, and in many cases their assembly, can be affected by pathogenic mutations in both nuclear and mtDNA. Nuclear DNA mutations have been identified in structural subunits of complex I [3], II [4], III [5, 6], IV [7] and V [8] (although nuclear mutations in subunits of complexes III, IV and V are rare). Mutations have also been described in nuclear genes that encode proteins which are not structural subunits of the mature holo-enzymes but actually aid the biogenesis of the OXPHOS complexes. In many cases, mutations in these assembly factors result in disruption of complex assembly and the depletion of steady-state holo-complex levels. Mutations associated with human mitochondrial disease have been identified in assembly factors of complex I [9–17], II [18], III [19–21], IV [22–27] and V [28].

OXPHOS defects can also be caused by mutations in nuclear genes which encode proteins involved in the replication, transcription and translation of mtDNA [29–36] and in genes that are not directly related to OXPHOS function but cause OXPHOS deficiencies. For example, mutations in the *TAZ* gene cause cardiolipin remodeling defects which result in the destabilization of OXPHOS complex structure in Barth Syndrome [37, 38].

In contrast to nuclear DNA, the mtDNA genome is a very different molecule, existing as a double-stranded, circular structure of 16,569 base pairs. It comprises a control region of approximately 1,000 base pairs (D-loop) which contains the heavy (H) and light (L) strand promoters (P_H and P_L) and the H-strand origin of replication (O_H) [39]. MtDNA also encodes 22 tRNAs and two rRNAs which are specific for mitochondrial translation, and 13 polypeptides, all of which are structural subunits of the OXPHOS complexes; ND1-6, ND4L (complex I), Cyt b (complex III), CO1-3 (complex IV), ATP6 and 8 (complex V).

Over 500 different point mutations in mtDNA have been reported, with a number of large-scale deletions and rearrangements also observed (http://www.mitomap.org). The first mtDNA mutations were described over 20 years ago in the late 1980s, with a point mutation in the complex I subunit gene *MTND4* identified in a patient with Leber Hereditary Optic Neuropathy (LHON) [40] and

large-scale deletions detected in patients with mitochondrial myopathy [41], Kearns-Sayre Syndrome [42] and progressive external ophthalmoplegia [43]. MtDNA mutations have now been reported in all of the 13 protein coding genes, in both the 12S and 16S rRNA genes, in each of the 22 tRNA genes, and in the non-coding D-loop (although many of these mutations are yet to be confirmed as truly pathogenic).

Mutations in the *MTCYB* gene, which encodes the only mtDNA protein of complex III's 11 subunits, have been described in a number of different mitochondrial disorders, including exercise intolerance [44–47], mitochondrial encephalomyopathy [48], cardiomyopathy [49], and multisystem disorders [50, 51]. These mutations generally result in complex III deficiency (or a combined complex I and III deficiency [52, 53]), and in some cases may also disrupt the biogenesis of the complex by altering its assembly kinetics [54, 55]. Mutations have been described in the mtDNA genes *MTCO1* [56, 57], *MTCO2* [58] and *MTCO3* [59–62], which encode for three of complex IV's 13 subunits. These mutations not only result in complex IV deficiency but can also reduce the levels of mature holo-enzyme by disrupting its biogenesis [62]. Mutations in *MTATP6* and *MTATP8*, genes which encode subunits of the hydrophobic F_o module of complex V, have also been described in patients with mitochondrial disease [63]. Depending on the mutant load, mutations in *MTATP6* result in either a progressive, adult-onset disorder known as neuropathy, ataxia, and retinitis pigmentosa (NARP) or the severe infantile disorder Maternally Inherited Leigh Syndrome (MILS) [63]. Alternatively, mutations in *MTATP8* have been reported in patients with hypertrophic cardiomyopathy and neuropathy or severe mitochondrial disease [64, 65]. All of these mutations can disrupt complex V assembly, resulting in the accumulation of stalled subcomplexes and deficiencies in enzymatic activity [64, 66–70].

Pathogenic mutations in all seven mtDNA-encoded complex I subunits have been reported, and can result in a wide variety of different clinical phenotypes. This chapter will describe how these mutations affect complex I activity and contribute to mitochondrial disease pathogenesis. Our current understanding of complex I structure, function, and assembly will also be discussed, with particular reference to how mtDNA mutations affect the biogenesis of the complex.

2.2 Complex I Structure

Electron microscopy studies of purified complex I from a variety of species have revealed that the enzyme has an L-shaped structure consisting of a hydrophobic membrane arm and a peripheral arm that protrudes into the mitochondrial matrix [71]. In mammals, complex I is approximately 980 kDa in size and comprises 45 different subunits [72]. Seven of these subunits are encoded by mtDNA, with the remaining 38 subunits encoded by the nuclear genome. The position of each subunit within mammalian complex I has not yet been fully defined, however, treatment of bovine complex I with mild chaotropic agents dissociates the

holo-enzyme into four subcomplexes (Iα, Iβ, Iλ and Iγ), allowing the identification of subunits within each subcomplex [73–75]. The human complex I subunit nomenclature is based in part according to its subcomplex position, with nuclear encoded subunits designated as either NDUFA (Iα subcomplex), NDUFB (Iβ subcomplex) or NDUFC (Iγ subcomplex). Other subunits are named NDUFS for 'Fe–S protein' (although only the subunits NDUFS1, 7 and 8 contain Fe–S clusters) or NDUFV for 'flavoprotein' (with NDUFV1 and 2 also containing Fe–S clusters). The seven complex I mtDNA-encoded subunits are given the prefix "ND" (NADH-dehydrogenase).

Our understanding of mammalian complex I structure has also been aided by studying its homolog in bacteria. In *E. coli,* complex I is approximately 550 kDa in size and consists of only 14 subunits. These subunits are considered 'core' subunits, in that they are able to form the minimal structure required for efficient electron transfer and proton translocation. Bacterial complex I is formed from three evolutionarily conserved modules; the electron input module (N) and the electron output module (Q), which protrude into the bacterial cytoplasm (mitochondrial matrix in mammals), and the proton translocation module (P), which is embedded within the membrane [76]. The N module consists of the subunits NuoG, NuoF, NuoE (NDUFS1, NDUFV1, NDUFV2) and a flavin mononucleotide (FMN). The module binds and oxidizes NADH, liberating electrons which pass via the FMN to a chain of Iron–Sulfur (Fe–S) clusters. This module has evolved from two separate origins; a soluble NAD^+-reducing hydrogenase found in purple bacteria [77, 78] and cyanobacteria [79] and a formate hydrogenlyase complex of *E. coli* [80].

The Q module is composed of the NuoI, NuoB, and NuoCD subunits (NDUFS8, NDUFS7, NDUFS3, and NDUFS2), with the genes for *NuoC* and *NuoD* fused in bacteria. This module transfers electrons, which have passed through the N module via the Fe–S clusters, to ubiquinone. The Q module is homologous to present-day soluble Nickel–Iron (Ni–Fe) hydrogenases, with the loss of the Ni–Fe active site and the possible addition of a quinone binding site [81].

The P module is composed of the seven subunits NuoH, NuoN, NuoA, NuoM, NuoK, NuoL and NuoJ, which correspond to the mtDNA-encoded subunits ND1, ND2, ND3, ND4, ND4L, ND5 and ND6 in mammalian complex I. This module is involved in proton translocation across the membrane, with the proton pumping subunits ND2, ND4 and ND5 having evolved from bacterial Na^+/H^+ and K^+/H^+ antiporters [82].

Recent structural data have provided insights into how the three modules (N, Q and P) act together to couple NADH oxidation, electron transfer, and proton translocation [83, 84], with a two-state, redox driven mechanism proposed [85]. Following oxidation of NADH at the N module, electrons are transferred via a chain of Fe–S clusters to cluster N2, which resides $\sim$10 Å from the ubiquinone binding site (Q-site) in the Q module. Upon reduction of ubiquinone at this site, membrane arm proton pumping is induced by long-range conformational energy transfer through an amphipathic α-helix of the ND5 subunit. This helix lies in a perpendicular direction to the membrane arm, and its mechanism of action has been likened to that of the pumping of a steam engine coupling rod.

The amphipathic α-helix is coupled to three discontinuous α-helices belonging to the antiporter-like subunits ND3, ND4, and ND5. Movement of these helices alters the conformation of ionisable channel residues in ND3, ND4, and ND5, thereby inducing proton translocation across the membrane [83, 84, 86].

2.3 Complex I Enzyme Deficiencies in Human Disease

Isolated complex I deficiency is the most common cause of respiratory chain dysfunction, accounting for around 50 % of cases [1, 87]. Pathogenic mutations have been identified in nuclear genes that encode both complex I structural subunits and complex I assembly factors [for review see [3, 88, 89]] and in all seven of the complex I mtDNA-encoded subunit genes (Table 2.1).

Patients with complex I deficiencies can present with isolated symptoms or may exhibit multiple tissue involvement. Of note, a single mtDNA mutation can result in different clinical phenotypes in different patients, and conversely, patients who harbor different mtDNA mutations may all present with the same disorder. For example, LHON is a form of blindness which presents in mid-life as acute or subacute central vision loss due to specific defects of the optic nerve [90]. This disorder has been associated with a number of different mtDNA mutations in the complex I subunit genes *MTND1* [91], *MTND4* [40], *MTND4L* [92] and *MTND6* [93]. However, some mtDNA mutations which cause LHON have also been described in patients with dystonia, a disease that presents in early life (usually childhood) with mental retardation, movement disorders, short stature, and degeneration of the basal ganglia. In some cases a correlation has been observed between the percentage of mutant mtDNA molecules (heteroplasmy) and the severity of disease, that is, a lower percentage results in LHON, whereas a higher percentage results in the more severe dystonia [93–95]. However, this correlation does not always hold, as some individuals who have (near) homoplasmic LHON mtDNA mutations do not develop either LHON or dystonia [96].

Mutations in complex I mtDNA genes are also associated with the multisymptomatic disorders mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) and Leigh Syndrome. *MTND1* [97], *MTND5* [98] and *MTND6* [99] mutations have been described in patients with MELAS, a heterogeneous mitochondrial disorder with a variable clinical phenotype. Patients can present with myopathy, encephalopathy and features of central nervous system involvement, including seizures, hemiparesis, hemianopia, cortical blindness, and episodic vomiting [100]. In addition, mutations in the *MTND1* [101] or *MTND5* [102] genes have been identified in patients with LHON/MELAS overlap syndrome, highlighting the possible combination of clinical outcomes due to single mtDNA point mutations.

Mutations in *MTND1* [103], *MTND2* [104], *MTND3* [105], *MTND4* [106], *MTND5* [107] and *MTND6* [108] have been described in patients with Leigh Syndrome, an early-onset progressive neurodegenerative disorder characterized by

Table 2.1 Mitochondrial DNA mutations in complex I deficiency

Gene	Disease	Mutation	References
MTND1	LHON	3460G > A, 3635G > A	[91, 116, 158]
	MELAS	3481G > A, 3697G > A, 3946G > A, 3949T > C	[97, 137]
	TIID/CM	3310C > T	[159]
MTND2	LHON	4640C > A	[158]
	LS	4681T > C	[104]
MTND3	LS	10158T > C, 10191T > C	[105, 160–162]
	DYS	10197G > A	[162]
MTND4	LHON	11778G > A	[40, 116, 117]
	LS	11777C > A	[106]
	PEO	11232T > C	[163]
	EXIT	11832G > A	[164]
MTND4L	LHON	10663T > C	[92]
MTND5	LS	12706T > C, 13513G > A	[107, 136, 165]
	MELAS	13063G > A, 13514A > G, 13042G > A	[111, 137, 165–167]
	MELAS/LS	13084T > C	[110]
	LHON/MELAS	13513G > A	[102]
	MELAS/LS/LHON	13045A > C	[112]
	AT/PEO	13094T > C	[103]
MTND6	LHON	14484T > C, 14459G > A	[93, 115, 116, 168]
	LS	14600G > A	[108, 137]
	TIID	14577T > C	[169]
	LS/CM	14487T > C	[137]
	DYS	14459G > A	[93, 115, 170]
	LHON/DYS	14459G > A	[95, 115]

LHON Leber Hereditary Optic Neuropathy; *MELAS* Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like Episodes, *TIID* Type two Diabetes; *CM* Cardiomyopathy; *LS* Leigh Syndrome; *DYS* Dystonia; *PEO* Progressive External Ophthalmoplegia; *EXIT* exercise intolerance; *AT* ataxia. Mutations listed were scored as pathogenic by Bridges and colleagues [171] using criteria based on biochemical deficiencies, number of independent reports, heteroplasmy, matrilineal variant segregation and conservation [172]. Diseases listed include combined disorders (e.g. LHON/MELAS = LHON/MELAS Overlap Syndrome)

bilateral lesions in one or more areas of the central nervous system. This disorder can present with a range of clinical symptoms, including cardiomyopathy, ataxia, hypotonia and deafness [109]. Leigh Syndrome can also present in conjunction with other mitochondrial diseases, with mutations in *MTND5* associated with MELAS/Leigh Syndrome [110, 111] and MELAS/Leigh/LHON overlap Syndrome [112].

The effects of different mtDNA mutations on complex I activity have been examined by using cytoplasmic hybrids (‘cybrids’) [113], which are created by fusing cytoplast fractions containing mitochondria with ethidium bromide treated

human mtDNA-less ρ^0 cells [114]. Using this technique it was found that the 14459G > A *MTND6* mutation, which is associated with LHON, dystonia and Leigh Syndrome, caused a 60 % complex I enzymatic deficiency but only mild respiratory dysfunction on polarographic analysis [115]. Conversely, the 11778G > A *MTND4* LHON mutation resulted in decreased respiration but complex I enzymatic deficiencies were either mild [116] or undetectable [117]. The 14484T > C *MTND6* LHON mutation also resulted in a mild respiratory defect, but again no complex I deficiency was detectable [116].

Although biochemical defects have been assigned to many of the complex I mtDNA mutations, the exact pathophysiological mechanisms involved remain puzzling. In particular, why does one specific mtDNA mutation result in such a range of clinical severity and phenotypes? It is possible that secondary effects may modulate the biochemical defect caused by the primary mtDNA mutation. For example, the altered binding of CoQ_{10} to complex I when the 14459G > A *MTND6* or 3460G > A *MTND1* mutations are present has been suggested as one possible factor that may contribute to LHON pathogenesis [115, 116]. Impaired CoQ_{10} reduction results in increased levels of ubisemiquinone and subsequently increased ROS generation, a process which may contribute to the premature death of the optic nerve [118]. This theory is supported by the observed increase in ROS generation in differentiated NT2 neurons containing the 11778G > A *MTND4* or 3460G > A *MTND1* LHON mutations [119]. These LHON mutations may also alter mitochondrial apoptotic signaling, with cybrid cells containing these mutations exhibiting increased sensitivity to Fas-induced apoptosis [120]. In addition, growth of LHON mutant cybrids on galactose media, which forces the cells to utilize OXPHOS to generate ATP, also induced apoptotic cell death [121].

Mutations in mtDNA-encoded complex I subunits have been reported to alter mitochondrial permeability transition pore (PTP) opening. Cell lines harboring either the 14484T > C and 14279G > A *MTND6* LHON mutations, or a *MT-ND1* frame-shift mutation, exhibit complex I enzymatic deficiencies and reduced levels of mature complex I (in the case of the *MTND1* mutation) [122]. In addition, the threshold voltage for PTP opening was shifted in these mutant cell lines, being induced close to the resting potential. This suggests that increased cell death due to PTP opening may also contribute to disease pathogenesis in patients with LHON mtDNA mutations [122].

Different mtDNA haplogroups may also influence the pathogenicity of certain mtDNA mutations. Studies have shown that Eurasian haplogroup J is preferentially associated with the 11778G > A *MTND4*, 14484T > C *MTND6*, and 10663T > C *MTND4L* LHON mutations, suggesting that this haplogroup exerts an effect on LHON mutation expression [123–128]. Indeed, it has been reported that cell cybrids containing haplogroup H or haplogroup UK mtDNA have different mtDNA and mtRNA levels, resulting in altered mtDNA-encoded protein synthesis and subsequently altered OXPHOS function [129]. Thus, mtDNA haplotype can affect the disease penetrance of the primary pathogenic LHON mutation, in some cases disrupting Complex I activity by modulating its assembly kinetics [130] (see below).

2.4 Defects in Human Complex I Assembly

Much of what we now understand about the assembly of human complex I has come from studies of complex I deficient patient cells. Early experiments using immunoprecipitation in conjunction with pulse-chase analysis of mtDNA-encoded subunits revealed that the loss of the subunit ND4 results in increased turn-over of other mtDNA-encoded complex I subunits and reduced assembly of these subunits into the mature complex [131]. In addition, although NADH: Q1 oxidoreductase activity was completely absent, NADH:$Fe(CN)_6$ oxidoreductase activity was similar to controls. This suggested that nuclear-encoded subunits were still able to assemble enough of the matrix arm for NADH oxidation to proceed, and that this assembly is independent to that of the membrane arm (which contains the mtDNA-encoded subunits) [131]. Similar results were obtained in mouse cells which harbored a *MTND6* mutation, with complex I activity and assembly of mtDNA-encoded subunits disrupted, but NADH:$Fe(CN)_6$ oxidoreductase activity remaining intact [132], providing further evidence that the assembly of the matrix arm N module is independent to membrane arm assembly.

Mutations in *MTND5*, which result in complete or near-complete loss of the ND5 subunit, were also shown to affect complex I activity [133]. However, unlike the assembly defect resulting from the loss of ND4, the mtDNA-encoded subunits were still able to assemble into the membrane arm and mature complex I (albeit with reduced efficiency) when ND5 was absent [133]. This suggested that different pathways for the assembly of complex I mtDNA-encoded subunits may exist, and that each subunit plays a different role in complex I activity, assembly and/or stability.

These early experiments provided indirect evidence that mutations in mtDNA-encoded complex I subunits could disrupt assembly, however, the development of blue native (BN)-PAGE allowed for the direct analysis of mitochondrial respiratory complex assembly. By using Coomassie Blue G in conjunction with mild, non-ionic detergents, respiratory complexes could now be resolved on polyacrylamide gels in their native form [134, 135]. Using this technique, a novel, pathogenic mutation in the *MTND6* gene, 14487T > C, was shown to alter the mobility and decrease the amount of fully assembled complex I in fibroblasts from a patient with Leigh Syndrome [108]. Stalled assembly intermediates of ~500 and 800 kDa, which contained nuclear-encoded complex I subunits, were also detected.

In a separate study, pathogenic mutations in the *MTND5* (13513G > A) and *MTND6* (14459G > A) genes were also shown to disrupt complex I assembly, with mature complex I, as detected by BN-PAGE, reduced to ~40 and 20 % of control values, respectively [136]. Of note, the *MTND5* mutation was present at mutant loads of approximately 50 % or less in all patient tissues tested, with only 20 % fully assembled complex I detected in fibroblasts. This suggests that the 13513G > A *MTND5* mutation disrupts complex I assembly and function when present at unusually low mutant loads and may act dominantly. This is in contrast to the studies in mouse cells by Hofhaus and Attardi [133], where an *MTND5* mutation had a relatively mild effect on complex I assembly. Thus, it has become

evident that different mutations in the same mtDNA-encoded subunit can have very different outcomes on the activity and assembly of complex I, which in turn may modulate the severity of the resulting clinical phenotype.

More recent studies have also used BN-PAGE to analyze complex I assembly in the presence of mtDNA mutations. A novel mutation in the *MTND2* gene from a patient with Leigh Syndrome was found to result in complex I enzymatic deficiency and disruption of assembly [104]. Levels of mature complex I were reduced, with the accumulation of stalled membrane arm and matrix arm intermediates. Pathogenic mutations in *MTND1*, *MTND5* and *MTND6* were also shown to result in complex I enzymatic deficiencies, however the assembly of complex I was affected to different degrees [137]. Levels of mature complex I were severely decreased in cybrids derived from patient mitochondria which harbored *MTND1* or *MTND6* mutations, whereas the *MTND5* mutation had little effect on the amount of mature complex I [in this case similar to the study by Hofhaus and Attardi 133]. However, all mutations appeared to increase the amount of stalled assembly intermediates, in particular subcomplexes which contain the nuclear-encoded membrane arm subunit NDUFB6 [137].

Studies of three common complex I subunit mutations associated with LHON (3460G > A *MTND1*, 11778G > A *MTND4* and 14484T > C *MTND6*) have revealed that although the steady-state levels of mature complex I are normal, assembly kinetics are affected [130]. Cybrids containing these mutations exhibited increased turn-over of complex I after doxycycline treatment and different synthesis rates of newly formed complex I. In addition, this process was modulated by the mtDNA background, with altered assembly rates and stability of the OXPHOS complexes III and IV. Defects in complex III and IV biogenesis may modulate the assembly/stability of complex I, as complexes I, III and IV are associated together in a large supercomplex or 'respirasome' [138, 139]. Loss of either complex III or IV has been shown to disrupt supercomplex formation, which in turn affects complex I assembly/stability [53, 140–142]. Thus, LHON pathogenic mutations can shift the assembly kinetics of complex I, with the mtDNA haplotype modulating this defect by altering supercomplex (and subsequently complex I) biogenesis [130].

2.5 Assembly Models of Complex I

The first model of complex I assembly was derived from the aerobic fungus *Neurospora crassa* by utilizing pulse-chase labeling of assembly intermediates and the characterization of subcomplexes in mutant strains [143–145]. Compared to human complex I (which is composed of 45 subunits), complex I in *N. crassa* is comprised of only 35 subunits, three of which are not found in the human complex. Therefore, the assembly of complex I may differ substantially between these two species.

To address this issue, human complex I assembly was studied by screening a cohort of complex I deficient patient cells using BN-PAGE, with a set of stalled assembly intermediates identified [146]. From these findings a model of complex I assembly was proposed, whereby both matrix and membrane arm subunits are

found together in early-stage intermediates. Interestingly, this model for human complex I assembly did not correspond to the modular, evolutionarily conserved system proposed for complex I assembly in the fungus *N. crassa,* where matrix and membrane arm subunits are found exclusively in separate intermediates during the early stages of assembly [143].

A subsequent model of complex I assembly utilized a conditional assembly system by blocking, then re-introducing, mtDNA-encoded protein translation [147]. This allowed for the depletion of complex I and the analysis of de novo complex I assembly. Using this system, it was proposed that the peripheral matrix arm and the membrane arm are assembled separately in a semi-sequential process, in this case consistent with the modular assembly found in *N. crassa* [147].

Other studies have utilized a GFP-tagged form of the complex I subunit NDUFS3 to monitor the progression of this subunit into the mature holo-enzyme [148]. Interestingly, this system identified intermediates containing both membrane and matrix arm subunits, resulting in a model for complex I assembly that was similar to that originally proposed by Antonicka and colleagues [146]. The assembly of individual, nuclear-encoded subunits has also been monitored using an in vitro mitochondrial import and assembly assay [149]. In the presence of endogenous complex I a number of subunits assembled via different intermediate complexes into the mature holo-enzyme. Conversely, some subunits appeared to assemble directly into mature pre-existing complex I (and its supercomplex forms) [149].

The assembly of mtDNA-encoded subunits into human complex I has also been examined directly by pulse-chase radiolabeling studies [149, 150]. At early chase times, the mtDNA-encoded complex I subunits ND1, ND2, ND3 and ND6 were detected in assembly intermediates in the range of ~400–830 kDa [149]. At later chase times, the subunits ND4 and ND5 assemble into the (almost) mature holo-enzyme, with complete assembly of all seven mtDNA-encoded subunits requiring at least 24 hours [149]. Pulse-chase studies in patient cells which harbor mutations in the nuclear-encoded complex I assembly factor *NDUFAF1* have revealed that loss of NDUFAF1 results in increased turn-over of ND2, loss of the ~460 kDa assembly intermediate which contains ND2, and the accumulation of a ~400 kDa intermediate which contains ND1 [9]. These findings suggest that ND1 and ND2 are in separate intermediates during the early stages of complex I assembly (corresponding to the proposed assembly pathway of these two subunits in *N. crassa* complex I). This is supported by data from patient cells where mutations are present in the nuclear-encoded complex I assembly factor *C20ORF7* [12]. In this case the opposite is observed; the ~400 kDa intermediate which contains ND1 is lost, with the accumulation of the ~460 kDa ND2-containing intermediate.

The assembly of mtDNA-encoded complex I subunits has also been studied by pulse-chase analyses and the identification of steady-state intermediate complexes in mouse cells with various complex I mtDNA mutations [151]. Using these techniques, five entry points of mtDNA-encoded subunits into the complex I assembly pathway were recently proposed [151]. ND1 was found in an early intermediate complex (point 1), while ND2, ND3 and ND4L were found in a separate early intermediate (point 2). ND4 is subsequently assembled (point 3), followed by ND6 (point 4),

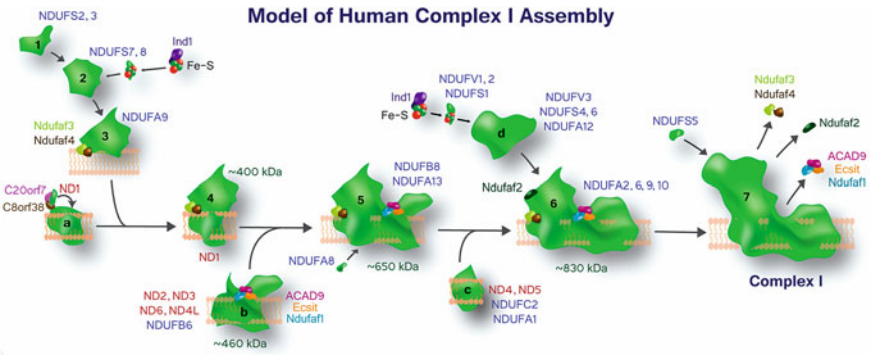

Fig. 2.1 Current model of human complex I assembly. Intermediates 1–6 correspond to the NDUFS3 containing intermediates described by Vogel and colleagues [148]. Entry points of structural subunits (*blue*, nuclear-encoded; *red*, mtDNA-encoded) and the various assembly factors involved are indicated. During the early stages of assembly NDUFS2, 3, 7, and 8 form an evolutionarily conserved hydrogenase module (Q module) as part of intermediate 2, with the assembly factor Ind1 inserting Fe–S clusters into NDUFS7 and 8. With the addition of NDUFA9 (and possibly other subunits), intermediate 3 is assembled and subsequently anchored to the membrane by the assembly factors Ndufaf3 (C3orf60) and Ndufaf4 (C6orf66). An early membrane arm intermediate, intermediate 'a', is assembled from subunits including ND1, whose biogenesis is aided by the assembly factors C20orf7 and C8orf38. Intermediate 'a' and intermediate 3 assemble to form the ~400 kDa membrane arm intermediate 4, while a second membrane arm intermediate of ~460 kDa, intermediate 'b', is assembled from the subunits ND2, ND3, ND6, ND4L and NDUFB6. This intermediate has been found associated with the assembly factors Ndufaf1 (CIA30), Ecsit and ACAD9. During the middle stages of complex I assembly, intermediate 4 and intermediate 'b' assemble together with NDUFA8, NDUFA13 and NDUFB8 to form the ~650 kDa intermediate 5. At this stage ND4, ND5, and possibly NDUFC2 and NDUFA1 (intermediate 'c'), are added to form the ~830 kDa intermediate 6. The subunits NDUFA2, 6, 9, and 10 are also added at this stage, with the assembly factors Ndufaf1, Ndufaf2 (B17.2L), Ecsit, ACAD9, Ndufaf3 and Ndufaf4 remaining associated with this intermediate. During the last stages of assembly the N module is formed from the subunits NDUFV1, 2, 3, NDUFS1, 4, 6 and NDUFA12 (intermediate 'd'), with Ind1 inserting Fe–S clusters into NDUFV1, 2 and NDUFS1. Lastly, intermediate'd', the subunit NDUFS5, and the ~830 kDa intermediate 6 are assembled together to form mature complex I

which appears to facilitate the assembly of the ND1 and ND2 containing intermediates. ND5 is assembled last, defining the 5th entry point [151].

From the studies described here, in conjunction with analyses of patient cells that harbor nuclear gene mutations in complex I subunits or complex I assembly factors, a model for human complex I assembly can be proposed (Fig. 2.1). During the early stages of complex I biogenesis, an intermediate is formed from the matrix arm subunits NDUFS2 and 3 (intermediate 1) [148]. NDUFS7 and 8 are added to form intermediate 2, with the assembly factor Ind1 involved in the insertion of Fe–S clusters into these two subunits [152]. Further subunits are added (including NDUFA9) to form intermediate 3 [146, 147]. This intermediate is associated with the assembly factors Ndufaf3 and Ndufaf4, proteins which may act to anchor intermediate 3 to the membrane [11].

The mtDNA-encoded subunit ND1 is assembled into membrane arm intermediate 'a', possibly with the aid of the assembly factors C20orf7 and C8orf38 [12, 17, 89]. Intermediate 'a' is then combined with intermediate 3 to form intermediate 4, an ~400 kDa complex which contains parts of both the membrane and matrix arms [88, 146]. A separate membrane arm intermediate (intermediate 'b') is formed from mtDNA-encoded subunits ND2, ND3, ND6 [149], ND4L [151] and possibly the nuclear encoded subunit NDUFB6 [89, 147, 152], and is associated with the assembly factors Ndufaf1, Ecsit, and ACAD9 [9, 13, 153, 154].

During the middle stages of complex I assembly, intermediate 'b' and intermediate 4 combine to form intermediate 5 [148], with the subunits NDUFA13 [148] and NDUFA8 (which inserts from the intermembrane space (IMS) side of the inner membrane [155]) also assembled. The mtDNA-encoded subunits ND4 and ND5 are added [88], with co-evolution analyses predicting an interaction between these two subunits and NDUFC2 [156]. This would suggest that NDUFC2 is also assembled at this point with ND4 and ND5 (intermediate 'c'). Furthermore, NDUFA1 is predicted to interact with ND1, ND4 and ND5 [156], and may also assemble at this stage to help anchor ND4 and ND5 to the growing membrane arm. Indeed, in vitro import studies with isolated mitochondria suggest that NDUFA1 assembles at this stage, along with the subunits NDUFA2, 6, and 10 to form the ~830 kDa intermediate 6 [149]. The assembly factors Ndufaf2 [10], Ndufaf1 [9, 154], Ndufaf3 [11], Ndufaf4 [11], Ecsit [153] and ACAD9 [13] are also associated with this ~830 kDa intermediate.

During the latter stages of complex I biogenesis the ~300 kDa matrix arm intermediate 'd', which contains the N module, is formed from the subunits NDUFV1, 2, 3, NDUFS1, 4, 6, and NDUFA12 [149], with the assembly factor Ind1 involved in insertion of Fe–S clusters into NDUFV1, 2 and NDUFS1 [152]. Intermediate 'd' and intermediate 6 combine, with the addition of further subunits (including NDUFS5 [147]) to form the mature complex.

2.6 Concluding Remarks

By piecing together information from the studies described in this chapter, we have been able to gain insights into the assembly process of complex I and how this relates to its function. Furthermore, we are now developing an understanding of how pathogenic mutations can disrupt complex I assembly during different stages of its biogenesis, thus affecting the activity of the enzyme complex. This new knowledge may prove invaluable for future therapeutic design, where treatment of mitochondrial disorders will require restoration of complex I function by manipulating its biogenesis. This will be of benefit for patients not only with classical mitochondrial diseases but also individuals who develop neurological disorders, such as Parkinson's Disease [157], where complex I dysfunction has been implicated.

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Chapter 3
Embryonic Stem Cells: A Signalling Perspective

Marios P. Stavridis

Abstract Since their discovery more than 30 years ago, embryonic stem (ES) cells have been propelled from relative obscurity into the centre stage of international attention. They have enabled the study of gene function in animal models, provided a platform for the study of early developmental processes and now their enormous promise for the study and treatment of many diseases is being tested, although to date very few clinical studies have been initiated to translate this promise into therapeutic outcome. Here, I review the progress made in understanding how signals from the environment influence pluripotent cell self-renewal and differentiation and discuss some of the differences encountered between pluripotent cells from various species and distinct developmental origins. The interaction between these signal transduction pathways is of critical importance, as it ultimately orchestrates the behaviour of ES cells by controlling the expression of pluripotency determinants as well as lineage effectors. Induced pluripotent stem cells (iPS cells) generated by reprogramming of somatic cells are also discussed and related to the pluripotent cell states which can be captured during normal embryonic development.

3.1 Introduction

The derivation of the first mouse embryonic stem (ES) cell lines more than 30 years ago [16, 44] was a landmark event which established the basis for what is now a very broad field of research. Despite their massive impact on science since,

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the original publications were of interest to a relatively limited audience at first. Studies on the properties of teratocarcinomas and embryonal carcinoma (EC) cells had set the scene decades before and the earliest papers held few clues about the importance of this new cell type. ES cells really got their break when it became clear that they could be used to generate germline chimaeras, followed by the demonstration of homologous recombination and targeted gene inactivation in these cells [4, 80]. Even so, for many years in most publications ES cells were simply tools to generate knockout mice for further study and few laboratories were concerned with the study of the pluripotent cells themselves.

After the derivation of stem cells from the blastocysts of rhesus monkey [82] and human [81] though, the properties and promise of these cells captured the imagination of scientists and lay public alike. In the intervening period, ES cells have stimulated intense debates on the ethics of animal and human experimentation, caused a number of countries to rewrite their laws regarding the status of the human embryo and been the subject of court cases with far-reaching implications [59]. The advent of induced pluripotent cells is but the latest chapter in the life story of pluripotent cell lines [76]. Within a very short period of time these cells have been the object of intense study (probably by more people than have ever before been involved in EC and ES cell research) which has already provided valuable insights into the establishment and maintenance of pluripotency during development, reprogramming and more traditional ES cell culture.

In this chapter, I will describe some of the mechanisms regulating pluripotent stem cell self-renewal and differentiation. The focus will be mostly on mouse ES cells, as they are the best understood system, although some sections will also deal with mouse epiblast stem cells (EpiSC) and human ES (hES) cells, as well as induced pluripotent stem cells (iPS cells).

3.2 Embryonic Stem Cells

ES cells are pluripotent cells derived from the inner cell mass of the preimplantation blastocyst (Fig. 3.1a–c). They were initially derived from mouse embryos of the 129 strain of mice, using conditions previously optimised for the culture of undifferentiated mouse EC cells. The efficiency of mouse ES cell derivation in the early days was relatively low and very dependent on the strain of mice used. Even in the best cases, efficiency of ES line derivation was not higher than 30 % [63] (and for most people closer to 10 %) [50]. 129 mice were easiest to derive from, C57BL/6 were possible also [37] as were CD1 [72] and C57BL/6 X DBA crosses [37] but most other strains proved refractory to ES cell derivation, hinting at the possibility that these cells were strange artefacts of in vitro adaptation rather than representing a true pluripotent state in vivo. Improvements in techniques and culture media eventually enabled the derivation of ES cell lines from refractory

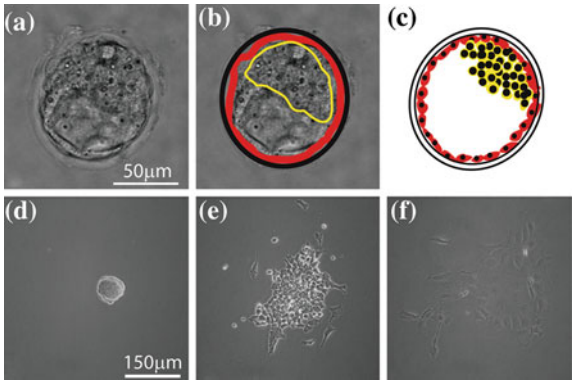

Fig. 3.1 Morphology of mouse embryonic pluripotent cells. **a** A mouse early blastocyst at 3.5 days post fertilisation. **b** Photograph from (**a**), highlighting the structures within the blastocyst. *Black line*: the Zona Pellucida; *red*: trophectoderm cell layer; *yellow*: outline of the inner cell mass. **c** Schematic representation of a mouse blastocyst. Colours as in (**b**). **d** Naïve mouse ES cells under 2i + LIF conditions note the compact, domed shape of the colony. **e** ES cells in conventional LIF supplemented media. Cells appear flatter and some differentiation around the edge of the colony is apparent, suggesting the presence of primed ES cells. **f** The same ES line as in (**d**), (**e**) grown in the absence of LIF, showing the morphology of differentiated cells under these conditions. Scale bars for (**a**–**b**): 50 μm, (**d–f**): 150 μm

strains such as CBA/Ca as well as from 100 % of embryos of the 129 strain [6] demonstrating that ES cell derivation potential is likely a general property of all mouse strains. Key to this was the discovery that ES cell derivation efficiency could be dramatically improved when blastocysts were isolated following a period of delayed implantation known as diapause [6]. In several mammalian species embryonic development can be experimentally delayed just prior to implantation if the mother is ovariectomised following fertilisation. In the wild, the same effect is achieved when a female is suckling a previous litter or under adverse environmental or nutritional conditions [47]. During this period in the mouse, blastocysts develop and hatch from the zona pellucida, but then arrest their development until more favourable conditions return; it is unclear why this suspension of developmental progression assists ES cell derivation, but it is certainly not sufficient to allow derivation from non-permissive strains. Nowadays, ES (or ES-like) cells have been derived from numerous species and the question of their origin seems long since settled. However, closer examination of the properties of various pluripotent cell lines can still reveal insightful information about lineage specification and early development.

3.3 Signals Controlling Pluripotency

3.3.1 LIF/Stat3

In the early days, mouse ES cells were derived and cultured either on feeder cells or in the presence of feeder or EC cell-conditioned media, always in the presence of 10–20 % serum [4, 16, 44]. The identification of Leukaemia Inhibitory Factor (LIF) as the molecule responsible for this activity [66, 94] enabled a more careful dissection of the signalling mechanisms supporting the undifferentiated state. LIF is a cytokine of the interleukin-6 (IL6) superfamily. It binds to and activates its cognate receptor (LIFR) which (in complex with the co-receptor gp130) leads to activation of the Janus kinase JAK and the latent transcription factor Stat3, which in turn promotes self-renewal (Fig. 3.2) [54]. Surprisingly, the core ES cell transcription factors Oct3/4 (also known as Oct3, Oct4 or Pou5f1), Sox2 and Nanog are not transcriptional targets of Stat3. It has been suggested that Stat3 promotes self-renewal by stimulating the expression of c-myc [11], however, this transcription factor's activity is regulated post-translationally by multiple signalling pathways (see below) and is usually present at very low numbers within cells [28], so other signalling pathways likely contribute or control c-myc's effect on pluripotency. Other LIF targets include Klf4 which, together with the Oct4 target Klf2 contribute to the ES cell self-renewal transcriptional programme [22].

Paradoxically, neither LIF [70], LIF receptor [40, 89] nor the LIFR dimerisation partner gp130 [101] knockouts have abnormalities in blastocyst formation in vivo. These findings suggested that the emergence of pluripotency and its (brief) maintenance necessary for expansion of the epiblast compartment during preimplantation development are different from the maintenance of ES cells in vitro, and that ES cells therefore represent a different population of cells than those of the early blastocyst. Nevertheless gp130 signalling is required for the maintenance of the mouse blastocyst during diapause [51]. This establishes a relationship between the in vitro maintenance of pluripotency by LIF and the in vivo state from which ES cells are most readily derived and suggests that implantation delay facilitates ES cell derivation by providing the opportunity for adaptation to the signalling mechanisms which maintain these cells in culture.

3.3.2 BMPs/Id

For many years, all ES cell culture and differentiation were performed in serum-containing media [69], and the contribution of serum components to ES cell maintenance was largely ignored. This led to the mistaken conclusion that Stat3 activation is sufficient to prevent mouse ES cell differentiation [45]. However, LIF is not able to inhibit mouse ES cell differentiation in the absence of serum and neural differentiation ensues with little delay [99]. For full inhibition of differentiation, LIF

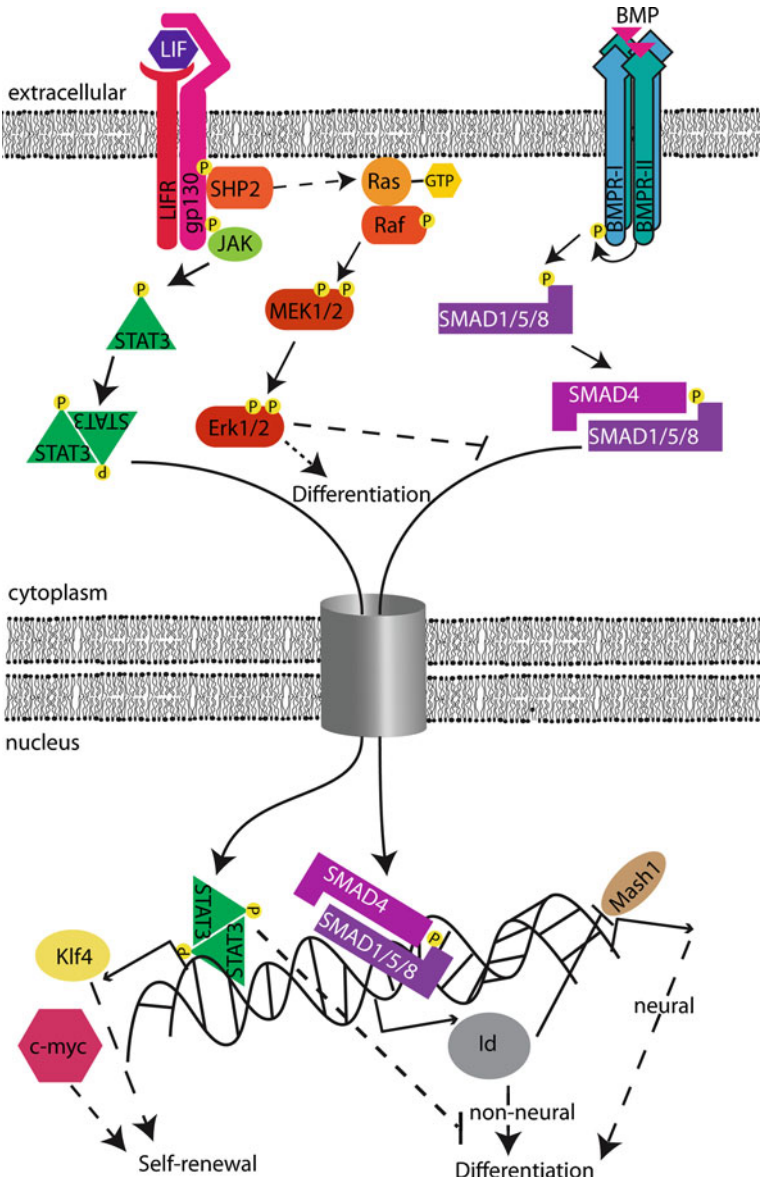

Fig. 3.2 LIF/BMP mediated self-renewal in mES cells. LIF stimulation activates both positive (Stat3) and negative (Erk) regulators of self-renewal. BMP stimulation induces expression of Id proteins which inhibit the neural bias imposed by proneural factors like Mash1, and promote non-neural differentiation. Stat3 regulates the expression of transcription factors like Klf4 and c-myc which contribute to self-renewal and can block the non-neural fates induced by BMP. In the absence of BMP/Id, Stat3 is not sufficient to prevent neural differentiation

requires that Id proteins are activated, inhibiting the proneural effect of basic helix-loop-helix transcription factors such as Mash1 (Ascl1) [97]. The induction of Id genes can be accomplished either by stimulation with serum or transforming growth factor beta (TGFβ) family members BMP2, BMP4 or GDF6 [97]. By the coordinated action of Stat and Id transcription factors derivation and clonal expansion of mouse ES cells can be accomplished with high efficiency, through the suppression of differentiation. The resulting clones are capable of extensive contribution to chimeras including the germline, demonstrating full pluripotency. Overexpression of the homeobox transcription factor Nanog is sufficient to prevent differentiation in the absence of Id and Stat3 activity [97], however, Nanog itself is a transcriptional target of neither, suggesting an alternative mechanism also able to promote self-renewal. Although Id genes appear to be the main contributors to the self-renewal downstream of BMP stimulation, there may well be other targets downstream which play important roles.

3.3.3 Fgfs/Erk

Activation of the gp130 receptor results, apart from Jak/Stat activation, in phosphorylation (activation) of the classical Erk1/2 mitogen activated protein kinase signalling pathway (Figs. 3.2, 3.3). However, unlike the actions of Stat which promote self-renewal, activation of Erk downstream of LIF was shown to antagonise it, promoting differentiation instead [9]. Pharmacological inhibition of the Erk activating kinase Mek resulted in reduced differentiation efficiency in embryoid bodies and maintenance of expression of the ES cell marker Oct3/4. Even in the absence of serum and other mitogens, ES cells exhibit basal levels of Erk phosphorylation in response to autocrine fibroblast growth factor (Fgf) stimulation [68]. When Fgf receptor activation is prevented (either through pharmacological inhibition, expression of a dominant-negative Fgfr or genetic ablation of the main ES cell ligand Fgf4), mouse ES cells are unable to differentiate and retain expression of the ES cell marker Oct3/4 [35, 68, 99]. Deletion of the enzyme Ext1 (required for the formation of heparan sulphate, a co-receptor for several growth factors including Fgf) also results in reduced Erk phosphorylation and loss of differentiation ([32], [60]). In the absence of Erk activity expression of differentiation markers is lost, with the exception of the epiblast marker Fgf5 [68] which is normally induced transiently early during differentiation. It is unclear whether the upregulation of Fgf5 has any functional role, as other epiblast markers are not expressed and the cells increase the expression of Nanog (which is normally expressed at low levels in the epiblast compared to ES cells or the inner cell mass).

The targets of Erk responsible for initiating the differentiation process are not yet fully understood. The duration of Erk activation appears to be important in determining whether cells will differentiate [68], and this indicates the presence of a downstream relay involving unstable protein products of immediate-early genes (IEGs) which are first induced and subsequently stabilised through

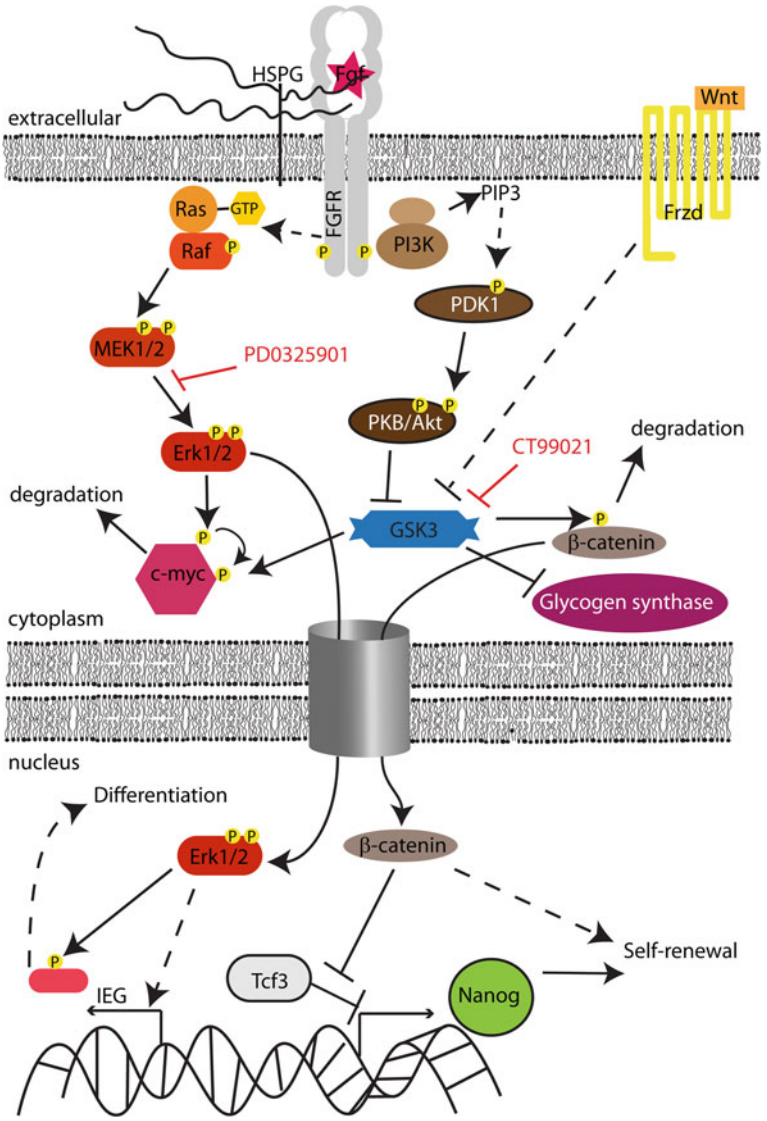

Fig. 3.3 Regulation of mES cell self-renewal by growth factor signalling. Growth factors like FGF promote mES cell differentiation by activating Erk signalling. The precise mechanism for Erk action is unclear but may involve induction and phosphorylation of immediate-early genes (IEG). Inhibition of Mek (e.g. by PD0325901) prevents Erk phosphorylation and differentiation onset but is not sufficient for long-term ES cell expansion, requiring inhibition of GSK3. This can be achieved either by stimulation with Wnt, via PI3 kinase (PI3 K) or pharmacologically (e.g. using CT99021). GSK3 normally negatively regulates β-catenin targeting it for degradation. In the absence of GSK3 activity, β-catenin can accumulate and regulate the expression of ES cell genes (either directly or, like Nanog, indirectly by displacing the repressor Tcf3). These pathways interact at multiple points (e.g. Erk and GSK3 coordinately regulate c-myc degradation) and their relative activity influences cell behaviour

phosphorylation by Erk when it is activated in a sustained rather than transient fashion [49] (Fig. 3.3). IEG induction involves phosphorylation of complexes containing the serum response factor (Srf) which bind to and induce transcription from serum response element (SRE) containing promoters. The hypothesis above is therefore consistent with the defect in differentiation of Srf-null ES cells [92], however, the precise identity of such downstream effectors remains elusive. Interestingly, Erk can phosphorylate S62 of the transcription factor c-myc which is thought to be contributing to the self-renewal programme [86] (see Fig. 3.3).

3.3.4 Wnt/Gsk3

Despite the lack of expression of differentiation markers, mouse ES cells do not thrive in conditions of low Erk activity, suggesting that Erk plays additional roles contributing to cell viability, or that other signals cooperate with Erk to promote it. This deficiency can be overcome by inhibition of the glycogen synthase kinase 3 (GSK3), leading to long-term expansion of germline competent cells [100]. GSK3's role in ES cell differentiation was first indicated by experiments showing that canonical Wnt signals (mediated by GSK3 inhibition) antagonise neural fates [2]. More directly GSK3 inhibition was shown to promote self-renewal of mouse and hES cells, allowing maintenance of mouse cells in the absence of LIF [64], although these findings are confounded by the lack of specificity of the inhibitor used [55]. Inhibition of Mek and GSK3 together (2i media) enabled for the first time the derivation of germline competent ES cells from refractory strains of mice such as NOD [52] as well as rats [8, 41]. In these conditions, LIF is not required for the maintenance of pluripotency, and its mediator Stat3 can be deleted [100]. Nevertheless, inclusion of LIF in 2i conditions improves the growth parameters of the cultures (MPS unpublished observations) and LIF can synergise with GSK3 inhibition for long-term maintenance of mouse ES cells without use of a Mek inhibitor [55, 100].

GSK3 is an enzyme with diverse roles in many cellular contexts. It is found to be active in most cell types and phosphorylation of its targets often leads to their inactivation or degradation via the proteasome pathway [18]. One such target is the transcription factor c-myc; phosphorylation of c-myc by Erk (or other kinases) at S62 primes it for subsequent phosphorylation on T58 by GSK3. Dual phosphorylation recruits phosphatases which then dephosphorylate S62, and the singly T58 phosphorylated form of c-myc is then targeted for degradation by the proteasome pathway [87]. Expression of a mutant c-myc (T58A) which cannot be phosphorylated by GSK3 can promote ES cell self-renewal in the absence of LIF [11].

GSK3 can be inhibited by phosphorylation by protein kinase B (also known as Akt) downstream of Insulin and growth factor stimulation which activates the PI3 kinase pathway [15]. This pathway is thought to contribute to ES cell self-renewal via regulation of Nanog expression [71]. Another mechanism of inhibiting GSK3 operates downstream of canonical Wnt signalling and results in the disruption of a

protein complex which includes GSK3 and its substrate β-catenin, enabling the latter to accumulate in its unphosphorylated form and mediate transcription (reviewed in [18]). It has been suggested that GSK3 inhibition in ES cells is required mainly for increasing growth and viability although it also biases cells against neural differentiation in the presence of Erk activity [100]. However, a number of alternative explanations for GSK3's role have also been proposed, including Wnt/β-catenin dependent and independent functions [11, 43, 77, 95, 96]. From the most recent reports, a consensus emerges on an important role of a Wnt-orchestrated transcriptional control of the pluripotency-sustaining transcription factor network via β-catenin and other related transcription factors, although GSK3 almost certainly has other targets in ES cells which regulate pluripotency or viability (Fig. 3.3).

3.3.5 Oxygen/HIF

Preimplantation development naturally occurs in low oxygen conditions, as the circulation is not yet established. In the mouse, it has been known for over 40 years that in vitro preimplantation development can be adversely affected by high (10–21 %) O_2 concentrations [93], but traditional cell culture is done at atmospheric O_2 (21 %) and stem cell culture has been no different for the most part. However, there are several reasons why ES cells may benefit from a reduced O_2 environment not least because increased O_2 can lead to the production of free radicals (e.g. reactive oxygen species, ROS) which can cause molecular damage to cells with the fast cell cycle of ES cells. It turns out that lowering O_2 levels to 5 % can improve human ES cell cultures by reducing background differentiation [17]. It is possible that this is an effect of reduced ROS production, although more recent work suggests that it may be (at least in part) due to regulation of transcription by the hypoxia induced transcription factors HIF. The transcription factor Hif2α has been shown to regulate the expression of Oct3/4 [14], and Hif1α was recently implicated in the regulation of β-catenin target expression in ES cells [46]. Hif1α null mouse ES cells also proliferate more slowly than wild types [30] (although these cells were derived form heterozygotes under high G418 selection which can result in karyotypic abnormalities and a rescue experiment to exclude such a possibility has not been published).

It may also be that reduced oxygen availability may contribute to a "quiet embryo" phenotype, proposed to be important for successful preimplantation development [38]. Taken together, these studies clearly demonstrate a role for oxygen concentration in the regulation of stem cell behaviour, and the mechanisms for this are likely multiple.

3.3.6 Lineage Effectors

In the sections above, I discussed mainly some of the key signalling pathways responsible for the maintenance of the undifferentiated cell state in mouse ES cells. Some of them (e.g. Erk) appear to be directly involved in the process of self-renewal, but others likely operate by promoting or inhibiting differentiation into specific fates (e.g. BMP4). Signals of this category can promote differentiation into some lineages at the expense of others, and their actions can be combined to result in the complete block of differentiation and promotion of self-renewal (see LIF and BMP4 example above and Fig. 3.2). There are also other signalling molecules which affect ES cell behaviour in vitro, although their precise roles may be less clear. For example, both mouse and human ES cell neural specification is promoted by activation of the Notch signalling pathway, although this does not appear to be an absolute requirement [42]. It may be that Notch is accelerating this particular fate, or exerting a bias towards a neural as opposed to non-neural fate before cells become committed. Other signalling mechanisms operate to influence mesodermal and endodermal fates. For example, addition of Activin can bias the fate of mES cells towards a mesendodermal fate, from which more mature mesodermal and ectodermal derivatives can be obtained [74]. On the other hand, other molecules such as retinoic acid can accelerate differentiation towards multiple fates [67]. It is however beyond the scope of this review to detail all such signals and their mechanism of action.

3.4 The States of Pluripotency

3.4.1 Species Differences

The derivation of hES cells in 1998 [81] was achieved in conditions not dissimilar to those used for the initial derivation of mouse ES cells (feeder cells and serum). It was immediately clear, however, that these cells have very different growth properties and growth factor requirements from their mouse counterparts. To begin with, they exhibit different morphologies, with the human cells having a flatter, more epithelial colony shape compared to the domed shape of mouse ES cell colonies. They also express slightly different markers: mouse cells express SSEA1, human cells SSEA4. HES cells express antigens Tra-1-81 and Tra-1-60 unlike mouse cells, and there are differences in the expression of various other genes [19].

A further fundamental difference is that the feeders used for human ES cell culture cannot be substituted with LIF [81]. In fact, human cells may have lower levels of the LIFR and gp130 molecules responsible for LIF signalling [19], rendering them insensitive to this cytokine. This difference could be because humans are not thought to undergo diapause (see above) and have therefore not evolved the same mechanisms for preventing differentiation in vivo as rodents. The search for

molecules supporting self-renewal eventually identified two secreted growth factors able to replace the feeders in human ES cultures: Fgf2 and Activin A (or Nodal) [1, 84]. The requirement for Fgf2 is a surprise in light of this growth factor family's role in mouse ES cell differentiation, and highlighted the differences between human and mouse lines. Activin/Nodal have since been shown to regulate the expression of Nanog in hES cells, which in turn prevents neural differentiation in response to Fgf2 and mesodermal differentiation downstream of Smad2/3 activation [85]. The role of Fgf2 and downstream signalling pathways in hES cells seems to be complex, as it has been reported to both promote expression of pluripotency genes, inhibit caspase-mediated apoptosis as well as indirect effects via feeders or differentiated cells present in the cultures (for a review see [36]).

Another difference between the two species is that hES cells survive poorly at clonal density (making them very difficult to genetically engineer) and often exhibit bias in their differentiation (restricting their usefulness as sources of various cell types) [65, 103]. The cloning efficiency of hES cells can be dramatically improved by the inhibition of the Rho-associated protein kinase (ROCK) [91]. Subsequent work identified a myosin-dependent apoptosis pathway downstream of ROCK activation in dissociated hES cells [13, 23, 56], operating through the mitochondrial apoptosis pathway [56].

Apart from these differences in the mechanisms maintaining the undifferentiated state in the ES cells of human and mouse origin, these cells have another, perhaps more fundamental difference. During blastocyst formation, female embryos activate the paternal X chromosome (which was inactivated following fertilisation) in cells of the inner cell mass. This is a transient state in mammalian development, as shortly afterwards one of the X chromosomes becomes randomly inactivated in somatic cell lineages to ensure genomic balance between female and male embryos (for review see [48]). Female mouse EC and ES cells have two active X chromosomes and begin X inactivation upon initiation of differentiation. However, unlike mouse, female hES cell lines have undergone X chromosome inactivation suggesting they are developmentally more advanced than mES cells. This difference indicates that the two cell types, despite their common features, represent distinct epigenetic states of development which perhaps explains other differences in the mechanisms of their self-renewal.

3.4.2 Naïve and Primed States

It is now well established that the expression of a number of ES cell markers is heterogeneous in mouse ES cell cultures. Expression of Nanog, Rex1 (also known as Zfp42) and Stella (also known as Dppa3) are not uniform in LIF/serum cultures of ES cells, although the proportions of positive and negative cells remain relatively stable. This is not due to the presence of differentiated cells within the cultures, as the expression of all these markers was shown to fluctuate [12, 27, 83]. Both positive and negative sorted cells can regenerate the initial heterogeneity,

although negative cells are more likely to differentiate. These findings have led to a description of a dynamic, bistable equilibrium in ES cell cultures, orchestrated by a transcription factor network involving Oct3/4, Sox2 and Nanog [33].

These two states have been called "naïve" (for the Nanog, Rex1 and Stella positive) and "primed" [53]. Primed cells are closer to the differentiation threshold whereas the naïve state can be stabilised in 2i media [100]. These two states can be distinguished on the basis of cell morphology as well as marker gene expression (see Fig. 3.1d–f). Such heterogeneity does not seem to exist in routine human ES cell cultures, which seem to be composed purely of primed cells.

Naïve hES cells were recently obtained from existing hES cells lines, following forced expression of Klf4 and reduced oxygen partial pressure [24, 39]. These appear to share important features with naïve mES cells (such as ability to grow in 2i, two active X chromosomes and high clonogenicity) [7, 24, 39]. There are also indications that such naïve hES cells will be more amenable to genetic engineering than traditional hES cells [7].

3.4.3 Epiblast Stem Cells

Cells with an epiblast-like gene expression profile were first derived from mouse ES cells upon culture in a conditioned medium [61]. These early primitive ectoderm-like (EPL) cells express the epiblast marker Fgf5 and self-renew in the presence of an undefined activity from HepG2 cell-conditioned media (MEDII media). Direct isolation of epiblast cells with the same characteristics in MEDII confirmed that the original ES-derived cells represent a population present in the early post-implantation mouse embryo [62]. Intriguingly, EPL cells revert to LIF-responsive ES cells when cultured in the presence of LIF without MEDII [61]. This suggests that early pluripotent cell populations are able to interconvert, at least in vitro.

The derivation of cells with hES cell growth requirements from later stage mouse embryos (termed epiblast stem cells or EpiSC) [5, 79] has reinforced the notion that hES cells represent a different cell type from mES cells. These mouse EpiSCs have a global gene expression signature closer to hES cells than to mES cells [5, 79]. EpiSC can also be derived from mES cells following culture in Activin A and Fgf2, demonstrating a close relationship between the two cell types [20]. Mouse EpiSC accelerate their differentiation upon FGFR inhibition [31], like mouse ES cells which have begun their differentiation [67], although these two populations may not be fully equivalent (MPS, unpublished).

EpiSC (like hES cells) require Fgf2 and Activin A rather than LIF for their continued propagation, they survive poorly at clonal densities and female lines have initiated X inactivation. A further striking difference between EpiSC and mES cells is that the former contribute very poorly to chimeras [5, 79]. It has been widely speculated that hES cells would exhibit similar characteristics in chimeras, however, such experiments cannot be performed for ethical reasons. Intriguingly,

recent evidence suggests that non-human primate ES cells do not contribute to chimeras following blastocyst injection or cleavage stage aggregation, consistent with the hypothesis that they are more similar to EpiSC than mouse ES cells [73]. It should be noted though that the number of embryos tested in this study is very small. More studies on primates may elucidate this issue further.

Like EPL cells, EpiSC can convert to LIF-dependent ES cells following a change in culture conditions [3], although this was initially reported to require forced expression of Klf genes [20, 25]. It is still unclear what the precise relationship between these three early embryo-derived stem cell populations is, it is however, noteworthy that EpiSC can be derived from the rat [5] as well as from the non-permissive mouse strain NOD, which can be converted to an ES-like state following continuous expression of Klf4 or c-myc [25]. It is therefore possible that the spontaneous EpiSC-to-ES cell conversion, like conventional ES cell derivation, is subject to uncharacterised genetic factors and can only occur in certain strains or species. EpiSC are thought to represent a stable primed pluripotent cell population (like hES cells), although the finding that EpiSC can also fluctuate the usage of an Oct4 enhancer [23], coupled with the reports of spontaneous interconversion between EpiSC and ES cell states suggests that pluripotency may be inherently unstable (at least in the mouse).

3.4.4 Induced Pluripotent Stem Cells

One of the reasons why ES cell research has attracted so much attention is its promise for the treatment of a host of currently incurable diseases through cell replacement-type therapies. The quest for personalised cell therapy was also the drive for understanding and harnessing mechanisms of reprogramming somatic cells to a pluripotent state. Gurdon first demonstrated in 1962 that somatic nuclei can be reprogrammed by the cytoplasm of oocytes [21], but it took until the birth of Dolly in 1996 to demonstrate that the same process can be achieved in mammals [10]. Reprogramming of somatic cells can also be achieved by fusion with ES cells [78, 98] as well as by incubation of permeabilised somatic cells in cell-free oocyte extracts [26]. By use of any of these methods, patient-specific ES-like cells can potentially be derived for the study of disease progression or even personalised cell replacement therapy. However, all the above methods of reprogramming somatic cells have some significant disadvantages which limit their potential for therapeutic use; nuclear transfer is very inefficient and technically challenging, requiring the use of a large number of donated oocytes for each line to be derived. Fusion results in the production of tetraploid cells with the risks associated with ploidy changes. Cell free extracts have many advantages over the previous two approaches, although they also require the use of either animal cell extracts (which carry the risks of infection with animal pathogens) or a large number of donated human oocytes, with the same ethical and logistical issues as nuclear transfer. A better approach was clearly needed.

In 2006, Takahashi and Yamanaka published a study which changed the field of stem cell biology forever. In their paper, they demonstrated that somatic cells could be reprogrammed to a pluripotent state simply by forced expression of four transcription factors: Oct3/4, Sox2, Klf4 and c-myc [76]. They called these reprogrammed cells induced pluripotent stem cells (iPS cells) to reflect the fact that they were induced to a pluripotent state unlike ES cells which develop pluripotency during normal embryonic development. A year later, iPS cells were successfully derived form human somatic cells by Yamanaka and Thomson's groups [75, 102], opening the way for a surge of interest in these cells and the mechanisms of reprogramming. Since then, many advances have been made and much more is understood about the process of reprogramming which also inform our understanding of the pluripotent state itself. It should be noted that this reprogramming technology is not limited to the generation of pluripotent cells, but can be used to reprogramme somatic cells to other somatic fates [88], removing the need for the intermediate state of pluripotency and the problems associated with it.

The major disadvantage of the original iPS cell technology is the use of virally transduced transgenes for reprogramming, which include a potent proto-oncogene (c-myc). Although the transgenes are usually silenced during the reprogramming process, reactivation can happen and this can lead to increased tumour formation in animals derived from these iPS cells [57]. There has therefore been a considerable drive to improve the efficiency of reprogramming, while either reducing the number of factors required, or introducing them in a safer way [58].

The most significant advances include the use of purified proteins [34] or mRNA [90] to deliver the reprogramming factors. These methods bypass the issues associated with the use of transgenes, as they do not involve genetic modification of the somatic cells to be reprogrammed. Irrespective of the methods used however, the iPS cells resemble the ES cells of the species from which they are derived. Mouse iPS cells generally become LIF dependent and can be grown in a naïve state in 2i media. Human iPS cells can be induced to this naïve state upon forced expression of some of the reprogramming factors (see Sect. 3.4.2), but seem to revert to the hES-like Fgf-dependent primed state following reprogramming factor silencing (probably due to our incomplete understanding of culture conditions required for naïve human cell culture). The last remaining challenge is to replace all reprogramming factors with small molecules, which will allow the derivation and maintenance of pluripotent cells at any state indefinitely and under completely defined conditions. This will result in a much better understanding of the mechanisms of reprogramming and will certainly reduce the costs and possibly the time required for this process.

3.5 Concluding Thoughts

In the last 30 years, ES cell research has transformed the way we think about embryonic development, cell biology and regenerative medicine. It has come from relative obscurity into the limelight, has provoked intense debate on science and morality, been awarded infamy and praise and has succeeded in dividing public opinion like few other areas of science. Regardless of whether pluripotent cell based therapies establish themselves in mainstream medical practise, they have a lot to offer to basic and applied research alike. ES and iPS cells provide a platform for the study of complex interacting signals which control cell fate within genetically stable cells. Systems-level analyses of the ES cell state have already begun, adding new perspectives and providing hypotheses to be tested. Advances in the understanding of ES cell biology summarised above have made it possible to grow such cells in exquisitely defined culture conditions, unlike most other cells in wide use today. It has recently been argued that popular cancer cell lines should be abandoned in favour of ES cells or ES-derived cells by all cell biologists [29]. Should this view become widely adopted, it will bring a new vigour to this already thriving field of science, helping it achieve its full potential. For a stem cell biologist, this prospect is exciting and stimulating like no other.

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Chapter 4
From Oocytes and Pluripotent Stem Cells to Fully Differentiated Fates: (Also) a Mitochondrial Odyssey

João Ramalho-Santos and Ana Sofia Rodrigues

Abstract In the pluripotent cellular state, characteristic of preimplantation embryos and embryonic stem cells (ESCs), metabolic activity in general, and mitochondrial function in particular, seems to be subdued; increasing upon differentiation, possibly to avoid oxidative stress-mediated damage. A crucial but overlooked aspect of development is related to how mitochondrial differentiation follows somatic differentiation in terms of producing specific cell fates with very distinct metabolic profiles and energy requirements, notably in two of the most sought after cell fates in the field of regenerative medicine, the neuronal and muscular lineages. Finally, recent evidence suggests that, although induced pluripotent stem (iPS) cells obtained from somatic cells show hallmarks of pluripotency from a mitochondrial standpoint, these characteristics are not as pronounced as those shown by ESCs. Thus, incomplete reprograming might also be reflected in terms of iPS mitochondrial status, with possible implications for the derivation of patient-specific cells.

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4.1 Mitochondrial Characteristics and Dynamics of the Oocyte and Early Embryo

Mitochondria are the most abundant organelles in the early embryo [1, 2] and, in normal situations, are transmitted exclusively from the oocyte [3]. Oocyte mitochondria will, thus, be passed on to embryonic stem cell lines generated from mammalian embryos. Although in mammals the entire sperm (and thus the midpiece-containing mitochondria) enters the oocyte at fertilization [4], contradicting a notion still commonly found in a number of textbooks, paternal mitochondria are subsequently diluted or destroyed inside the embryo [5]. It should, however, be noted that there may be exceptions to this mitochondrial transmission paradigm, associated with certain pathologies or involving hybrids between closely related species/subspecies (see [6, 7] for Review).

The cell cycle in the early mammalian embryo is basically comprised of a bare-bone alternation between M and S phases with no G1 or G2, resulting in the production of cumulatively smaller cells, while maintaining the same overall structure size, i.e., the size of a blastocyst is not that much greater than that of a mature oocyte, despite ongoing mitoses. As preimplantation development progresses each cell contains less cytoplasm and fewer organelles, and the original oocyte mitochondria must support this development until the resumption of mitochondrial replication and the implementation of a full cell cycle, which only occurs post-implantation (reviewed in [7, 8]).

A human oocyte contains around 10^5 mitochondria [9, 10] propagated from a restricted founder population present in primordial germ cells (PGCs) [3, 11] that colonize the ovary, ensuring homogeneity in the mature oocyte, and therefore in dividing blastomeres. During oogenesis, increments in mitochondrial numbers parallel the increase in cytoplasmatic volume. Premigratory PGCs have less than 10 mitochondria, while 100 mitochondria are present in ovarian PGCs and 200 can be found in oogonia. Primordial follicle oocytes contain 10,000 mitochondria, a number which ultimately increases ten-fold. In the mature oocyte, each mitochondrion possesses a single copy of mtDNA (reviewed in [6, 7, 10]). The increase in mitochondrial number during oocyte growth is accompanied by ultrastructural changes in terms of shape, matrix, and number of cristae [1, 12, 13].

During oocyte maturation and in early embryos, mitochondria are relocated to different regions, probably in response to localized energy demands (reviewed in [14]). In addition, mitochondrial populations present heterogeneity in terms of mitochondrial membrane potential (MMP), with two identifiable populations: one with low MMP which is more abundant, and the other with high polarization. Clusters of highly-polarized mitochondria are found in the subplasmalemmal/pericortical cytoplasm of oocytes and in early blastomeres. Loss of these mitochondrial domains affects division, which may be associated with the focal ionic and metabolic regulation involved in oocyte activation and early development [15–18].

The total number of mitochondria in a normal human blastocyst, from which embryonic stem cells are derived, is about 14,000, and the average number of

mitochondria per cell is about 150 [19]. There is some controversy regarding the morphological homogeneity of the mitochondria found at the blastocyst stage. While some authors claim that mitochondria in mouse and human blastocysts are homogenous and elongated [2], the existence of two types of mitochondria in the mouse blastocyst has been reported: spherical mitochondria in the inner cell mass (ICM; which will give rise to the embryo, and, if removed from context, to pluripotent ESCs) and elongated mitochondria in the trophectoderm (TE), which will give rise to the embryonic contribution to the placenta [19, 20]. Interestingly, while the ICM cells have low MMP and are almost quiescent, the TE cells are highly polarized and very active, producing more ATP, and consuming more oxygen (O_2) [20–22].

4.2 Bioenergetics in the Oocyte and Early Embryo

During female gametogenesis, a combination of metabolic pathways is found in the follicle, comprised mainly of the oocyte proper and granulosa cells, which establish functionally important connections with the developing gamete, both via signaling pathways and gap junctions. A subpopulation of granulosa cells accompanies the oocyte upon ovulation (at that stage these cells are known as the cumulus cells), maintaining these connections. In terms of carbon sources, pyruvate and glucose are used by primordial follicles, suggesting that both oxidative phosphorylation (OXPHOS) and glycolysis are involved [23–25]. Furthermore, glucose used by the cumulus cells may lead to pyruvate production that is subsequently utilized by the oocyte [26].

An increase in pyruvate uptake, accompanied by a boost in O_2 consumption takes place during oocyte maturation [27], and the mature oocyte displays a high ATP turnover, supplied by mitochondrial respiration [28] and by pyruvate uptake [29]. Pyruvate is also the main substrate used by zygotes [23, 30], and is therefore essential for both meiotic maturation and to support the first cleavage division [23]. From zygote to morula expenditure levels of ATP and O_2 remain basically constant, and it is essentially substrates for OXPHOS that are metabolized [31, 32]. In later stages, the pattern of energy metabolism for the cleaving embryo changes (reviewed in [33]). At the morula stage, mitochondrial and metabolic changes occur gradually, and a shift in ATP production to glycolysis is evident [29, 32, 34]. Glucose is the predominant substrate that supports late embryo development [23, 35–37], but the increase in glucose uptake at the blastocyst stage is accompanied by a substantial increment in ATP generation and O_2 consumption [22], suggesting OXPHOS also takes place [33]. After implantation, levels of O_2 use decrease to those found in pre-blastocyst stages [22].

Importantly, intra and inter-individual variations in oocyte ATP content have been described, and there is a close association between oocyte ATP concentration and developmental competence of the resulting embryo [32]. Additionally, blastocysts that implant and develop to term have a significantly higher glucose uptake

prior to transfer than those that fail to develop [38]. It is also possible that constant changes in metabolism are simply adjustments to the substrates available in distinct regions of the female reproductive tract [26]. Regardless, taking cellular volume into account, Harris and coworkers [27] found that metabolism is higher in primary follicles, indicating that energy demands are greater at this stage. On the other hand, a relatively low metabolism is found in embryos, which seems associated with low oxidative stress and thus with embryo vitality [39, 40].

4.3 Mitochondrial Dynamics in Embryonic Stem Cells

The essential take-home message from mature oocytes and early embryos is that, even though pathways may vary, these structures maintain an overall low-level (i.e., "quiet") metabolism, thus possibly minimizing mitochondrial production of reactive oxygen species (ROS) and oxidative stress, but generating the necessary ATP to fulfill cellular functions [40, 41]. Considering that within a blastocyst the ICM seems to have much lower activity than the trophoblast/TE it follows that these properties are likely to be shared by pluripotent ESCs, which, as noted earlier, are ICM cells removed from the blastocyst structural context and put in culture. ESCs can be maintained in vitro in colonies for prolonged periods without losing the properties of indefinite self-renewal or differentiation into tissues from all three germ layers, as would have happened with ICM cells [42–46]. With human embryonic stem cells (hESCs) this differentiation property can be assessed in vitro (through embryoid body generation) or in vivo (by teratoma formation). Because of these biological properties, hESCs have enormous potential as models to study cell differentiation and for possible cell replacement therapies. Indeed, several groups have shown that under specific culture conditions hESCs can differentiate into various somatic cell types (for review see [47–49]), and cells differentiated from both mouse ESC and hESCs have been shown to ameliorate symptoms in several animal models of cell-based disorders [50].

Although there are line-specific differences it has been shown that, similar to ICM cells, undifferentiated hESCs have few ovoid mitochondria arranged in small perinuclear clusters and immature morphology, as evidenced by the presence of few cristae and low electron lucid matrix [51–53]. The fact that ESCs seem not to rely on OXPHOS, and thus that their mitochondria may not be very active, seems to be reinforced by data suggesting that a high glycolytic flux supports stem cell proliferation and that hypoxia may facilitate cell growth and pluripotency maintenance [54]. The rationale for using low O_2 tension is related to the conditions found in the female reproductive tract, thus mimicking physiological conditions for ICM cells. This is not a straightforward issue, because although O_2 may vary throughout the tract, it also seems sufficient to maintain active OXPHOS (see [55] for review).

ESC colonies are characterized by high nuclear cytoplasmatic ratios and tightly packed cells. Although there seems to be a paucity of intracellular organization (sometimes described as a "stemness" attribute), this could also just be a reflection

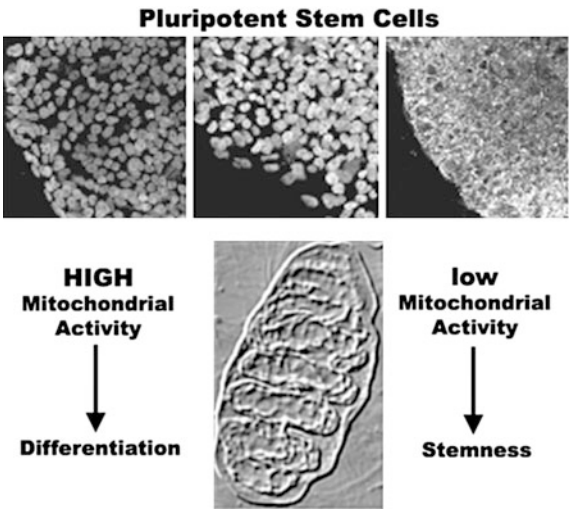

Fig. 4.1 Mitochondrial activity and Stemness. Limited electron transport and OXPHOS activity correlate with maintenance of cell pluripotency, while a boost in mitochondrial function is related with cellular differentiation to specific somatic fates

of reduced cytoplasm. Furthermore, it is well accepted that cells in the periphery of the colony are among the first cells to undergo spontaneous differentiation during in vitro culture. Interestingly, these cells have higher quantities of mitochondria [51]. In general, differentiation involves a shift from small individual oval mitochondria to dynamic tubular networks [53] with filaments that can reach several tens of microns (in an axon or dendrite) and an increase in the number of mitochondrial cristae, suggesting higher OXPHOS activity (Fig. 4.1).

In support of low OXPHOS activity in pluripotent ESCs, and that reduced mitochondrial activity may be considered a "stemness attribute", the hypoxic environment has been shown to prevent spontaneous hESC differentiation [56]. We have recently shown that mitochondrial inhibition at complex III leads to a shift of metabolism toward glycolysis boosting undifferentiated hESC pluripotency [57], and other authors have reported equivalent findings [58].

4.4 Mitochondria in Induced Pluripotent Stem Cells

Recent data suggest that the paradigms suggested above for ESCs are also true for the more recently characterized induced pluripotent stem (iPS) cells, in which a pluripotent ESC-like state is induced in both mouse [59] and human [60, 61] somatic cells. Although further research is warranted (both at the basic and applied levels), iPS cells have the long-term potential to complement and possibly even replace current human ESC lines in much of the research related to pluripotency, differentiation, and maintenance of a cell state (i.e. also relevant for putative cell dedifferentiation during cancer), in as much as they also represent a technology with the true potential for the generation of embryo and oocyte-free patient-specific cell lines for putative cell replacement therapies. However, the argument for

viewing iPS cells as more neutral and efficient entities in basic biology and regenerative medicine may not be as clear cut for several reasons. This includes the fact that oocyte-derived cells are still sought after as a "gold standard" for pluripotency; a need that arises from the fact that important differences between iPS cells and this gold standard still exist, including epigenetic hallmarks [62–64], and other resultant changes in reprogramed cells, such as somatic mutations [65] which may be very relevant for future clinical applications.

Regardless, and as noted above, available data point to the same profile of mitochondrial quiescence in pluripotent iPS cells, as has been described for ESCs. This includes similar morphological, metabolic, and transcriptomal profiles in terms of mitochondrial-related events, metabolism, and the management of oxidative stress [66–68]. Furthermore, conditions of low mitochondrial activity discussed as being important for pluripotency (including hypoxia) have been shown to improve the derivation of iPS cell lines [69], and antioxidants such as vitamin C also seem to have a beneficial effect [70]. However, some care must be taken in evaluating these data, given that the effect of vitamin C is unrelated to its antioxidant ability [70], and that a general role for antioxidants (or mitochondrial inhibition) in aiding cellular reprograming has not been established.

On the other hand, we have recently shown that inadequate/incomplete reprograming of somatic cells to a pluripotent phenotype (already described in terms of epigenetics), may also be reflected in mitochondrial properties. Indeed, mitochondrial structure (Fig. 4.2) and metabolic activity in iPS cells seem to be intermediate between ESCs and differentiated cells (although closer to the former). In addition, iPS cells cluster together, distinctly from ESCs, when mitochondrial-themed microarrays are performed [71]. Whether mitochondrial activity will be relevant in terms of the differentiation of iPS cells to somatic cell fates of interest remains to be determined.

4.5 Mitochondrial Bioenergetics in Target Somatic Tissues

In terms of bioenergetic characteristics, there are very distinct needs in different somatic tissues that might be targets for pluripotent cell differentiation and regenerative medicine. Interestingly, the literature contains many more references to mitochondrial dysfunction in pathological conditions, such as cardiac insufficiency and neurodegenerative disorders, than those describing characteristics of healthy tissue. Most of the current research in mitochondrial function in somatic cells focuses on processes thought to function as possible early indicators related to pathology and aging, and in the attempt to find molecular regulators and possible preventive/therapeutic targets. There are indications that mitochondrial enzyme activities, OXPHOS, and other MMP-dependent functions, are impaired in many conditions (possibly with an increment in ROS generation), for example in models of heart insufficiency, diabetes, Huntington's, Alzheimer's, and Parkinson's diseases, as well as in aging [72–75]. However, much more prevalent in the

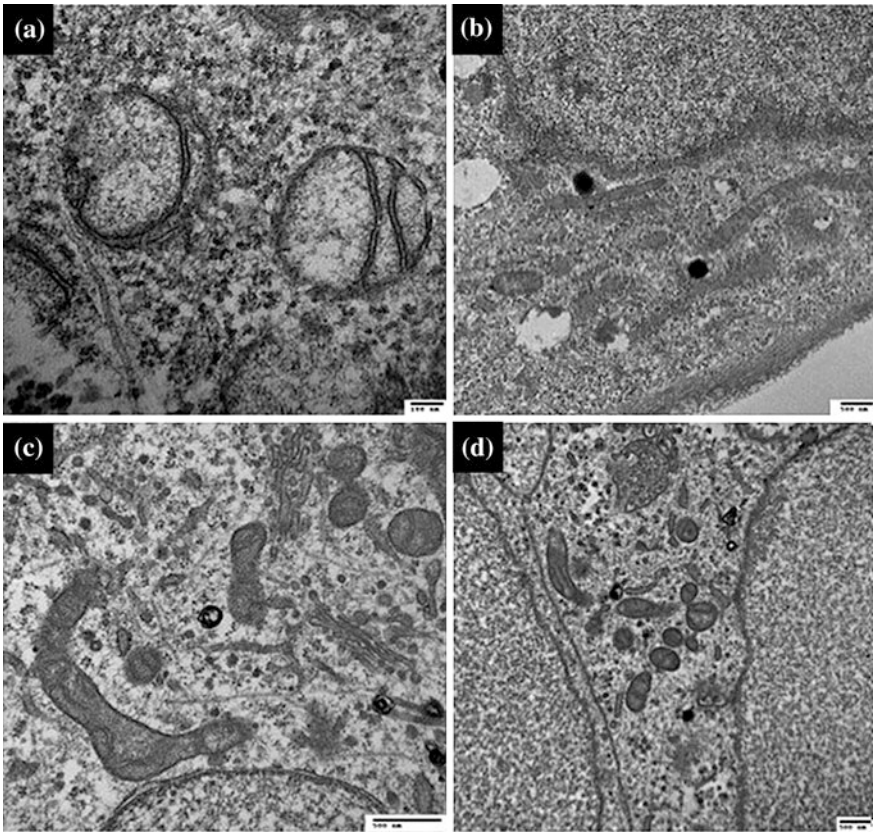

Fig. 4.2 Mitochondria in pluripotent and differentiated cells. **a** Mitochondria in pluripotent human ESCs (WA07 line) are small and oval shaped with a clear matrix and limited cristae, **b** while in fibroblasts differentiated from this line (H7TF) they show an elongated morphology with a dark matrix and abundant cristae. In two iPS lines **c** and **d** the morphology is more variable, but clearly intermediate between embryonic and differentiated cells. Scale bar: 500 nm

literature in terms of mitochondrial dysfunction are aspects related to mitochondrial morphology and dynamics including mitochondrial fission and fusion processes, mitochondrial turnover through autophagy, mitochondrial movement (especially in mature neurons), and mitochondrial-based apoptosis. One common working hypothesis in several conditions is that mitochondrial dysfunction involves disruption in the fission/fusion and autophagy machinery resulting in the fragmentation of the reticulate network, and in smaller oval-shaped mitochondria with abnormal cristae morphology. In essence, this seems to be the reverse of what happens during differentiation, with mitochondria reverting to a less functional state. But the impact of shifting the fission/fusion balance upon mitochondrial physiology remains relatively unexplored [72, 76].

Regardless, although mitochondrial bioenergetics are crucial for tissue homeostasis, mitochondrial content, and OXPHOS are thought to be more prevalent in muscle (and specifically heart) than in neural/brain tissue ([77]; see below). There are also some differences among tissues, which can be noted in specific mitochondrial proteomes, as well as in mitochondrial content, morphology, activity, and regulation [77, 78]. In terms of fuel, heart and skeletal muscle (type I muscular fibers) can use both carbohydrate and lipid sources of carbon, while other muscle types (Types 2A and 2B) and neurons preferentially use carbohydrates; although there can be some plasticity determined by available resources [72, 79].

Furthermore, energy demands change throughout the lifetime of the organism and bioenergetic properties are not fixed. In general terms, OXPHOS activity was found to greatly increase in postnatal human tissues, when compared to prenatal samples [80]. More specifically, prior to muscle differentiation, myoblasts possess mitochondria that contribute approximately 40 % of the ATP for energy metabolism [81]; and during the establishment of a myogenic program, mitochondrial proliferation and remodeling take place, with a major increase in mitochondrial enzymes. This process is furthered during differentiation, with an increase in cristae surface area [79], and fusion of mitochondria into a more continuous reticulum [82], as well as changes in tissue-specific isoforms of OXPHOS subunits and of other mitochondrial enzymes [83]. This energetic switch might be manipulated by modifying the copy number of regulators of mitochondrial fusion and fission. Therefore, mitochondrial dynamics could be critically involved in regulating the differentiation of stem cells into a functional muscle phenotype [84]. In mature heart tissue 30–50 % of volume is comprised of mitochondria and metabolism starts with carbohydrate in utero, but a lipid-based fuel metabolism becomes prevalent upon maturation, with the maintenance of carbohydrate fuel sources having pathological implications [85]. In the 12 day rat embryo, the mitochondrial morphology in heart muscle cells includes both rod like and spherical shapes [86] suggesting that myocytes are undergoing a transition from a glycolytic state with more fragmented mitochondria to an oxidative state with more fused mitochondria.

Besides fuel preferences, it should also be noted that there could be other biologically relevant distinctions. When assayed in the same laboratory, under the same sort of experimental settings and using direct electron transfer chain substrates (therefore bypassing fuel processing in glycolysis or the Krebs cycle), there are clear differences between mitochondrial populations from distinct rat organs (Table 4.1). With the caveat that both in vitro optimization and differences between in vitro and in vivo bioenergetical properties may always be an issue, heart and liver show high mitochondrial activity, although, for example, testicular mitochondria seem to have low but very efficient activity in terms of a high ADP/O ratio. Higher activity in heart in relation to brain (and of both in relation to other muscle types) is also paralleled by a greater number of cristae and higher mitochondrial enzyme quantity and activity [77, 87], as well as reduced glutathione concentration in both mitochondrial preparations and whole tissue [88, 89],

Table 4.1 Comparative analysis of mitochondrial bioenergetic parameters in different organs

	State 3 (natmsO/min/mg protein)	State 4 (natmsO/min/mg protein)	RCR	ADP/O	$\Delta\psi$ max (−mV)
Brain	98.82 ± 5,32	47.46 ± 2.63	2.28 ± 0.09	1.27 ± 0.08	177.3 ± 2.2
Heart	145.95 ± 3,56	61.72 ± 6.05	3.38 ± 0.09	1.22 ± 0.03	239.89 ± 0.79
Liver	77.7 ± 7.7	16.9 ± 0.6	4.7 ± 0.5	1.7 ± 0.1	224.0 ± 1.28
Kidney	125.47 ± 13.89	60.51 ± 12.37	2.23 ± 0.28	1.05 ± 0.12	213.09 ± 1.09
Testis	36.78 ± 1.95	24.75 ± 1.06	1.5 ± 0.053	1.74 ± 0.11	206.72 ± 1.74

Data show mean ± SEM, according to [102–104]; natmsO = nano-atoms of oxygen

although both brain and heart seem more susceptible to baseline oxidative stress (monitored in terms of both protein and lipid oxidation) than skeletal muscle [88].

Another issue is mitochondrial heterogeneity, as several populations with distinct activities may be found in the same organ. For example in the heart, mitochondria located around the nucleus, between the myofibrils, and beneath the sarcolemma, appear to have different morphology and distribution, as well as different oxidative metabolic activities [82, 90]. Of note, these differences are diluted in whole organ analysis, as spatial information is necessarily lost. Similarly, in neurons, mitochondria are typically located at sites of high ATP demand, including synapses, growth cones, Ranvier nodes, and myelination/demyelination interfaces (reviewed in [76]).

It is also important to be aware of differences between target human tissues and animal models. Compared to human skeletal muscle, the human heart shows a 3-fold increased mitochondrial content and electron transfer chain components, although this may not necessarily lead to increased OXPHOS activity due to differences in coupling; while in mice and rats OXPHOS capacity is higher in heart compared to skeletal muscle (see [74] for Review). This reinforces the importance of achieving a better understanding of the regulation of mitochondrial metabolism specifically in human target tissues.

4.6 Mitochondrial Bioenergetics During Differentiation

Mitochondrial dynamics have long been postulated to be involved in embryo patterning and early differentiation due to both asymmetric mitochondrial partitioning and differential activity (for review see [91]). Furthermore, the redox balance to which mitochondria contribute may more subtly influence cellular development by contributing to substrates used in epigenetic modulation of gene expression [92].

There is some controversy regarding the polarization of mitochondria in undifferentiated *versus* differentiated ESCs. Undifferentiated mouse ESCs have been reported to have highly polarized mitochondria, which decreases upon differentiation to cardiomyocytes [93]. On the other hand, no differences in MMP between undifferentiated and differentiated hESCs have been reported [94]. The

controversy might be due to the fact that the two groups work with ESCs from different species, mouse and human, respectively. In addition, mouse ESCs were specifically differentiated into cardiomyocytes, whereas spontaneous differentiation was investigated with hESCs, and as consequence a mixture of cell lineages would be present. Several studies have differentiated ESCs in vitro and observed changes in mitochondrial dynamics during differentiation.

As stated above, when ESCs differentiate the number of mitochondria increase, as well as the number of mitochondria with a more mature morphology [51, 53], similar to what is described for spermatogonial stem cells. Concomitantly, with an increase of mitochondrial number during ESC differentiation, the rates of O_2 consumption and ATP production in the cell increase as well, while lactate production decreases [93], suggesting a switch in energy metabolism from glycolysis to OXPHOS. Similar results have been reported for adult stem cells [95, 96]. The increase in the number of mitochondria and OXPHOS in differentiated cells also leads to an increase in ROS production [51, 94]. It should be noted that conflicting results have also been published in one recent study, suggesting that undifferentiated hESCs have higher mitochondrial activity, which decreases upon differentiation [97]. However, careful analysis of the data (Fig. 1A) a) shows clearly differentiating (i.e., not pluripotent) hESC colonies as starting points for the experiment, with no regular controls for pluripotency performed, underscoring the need for carefully controlled experiments to confirm the status of the cells at the time of analysis. This is especially pertinent in a field where cells are fickle and cellular status is permanently in flux, compared to the fixed status of more predominantly used cell lines or even primary cultures.

Given the distinct mitochondrial properties in undifferentiated *versus* differentiated ESCs, a role for mitochondria in differentiation may be postulated [55]. In addition, several groups have shown that functional mitochondria are necessary for differentiation. For example, inhibition of mitochondrial respiratory chain complexes I and III, by Rotenone and Antimycin A, respectively, results in reduced cardiomyocyte differentiation, due to an impairment of OXPHOS [93]. Furthermore, glycolytic metabolism is sufficient for maintaining mouse ESC homeostasis, however, in order for cells to differentiate there must be a switch from glycolysis to the more efficient OXPHOS [93]. In addition, inhibition of complex III of the mitochondrial respiratory chain by Antimycin A reduced the appearance of beating cardiomyocytes from ESCs, probably due to inhibition in calcium signaling [98]. Again, several authors have reported a similar role for mitochondria in adult stem cell differentiation [95, 99]. A correlation between MMP, metabolic rate, and the differentiation of mouse ESCs has been described, where cells with lower MMP showed more efficient mesodermal differentiation (but low ability to form teratomas), while a population with higher potential behaved in the exact opposite fashion (both populations were indistinguishable in terms of pluripotency markers) [100]. In addition, mitochondrial-based apoptosis may contribute to cell differentiation (see [55]), and mtDNA may also play an important role, given the deficient neuronal differentiation in ESCs carrying mtDNA mutations that resulted in severe biochemical deficiency [101].

4.7 Specific Mitochondrial Needs in Bioengineered Tissues from Pluripotent Cells

Pluripotent cell biology can be extremely informative in terms of providing a valid model to study the basic biology of mammalian differentiation and reprograming [55]. In fact, besides the differences between ESC and iPS cells, the picture that is currently available is much more complex than a simplistic dichotomy between pluripotent and differentiated cells. While the understanding that any differentiating cells follow consecutive functional steps from fully pluripotent to primed, to committed, to progenitor, to differentiated cells has been well established (although the plasticity and the definition of each of these entities may be problematic), to complicate the issue various stages of pluripotency have been proposed, each with distinct properties [102].

Whatever the choice of pluripotent cell and differentiation steps, the ultimate goal is to produce functional cells that will replace damaged or dead counterparts in vivo, or to model diseases in vitro, for both study and high-throughput screens. While there have been advances in the engineering of complex tissues and even whole organs [103–105], it seems more realistic that the first therapeutic applications will arise from very specific cell types transplanted in isolation or in small 3-dimensional aggregates (possibly also composed of biodegradable polymers) that would engraft onto preexisting structures, rather than attempting to engineer those structures de novo. For example, the specific lack of dopaminergic neurons in Parkinson's disease makes it a more compelling target than the more complex neurodegeneration pattern found in Alzheimer's disease [72].

In terms of differentiation as considered from a metabolic standpoint, the question is whether nuclear differentiation will be paralleled by equivalent mitochondrial differentiation. For example, if oval mitochondria in ESCs give rise to filamentous networks found in myofibers and axons [76], proper mtDNA replication is regulated [6, 7], and metabolism is switched from quiescent to more active OXPHOS. Furthermore, there is an issue of whether the metabolism of cells engineered from pluripotent precursors, usually differentiated and grown in high-glucose based media which are not encountered in vivo, show the same efficiency in situ. Having the same preferences in terms of fuels at the time of generation may, however, be secondary to being able to adapt to what is offered in a biological context. Interestingly, one characteristic of heart and skeletal muscle is the presence of red colored oxygen-binding myoglobin. Many tissue engineered cardiac tissue protocols derive force-producing cells with the proper stimulatory cues, that are, however, clearly not red, even when derived from fetal tissue [104, 106]. A similar observation of lack of proper gross morphology was made clear in a recent chapter discussing an engineered liver [104]. Another important point has to do with the fact that, as might have been anticipated, somatic cells differentiated from ESCs have characteristics that resemble fetal rather than adult cells, when the latter would be preferable for most applications. This has been clearly shown in the case of cardiomyocytes, suggesting that further in vitro maturation steps would be

required to obtain fully functional adult cells [107, 108]. Of course, it remains to be determined if the engineered tissue would adapt after engraftment both in terms of metabolism and proper maturation, and thus if the considerations noted above are moot points.

Two further aspects should be noted. On the one hand, there is the issue of cause or consequence in terms of mitochondrial contributions to organ homeostasis; namely, it is still unclear if altered mitochondrial dynamics, morphology, or bioenergetics actually drive pathological situations, or whether the monitored changes are injury-related epiphenomena, or responses to injury. It is also unknown to what extent the dynamic nature found in cultured cell models or isolated mitochondria can be extrapolated to a mature heart or brain as remodeling after the differentiation programs are complete is thought to be only modest in response to changes in activity levels or damage [76, 79].

As a final note it will be extremely interesting to determine how a mitochondrial perspective might inform *direct differentiation*, by which a somatic cell fate is transformed into another, skipping a pluripotent cell intermediate. This is especially relevant given that such procedures have produced cells of interest (muscle, neurons, hepatocytes) with distinct metabolic properties [109–111]. Although reproducibility might still be an issue in terms of direct differentiation protocols, this could well be the road both ESCs and iPS cells helped pave, by suggesting somatic cell fates are not final but open to transformation provided the right reprograming environment can be provided. In this case how will the conversion of mitochondrial types, for example from a mature fibroblast to a fully functional cardiomyocyte phenotype, occur? Will it be complete, involve intermediate mitochondrial forms, immediately form the correct adult cellular phenotype? And can creating differentiation conditions favoring different metabolic solutions employed by the two differentiated cell types possibly modulate the process?

4.8 Conclusions

In summary, although this remains a promising and novel area for research, overall results indicate that mitochondrial activity can help to characterize different cellular phenotypes, and that modulation of mitochondrial activity can be seen as a useful tool to maintain cells in a pluripotent state, or to drive differentiation toward a specific lineage. It is also important to note that, although small steps have been taken in the successful differentiation of cells into different tissues using either hESCs or iPSCs, there is still a knowledge gap to fulfill in terms of normal mitochondrial function and its link with the metabolic demands for the different tissues.

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Chapter 5
From Pluripotency to Differentiation: The Role of mtDNA in Stem Cell Models of Mitochondrial Diseases

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Abstract Embryonic stem cells (ESCs) are characterized by pluripotency, self-renewal and unlimited proliferation representing a limitless supply of cells for therapy. Moreover, ESCs represent a unique experimental model to investigate the basic principles of mammalian cell differentiation. ESCs are very useful for in-depth analysis of the development of the mitochondrial complement as the cells activate aerobic metabolism during differentiation. Induced pluripotent stem cells (iPSCs), which are reprogrammed somatic cells, appear to have identical properties to those of ESCs. They will certainly be a fundamental tool to establish human models for specific diseases. Nevertheless, the generation of iPSCs through reprogramming of mouse and human differentiated adult cells containing a mature mitochondrial complement requires a complete reprogramming of the cytoplasm to acquire the "pluripotent" mitochondrial network typical of undifferentiated ESCs.

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5.1 Introduction

Stem cells are characterized by their ability to endure cellular senescence, undergo prolonged proliferation or self-renewal in an undifferentiated state [1] as well as their potential to differentiate into at least a variety of cell lineages [2]. Stem cells can be broadly classified as embryonic stem cells (ESCs) and adult stem cells. ESCs are derived from the inner cells mass (ICM) of the blastocyst stage embryo and are pluripotent. These cells can give rise to all three embryonic germ layers; namely, the endoderm, mesoderm and ectoderm [3, 4] whereas adult stem cells are present in newborn and adult tissues. Compared to the pluripotent and almost immortal nature of ESCs, adult stem cells are typically characterized by more limited proliferation and more restricted differentiation potential (multipotent) as they are already partially committed to the lineages that constitute the tissues in which they reside [5]. Nonetheless, adult stem cells are emerging as a promising alternative therapy in regenerative medicine. In particular, mesenchymal stem cells (MSCs), present in the bone marrow and adipose tissues, can be differentiated into cardiovascular cell types that once transplanted facilitate both myocardial repair and neovascularization in models of cardiac injury [6].

Another type of stem cell, induced pluripotent stem cells (iPSCs), are non-naturally occurring cells that can be generated in the laboratory through manipulation of the expression of a number of factors that regulate pluripotency [7]. Human iPSCs (hiPSCs) were firstly generated in 2007 by Yamanaka's research group through overexpression of Oct4, Sox2, c-myc and Klf4 in adult human fibroblasts [8]. Generation of iPSCs has since been reported by a large number of investigators who have confirmed that iPSCs have gene expression profiles, patterns of methylation of the promoters of pluripotency genes, morphology and thus, differentiation potential and self-renewal properties similar to ESCs [9]. Further progress was accomplished when adult cells were reprogrammed using virus-free and vector-free approaches, eliminating the risk of development of viral-associated tumors upon transplantation [10, 11]. The generation of iPSCs raised the possibility of producing customized cells for the study and treatment of numerous diseases by autologous cell-transplantation, due to their distinct advantage of being derived from somatic cells, such as skin fibroblasts, from the patient themselves. In fact, iPSCs have already been derived from patients suffering from a variety of disorders [12–15]. iPSCs, which retain all the genetic information from patients, have been shown to recapitulate at least some of the disease phenotypes in vitro and are therefore an important tool for establishing disease-specific models and for subsequent use in screening new drugs [13, 16].

Despite the ability of ESCs and iPSCs to differentiate into a wide variety of functional cell types, as a consequence of their "stemness", it is important to recognize that their elevated proliferative capacity and high degree of pluripotency are also the main impediment in the therapeutic application of these cells [9, 17]. Although the somatic origin of iPSCs has minimized some of the challenges that have hindered the development of human (h)ESC-replacement therapies, the use

of pluripotent stem cells in regenerative medicine will depend on efficient lineage specific differentiation, purification of the desired phenotype to eliminate the risk of tumor development and generation of new methods of cell engraftment [9, 18]. Application in cell-replacement therapies will require further understanding of the most efficient stage of differentiation with respect to the assessment of the levels of maturation of individual intracellular organelles of the desired cell phenotype.

Increasingly, reports suggest that mitochondria play a crucial role in both the processes of maintaining pluripotency, as well as differentiation. Early evidence comes from mammalian pre-implantation embryonic development in which pluripotency was systematically associated with limited expression of the mitochondrial DNA (mtDNA)-specific transcription and replication factors Polymerase Gamma A (PolgA), Polymerase Gamma B (PolgB) and mitochondrial transcription factor A (Tfam), reduced expression of mtDNA-encoded subunits of the electron transport chain (ETC) and consequently restricted oxidative capacity [19–22]. In contrast, the onset of differentiation at the blastocyst stage, which gives rise to the trophectoderm, has been characterized by the upregulation of PolgA, PolgB Tfam and mtDNA-encoded genes and activation of mitochondrial aerobic metabolism [20, 23–27]. Consistent with this, undifferentiated mouse (m) ESCs and hESCs have also been shown to possess a relatively undeveloped mitochondrial network with only a few immature mitochondria, which express low levels of the mtDNA transcription and replication factors and contain low numbers of mtDNA copies/cell [28–32]. Once induced to differentiate, mESCs and hESCs upregulate the expression of PolgA, PolgB and Tfam resulting in an increase in mtDNA replication activities and subsequent mitochondrial maturation and activation of aerobic metabolism [29–32]. The potential role of mitochondria regulating molecular "switches" that control the maintenance of pluripotency and direct stem cell differentiation is yet to be unraveled. However, it has been hypothesized that production and controlled release of reactive oxygen species (ROS) might act as signaling molecules capable of regulating a variety of cellular functions [33].

5.2 Pluripotent ESCs Retain Quiescent Mitochondrial Metabolism

Pluripotent ESCs are characterized by a number of molecular and morphological properties which relate to their defining abilities of self-renewal and pluripotency [3, 4, 34]. Self-renewal is frequently monitored by colony morphology, growth rate and the expression of genes associated with pluripotency (for example, see Fig. 5.1). Pluripotency can be assessed in vitro by analysis of the potential to differentiate into endoderm, mesoderm and ectoderm lineages or in vivo through analysis of chimera (mESCs) or teratoma formation (non-murine ESCs; [3, 34].

Another particularly important feature of undifferentiated ESCs that has recently gained much interest relates to their mitochondrial metabolism. Undifferentiated mouse and human ESCs contain a relatively undeveloped mitochondrial network with

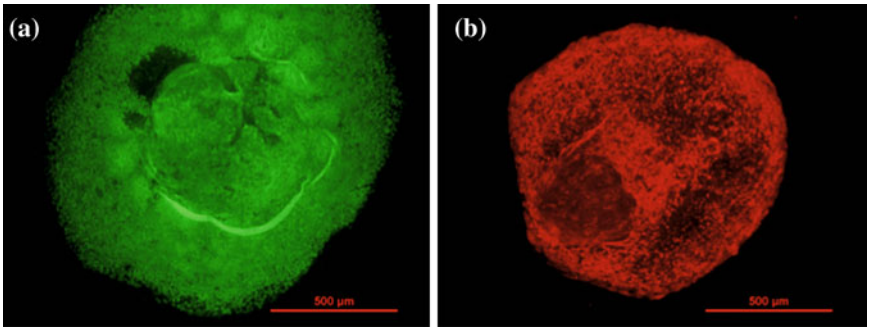


Fig. 5.1 Undifferentiated hESC colonies cultured on mouse embryonic fibroblasts expressing two keys markers of undifferentiated cells **a** the pluripotent gene, Oct4 and **b** Tra-1-60

only a few organelles with poorly developed cristae and a low electron lucid matrix [28–30, 32]. Indeed, mESCs have been shown to possess reduced numbers of mtDNA copies/cell and express low levels of the mitochondrial transcription and replication factors PolgA, PolgB and Tfam. Analysis of the number of mtDNA copies/cell in three mESC lines detected only between 31 and 44 mtDNA copies/cell [19, 31]. Similarly, a number of reports have also shown that undifferentiated hESCs contain a reduced number of mtDNA copies/cell [29, 35, 36]. Moreover, low levels of expression of the mitochondrial biogenesis regulators nuclear respiratory factor 1 (NRF1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and peroxisome proliferator-activated receptor gamma coactivator 1-beta (PGC1β; [36]) and subsequent reduced expression of PolgA, PolgB, Tfam and mitochondrial transcription factors b1 and b2 (Tfb1m and Tfb2m) have been described for undifferentiated hESCs [32, 36]. As a result, undifferentiated mouse and human ESCs express decreased levels of the ETC subunits [31, 32] and citric acid cycle enzymes [29, 30, 37] leading to poor oxidative capacity as demonstrated by low oxygen (O_2) consumption [37] and low levels of adenosine-5′ triphosphate (ATP) production [29, 30, 36]. Indeed, undifferentiated ESCs have been shown to express increased levels of glycolytic enzymes and possess high glycolytic flux resulting in high levels of lactate production in comparison to mature or differentiated cells [30, 36, 37].

Similar mitochondrial characteristics have also been observed in the pluripotent cells of the ICM of the blastocyst. Indeed, the ICM has been shown to contain low numbers of immature mitochondria and reduced levels of mtDNA copies/cell [20]. Despite the high mean values of mtDNA copies reported, varying between 114000 [38] and 249000 mtDNA molecules [39] per mature murine oocyte and between 193000 [40] and 314000 mtDNA molecules [41] per mature human oocyte, mtDNA replication is, in fact, very limited or even non-existent during preimplantation stages of embryonic development [20, 23, 38, 42, 43]. As such, the maternally inherited mtDNA of the embryo becomes progressively diluted at each cleavage division resulting in successive blastomeres containing fewer mtDNA copies and hence explains cells of the ICM having a limited number of mtDNA copies and reduced oxidative capacity [22, 44, 45]. Moreover, undifferentiated

ESCs and pluripotent cells in the early stages of mammalian embryo development also share similar patterns of mitochondrial network maturation and cytoplasmic localization [46]. In fact, mouse and human ESCs exhibit a limited mitochondrial network with spherical and immature organelles clustering around the nucleus [29, 30, 32], identical to that observed in mouse [47], cattle [48], pig [20], monkey [49] and human pre-implantation embryos [27].

In summary, maintenance of reduced levels of mitochondria and mtDNA along with reduced expression of oxidative phosphorylation (OXPHOS) enzymes and quiescent mitochondrial activity appear to be not only a particular feature of undifferentiated ESCs and pre-implantation embryonic cells but essential for the maintenance of pluripotency and/or self-renewal properties and should therefore be considered as another ESC marker [19]. Further supporting this hypothesis, it has been shown that hypoxic conditions (3–5 % O_2) promote normal and sustained cell proliferation and support maintenance of pluripotency of hESCs in comparison to the traditional culture conditions of 21 % O_2 [50]. The use of hypoxic conditions as a limiting modulator of oxidative phosphorylation is likely to prevent mitochondrial biogenesis and the acquisition of mature mitochondrial phenotypes possibly preventing the spontaneous differentiation that tends to occur in the periphery of ESC colonies cultured in standard conditions. In vivo, preservation of the immature mitochondrial network and low oxidative capacity in the pluripotent cells of pre-implantation and early post-implantation embryos might also be regulated by the hypoxic environment in the oviduct and in the uterus [51]. Moreover, it has also been reported that exposure of hESCs to antimycin A or myxothiazol, an inhibitor of the ETC, enhances the expression of pluripotency genes, represses lineage-specific genes and promotes the maintenance of compact morphology of undifferentiated hESCs [52].

5.3 The Onset of ESC Differentiation Regulates Activation of Mitochondrial Metabolism

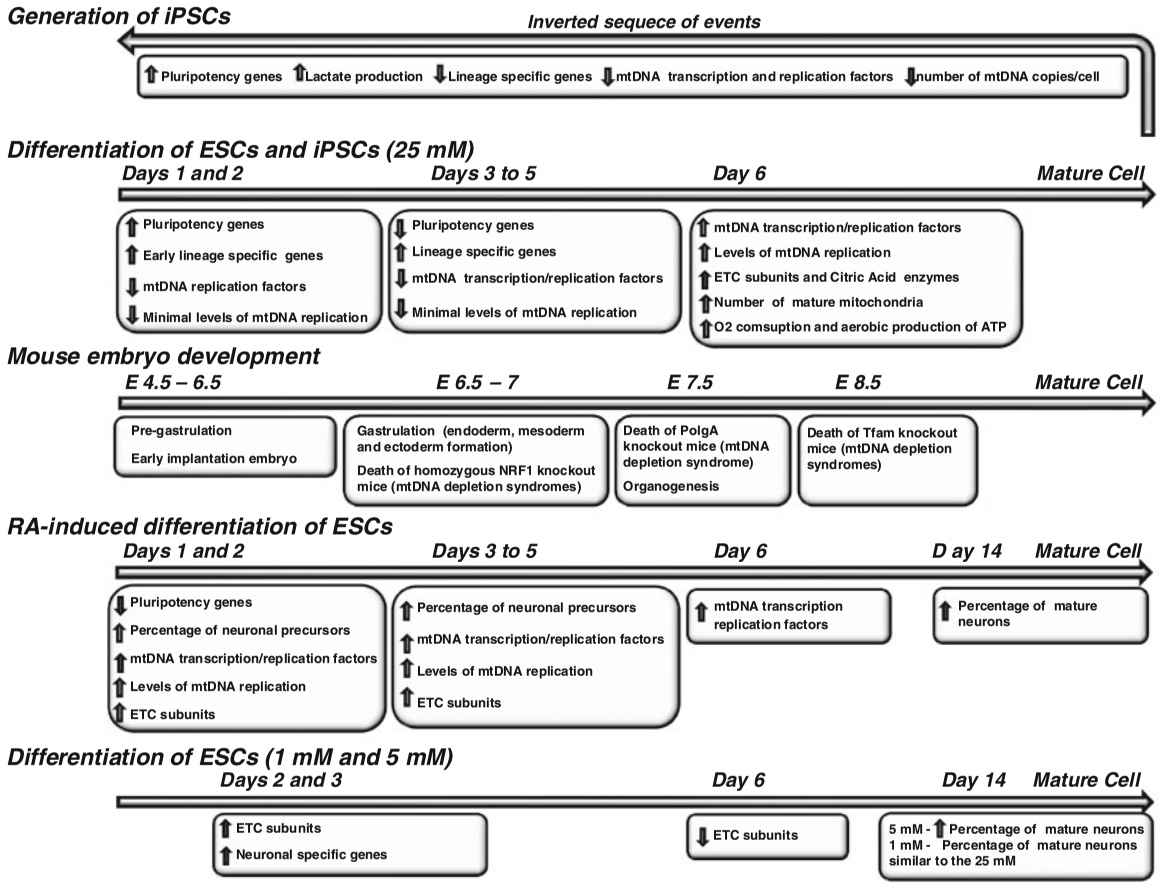
During differentiation of ESCs, profound alterations occur related to the stage of maturation of mitochondria and in the number of mtDNA copies/cell, once more suggesting a role of mitochondrial activity in the balance between pluripotency and differentiation. These alterations in the mitochondrial complement upon loss of pluripotency have been shown to reflect the switch from anaerobic metabolism to aerobic metabolism as a consequence of the increase in demand for ATP during differentiation [19, 30–32, 37, 53]. The efficiency of ATP production through OXPHOS is far superior compared to that through glycolysis [54, 55] and thus the immature mitochondrial network of undifferentiated ESCs must be differentiated into a more active and mature network in order to promote the synthesis of greater levels of ATP and the maintenance of homeostasis in the differentiated cell [54]. Indeed, fully differentiated cells such as neurons and cardiomyocytes express high

levels of the nuclear DNA (nDNA)- and mtDNA-encoded ETC subunits, produce ATP through OXPHOS [30, 32, 56] and contain enriched mtDNA content [57, 58].

Analysis of the expression of the pluripotency genes Nanog, octamer-binding transcription factor 4 (Oct4), developmental pluripotency-associated protein 5 (Dppa5), Prame-like 7 (Pramel7), the mesodermal marker brachyury and the neuroectodermal markers nestin, vimentin and β-tubulinIII showed that loss of pluripotency and the initial stages of cellular lineage commitment take place during days 3–5 of spontaneous differentiation of mESCs [19, 31]. This period coincided with low levels of expression of the mitochondrial transcription and replication factors PolgA, PolgB and Tfam and minimal levels of mtDNA copies/cell (see Fig. 5.2; [31]). Nevertheless, analysis of incorporation of bromodeoxyuridine (BrdU) into mtDNA confirmed that basal levels of mtDNA replication take place most probably due to replenish of the mtDNA content of proliferating mESCs [31]. At day 6, when cells had clearly committed to a specific lineage, the increase in expression of mitochondrial transcription and replication factors resulted in extensive mtDNA replication [31] and elevated levels of expression of nDNA- and mtDNA-encoded ETC subunits (see Fig. 5.2; [29, 31, 32]). Commitment to a specific lineage, regulated by lineage-specific transcription, is thus likely to initiate the expansion of the number of mitochondria and mtDNA molecules per cell according to the specific metabolic demands of individual cells [59, 60].

Moreover, the increase in the number of mtDNA copies/cell on day 6 of mESC differentiation coincides with the increase in the number of mitochondria and changes in mitochondrial structure, morphology and patterns of cytoplasmic localization detected during hESC differentiation [29, 30, 32]. Mitochondria migrate from the perinuclear regions into more wider cytoplasmic areas, acquire a tubular structure, numerous elongated cristae, dense matrices and high membrane potential, suggesting the initiation of metabolic activity through OXPHOS [29, 30, 32, 36]. Consistent with this, the expression of glycolytic enzymes decreases and the expression of ETC and citric acid cycle enzymes increases during cardiac differentiation of mESCs [30]. Furthermore, the levels of ATP production, O_2 consumption and cellular respiration are also increased whilst lactate production and anaerobic production of ATP are reduced (see Fig. 5.2; [29, 30, 36]).

Analysis of the expression of a panel of developmental marker genes used to determine how differentiation of mESCs correlated with early murine post-implantation development demonstrated a strict temporal and spatial relationship in the regulation of molecular events related to the loss of pluripotency and differentiation (see Fig. 5.2; [61]). Mouse embryos at embryonic day (E) 4.5–6.5 were shown to correlate with embryoid bodies (EBs) at day 1 and 2 of spontaneous differentiation, embryos at E6.5 to 7.0 were at a similar stage to EBs at days 3 to 5, and embryos at E7.5 had a similar expression pattern to EBs having undergone more than 6 days of spontaneous differentiation [61]. Embryos at E4.5 to E6.5 are at the pre-gastrulation stage, which correlates with the expression of the pluripotency genes during days 1 and 2 of mESC differentiation [19, 31]. At E6.5 to E7.0, the embryos undergo gastrulation, differentiating into the three embryonic germ layers [61].


Generation of iPSCs
Inverted sequece of events
Pluripotency genes
Lactate production
Lineage specific genes
mtDNA transcription and replication factors
number of mtDNA copies/cell
Differentiation of ESCs and iPSCs (25 mM)
Days 1 and 2
Days 3 to 5
Day 6
Mature Cell
Pluripotency genes
Early lineage specific genes
mtDNA replication factors
Minimal levels of mtDNA replication
Pluripotency genes
Lineage specific genes
mtDNA transcription/replication factors
Minimal levels of mtDNA replication
mtDNA transcription/replication factors
Levels of mtDNA replication
ETC subunits and Citric Acid enzymes
Number of mature mitochondria
O2 comsuption and aerobic production of ATP
Mouse embryo development
E 4.5 – 6.5
E 6.5 – 7
E 7.5
E 8.5
Mature Cell
Pre-gastrulation
Early implantation embryo
Gastrulation (endoderm, mesoderm and ectoderm formation)
Death of homozygous NRF1 knockout mice (mtDNA depletion syndromes)
Death of PolgA knockout mice (mtDNA depletion syndrome)
Organogenesis
Death of Tfam knockout mice (mtDNA depletion syndromes)
RA-induced differentiation of ESCs
Days 1 and 2
Days 3 to 5
Day 6
D ay 14
Mature Cell
Pluripotency genes
Percentage of neuronal precursors
mtDNA transcription/replication factors
Levels of mtDNA replication
ETC subunits
Percentage of neuronal precursors
mtDNA transcription/replication factors
Levels of mtDNA replication
ETC subunits
mtDNA transcription replication factors
Percentage of mature neurons
Differentiation of ESCs (1 mM and 5 mM)
Days 2 and 3
Day 6
Day 14
Mature Cell
ETC subunits
Neuronal specific genes
ETC subunits
5 mM - Percentage of mature neurons
1 mM - Percentage of mature neurons similar to the 25 mM

◀ **Fig. 5.2** General schematic representation of the main events involved in mitochondria biogenesis during differentiation of pluripotent stem cells and their relation to mouse embryonic development. Early stages of spontaneous differentiation in presence of standard concentration of glucose (25 mM) are associated with loss of pluripotency and upregulation of lineage specific markers (days 1–5). This stage is also characterized by quiescent mitochondrial metabolism as demonstrated by the low number of mitochondria and mtDNA copies/cell, reduced expression of mtDNA transcription and replication factors and ETC subunits and decreased levels of aerobic production of ATP. Interestingly, this period correlates with early mouse implantation development and the initiation of gastrulation with differentiation of the three embryonic germ layers. By day 6 of spontaneous differentiation (25 mM of glucose), the expression of mtDNA transcription and replication factors and ETC subunits is upregulated leading to replication of the mtDNA, increase in the number of mature mitochondria and activation of aerobic metabolism. Similar cellular differentiation events are also likely to take place between E7.5 and E8.5 of mouse development as homozygous Nrf1, PolgA and Tfam knockout mice arrest during development with severe mtDNA depletion syndromes at these stages. Differentiation of ESCs in the presence of retinoic acid (RA-induced differentiation) has been shown to result in more rapid loss of pluripotency, upregulation of neuronal specific genes and subsequently earlier activation of mitochondrial biogenesis with increased expression of mtDNA transcription and replication factors and ETC subunits and higher number of mtDNA copies/cell. Differentiation of ESCs in the presence of 1 and 5 mM of glucose also resulted in more rapid upregulation of ETC subunits in comparison to the mESCs differentiated in the presence of 25 mM of glucose. Furthermore, differentiation of mESCs in 5 mM of glucose has been shown to result in enhanced neuronal differentiation

At E7.5, mouse embryos are in the early stages of organogenesis [61], which coincides with the increase in expression of the mtDNA replication factors and ETC subunits, number of mtDNA copies/cell and number of mature mitochondria on day 6 of mESC differentiation (see Fig. 5.2; [19, 29, 31]). In addition, homozygous PolgA and Tfam knockout mice have been shown to have arrested development at days E7.5 and E8.5, respectively, due to severe mtDNA depletion and impaired OXPHOS, which ultimately led to complete failure of organogenesis [62, 63]. As day 6 of EB differentiation mimics the molecular events that occur during E7.5, it is very likely that the developmental arrest of homozygous PolgA and Tfam knockout mice is caused by the failure to replicate mtDNA in order to expand the number of mtDNA molecules per cell and activate aerobic metabolism (see Fig. 5.2). This is possibly similar to the activation of the mitochondrial genome, a process similar to embryonic genome activation [64], which results in fully transcriptionally active mitochondria. Further evidence of the importance of mitochondrial biogenesis for embryo survival and successful differentiation of ESCs arises from homozygous NRF1 knockout mice [65]. Consistent with its critical role during mitochondrial biogenesis, homozygous disruption of NRF1 resulted in earlier developmental arrest than homozygous disruption of PolgA and Tfam, with defective mitochondrial membrane potential and mtDNA depletion causing embryonic death between E3.5 and E6.5 (see Fig. 5.2; [65]).

Exposure to 10^{-7} M retinoic acid (RA) during the hanging-droplet stage has previously been shown to enhance neuronal differentiation of mESCs [66–68], inducing loss of pluripotency with faster downregulation of Oct4 and Nanog [69]

and promoting the upregulation of neuronal transcription factors such as Mash1 [70], Pax6 [71, 72] and Hoxa1 [69]. Interestingly, the use of RA during days 1 and 2 of EB formation (RA-induced differentiation) to stimulate cellular differentiation and enhance enrichment of neuronal phenotypes resulted in elevated and more consistent numbers of mtDNA copies/cell than the spontaneously differentiated mESCs (see Fig. 5.2; [31]). Perhaps, due to the more rapid onset of differentiation, RA-induced ESCs also expressed consistently higher levels of PolgA, PolgB and Tfam and had a higher percentage of cells with increased ATPase5b and COXI expression than spontaneously differentiated ESCs during days 1–5 [31, 73]; see Fig. 5.3). This has led us to conclude that RA exposure may stimulate earlier activation of OXPHOS compared to spontaneous differentiation, in mESCs [31].

Although the specific pathway activated by exposure to RA was not investigated, it is possible that the increase in the mtDNA content may have resulted from a direct effect of RA on retinoic acid response elements (RARE) in the mitochondrial genome [74, 75] and retinoic acid receptors (RARs) in the mitochondria [76]. RA has been previously reported to upregulate transcription of the mtDNA-encoded nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 5 (*mt-Nd5*), *COXI* and *16S rRNA* (*mt-Rnr2*) [77, 78]. Given that POLG requires an RNA primer to replicate mtDNA [79, 80], it is possible that the increase in mtDNA transcription also generated the observed increases in mtDNA replication. In addition, exposure to RA might also have resulted in upregulation of the mitochondrial biogenesis-related genes PGC-1α [81], NRF1 and NRF2 [81, 82]. As NRF1 and NRF2 are the major factors regulating mtDNA transcription, replication factors and nDNA-encoded ETC subunits [83, 84], activation of NRF1 and NRF2 upon RA exposure could certainly upregulate the expression of PolgA, PolgB and Tfam, which consequently induces mtDNA transcription and replication. The faster onset of differentiation and earlier increase in the number of mtDNA copies/cell in RA-induced ESCs further supports the concept that loss of pluripotency, cell fate commitment and activation of aerobic mitochondrial metabolism are sequential events during ESC differentiation.

Additional experiments assessing the effect of decreased concentrations of glucose on the onset of mitochondrial biogenesis during mESCs differentiation have shown that culture of mESCs with 1 and 5 mM of glucose resulted in a significantly higher percentage of cells with elevated expression of the ETC subunits ATPase5b and COXI than mESCs differentiated under standard concentration of glucose (25 mM) on days 2 and 3 ([73]; see Fig. 5.3). Culture of human hepatocytes in decreased concentrations of glucose (5 mM) and rat pheochromocytoma PC-12 cells in D-glucose free media has been shown to stimulate mtDNA transcription and increase the cell's oxidative capacity [85, 86]. Human ESCs differentiated in decreased concentration of glucose (5.5 mM of glucose), showed upregulation of neuronal markers and enhanced neurogenesis in comparison to the hESCs differentiated in the presence of 25 mM of glucose [87].

Similarly, our mESC differentiation experiments using 5 mM of glucose also showed enhanced neuronal differentiation with an increase in the percentage of β-tubulin III+ neurons to levels similar to those observed with RA-induced differentiation [73]. Conversely, comparative analysis of the potential of mESCs cultured in 5

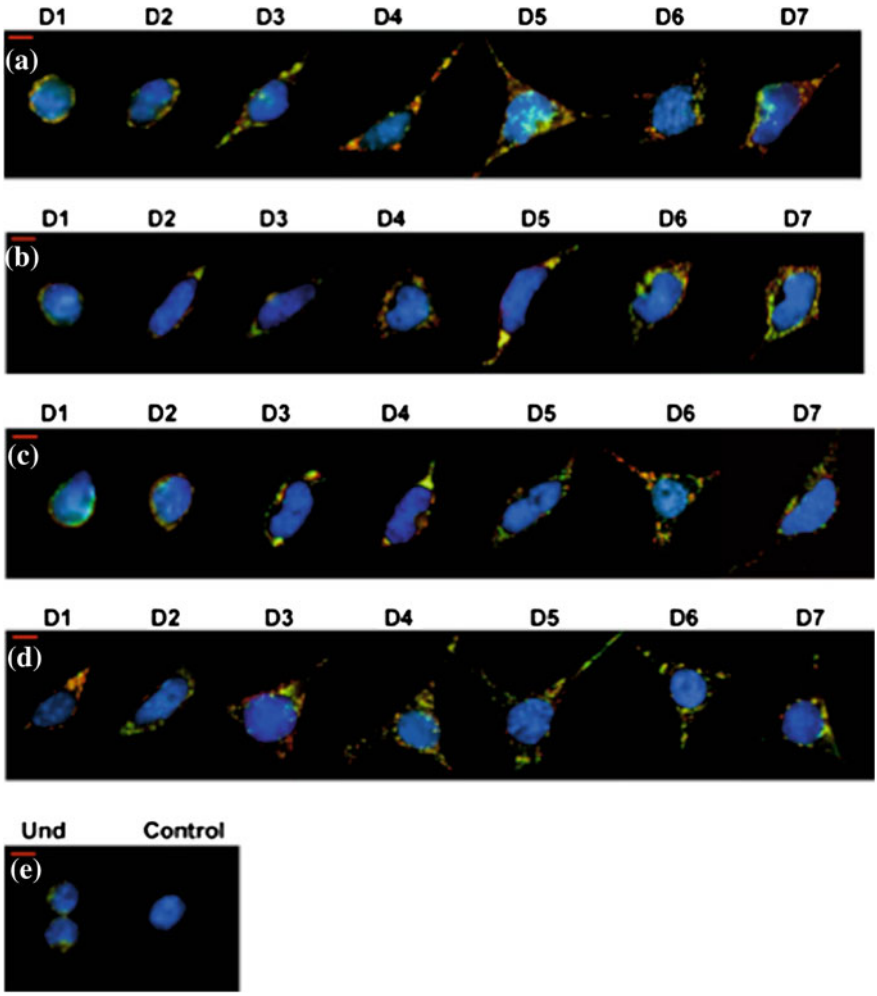


Fig. 5.3 Expression and localization of the nuclear-encoded ATPase5b and the mtDNA-encoded COXI in mESCs differentiated in the presence of 1 mM (**a**), 5 mM (**b**) and 25 mM (**c**) of glucose, RA-induced mESCs (**d**) and undifferentiated mESCs (**e**). In undifferentiated mESCs, the expression of ATPase5b and COXI was restricted to the perinuclear regions of the cytoplasm. However, as the mESCs differentiated, the cytoplasm expanded and the expression of ATPase5b and COXI gradually increased. Scale bar = 10 μm

and 25 mM of glucose to differentiate into cardiomyocytes has demonstrated that high glucose levels favour cardiac differentiation through the induction of increased levels of ROS ([88]; see Sect. 5.6). Similarly, differentiation of germ cells from mESCs was markedly inhibited by physiological levels of glucose (5.5 mM) in comparison to the mESCs differentiated in a high glucose concentration of 25 mM [89]. On the other hand, pancreatic endocrine cell differentiation from pancreatic progenitor cells has

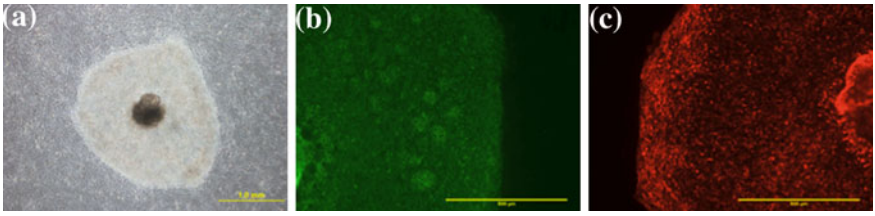


Fig. 5.4 Undifferentiated hiPS cells. **a** Phase contrast of a colony; **b** A colony expressing the puripotency gene Oct4 and **c** and Tra-1-60

been shown to be enhanced in media containing 10 mM glucose [90]. These results suggest that glucose concentration might have an important role in lineage specification during ESC differentiation and embryo development. Supporting this concept, human and murine EBs and murine blastocysts showed stage-specific and polarised expression of glucose transporters with different efficiencies [91–93], which could regulate differential access to glucose. If glucose levels are involved in regulation of lineage specification, then differential activation of OXPHOS and therefore transcription and replication of the mtDNA are likely to contribute to cell fate commitment.

The importance of the activation of aerobic respiration for successful differentiation is further elucidated by inhibition of the ETC with antimycin A or rotenone. Culture of differentiating ESCs in the presence of these mitochondrial poisons has been shown to lead to reduced volume and abnormal distribution of mitochondria, decreased number of beating areas (up to 80 %), atypical expression of cardiac specific proteins, deficient sarcomere formation, depleted sarcomere content and consequently compromised cardiac differentiation [30, 94].

5.4 Mitochondrial Reprogramming in iPSCs

The generation of iPSCs from murine to human adult somatic cells involves complete reprogramming of the somatic cell to acquire the pluripotent properties characteristic of ESCs [7, 9]. Nuclear reprogramming is achieved through ectopic manipulation of the expression of a number of transcription factors that regulate pluripotency. This has major implications for the field of regenerative medicine, allowing for the generation of patient-specific stem cells and also for in vitro modeling of complex human disorders using iPSCs derived from adult cells with genetically-associated diseases. Undifferentiated human iPSCs are morphologically very similar to hESCs and exhibit the same fundamental features of self-renewal and pluripotency ([8]; see Fig. 5.4). hiPSCs have proliferative properties similar to hESCs, exhibit similar patterns of methylation to the promoters of pluripotency genes and are able to differentiate into the 3 embryonic germ layers in vitro, and form teratomas in vivo [8]. In addition, genetic reprogramming of iPSCs also

results in telomere elongation mediated by increases in telomerase activity and acquisition of epigenetic marks typical of telomeres of ESCs, including a low density of trimethylated histones H3K9 and H4K20 and increased abundance of telomere transcripts [53, 95]. Nevertheless, comparative gene expression profiling has revealed significant differences in the expression signatures of iPSCs and ESCs derived from both mouse and human sources [96]. These differences appear to diminish as iPSCs are cultured for longer periods of time, suggesting that complete reprogramming of the cell requires additional adjustments that are possibly related to the acquisition of intracellular organelles with "stemness"-like characteristics [96].

Following the comprehensive analysis of the epigenetic modifications including DNA methylation, histone modification and chromatin remodeling and expression profiling of iPSCs [97–99], much interest has recently been drawn towards the state of maturation of cytoplasmic organelles, particularly the reprogramming of mtDNA and mitochondria. As previously mentioned, the pluripotent cells of pre-implantation embryos and undifferentiated ESCs have distinct similarities including low levels of mtDNA transcription and replication, poorly developed mitochondrial networks with perinuclear organization and great reliance on anaerobic metabolism, suggesting a strict correlation between these mitochondrial properties and the maintenance of the pluripotency state. On the other hand, most somatic cells contain well-developed mitochondrial networks composed of hundreds of mitochondria and high numbers of mtDNA copies/cell [58, 100–102]. Hence, it is evident that the generation of iPSCs capable of differentiating into physiologically mature and therapeutically significant cell types will require not only epigenetic modifications at the level of the nuclear DNA but also reprogramming of the mtDNA and mitochondria to an immature and "stemness" state. Failure to reprogram the mitochondria would most likely result in iPSCs exhibiting mitochondria and numbers of mtDNA which are phenotypically characteristic of the parental cell and might therefore lack the level of plasticity to differentiate and fulfill the multitude of ATP requirements of all cell types.

The reprogramming of mtDNA and mitochondria upon generation of iPSCs has been recently investigated by a number of independent research groups. It has been demonstrated that the mitochondria of the parental cell lines revert to an immature ESC-like state in respect to mtDNA content [35, 36, 103], mitochondrial mass [35, 53], patterns of cytoplasmic localization, mitochondrial morphology and overall metabolic activity [35, 36, 53]. The number of mtDNA copies in undifferentiated iPSCs is significantly lower than in the parental somatic cells and comparable to the levels of mtDNA copies/cell in hESCs [36]. Analysis of the expression of the mitochondrial biogenesis related factors, NRF1, PGC1α and PGC1β, and mtDNA transcription and replication factors including PolgA, PolgB, Tfam, Tfb2m, the mitochondrial RNA polymerase (mtRNApol), the mitochondrial transcription termination factor (mTERF) and the mitochondrial single-stranded DNA binding protein (mtSSBP1) showed decreased transcript levels similar to those detected in mouse and human ESCs [35, 36, 103]. Moreover, transmission electron microscopy analysis reveals that hiPSCs exhibit limited numbers of mitochondria with

underdeveloped cristae and low mitochondrial mass similar to those found in hESCs [35, 36, 53]. Similarly, a reduced number of mitochondria with oval-shaped morphology located in the perinuclear regions of the cytoplasm have also been found in undifferentiated mouse iPSCs (miPSCs; [103, 104]). In addition, undifferentiated hiPSCs exhibited higher levels of lactate than somatic fibroblasts or fully differentiated cells but low levels of ATP production, suggesting that most likely, undifferentiated mouse and human ESCs, and iPSCs rely on anaerobic metabolism as the predominant source of ATP [36, 53].

A recent analysis by Kelly et al. looked at how nuclear reprogramming techniques, such as induced pluripotency and cell fusion, have an impact on the ability of the hybrid or reprogrammed cell to revert to true pluripotency and thus, regulate its own mitochondrial DNA copy number as typically observed in embryonic stem cells [103]. By comparing early passage iPS cell lines generated by viral induction using the transcription factors Oct-4, Sox-2, Klf-4 and c-Myc [105], somatic cell-ESC hybrid lines generated by the fusion of murine ESCs with mouse embryonic fibroblasts [106], ESCs fused with ESCs and ESCs generated through nuclear transfer (NT–ES) [107], this study essentially examined how changes to the nuclear genome through induction of pluripotency via transient exposure to a defined group of factors or through the fusion of a somatic karyoplast containing a nucleus to a 'pluripotent' cytoplast could potentially disturb the strict maintenance and control of mtDNA copy number that occurs at fertilization and is regulated at key stages of differentiation.

While cell fusion resulted in up to a ninefold increase in mtDNA copy number in comparison to ESCs, iPSCs, interestingly, appeared to have copy numbers that matched ESCs in the undifferentiated stage, suggesting their capacity to mediate pluripotent gene expression and match mtDNA copy numbers found in ESCs. However, the similarity in copy numbers, as noted by the authors, could possibly be a consequence of other regulatory events such as mitophagy that may alter the final mtDNA copy number count, rather than the complete establishment of pluripotency. Reasons underlying this impression come from the observation of the immuno-staining results that indicate that mtDNA replication events are more prominent in undifferentiated iPSCs when compared to ESCs. The failure of all other types of reprogrammed cells to match ESC mtDNA copy numbers in the undifferentiated state also suggests incomplete reprogramming, possibly due to tetraploidy in the case of ESC–ESC and somatic cell-ESC fusions. Although a direct correlation between copy number and markers of pluripotency was found, with copy number being directly proportional to Nanog and inversely to Sox2, a similar correlation between pluripotent gene expression and mtDNA copy number could not be established during spontaneous differentiation. This, yet again, points to the interference of incomplete reprogramming with the proper re-establishment of the OCT4–SOX2–NANOG network [108–110] necessary not only for the maintenance of pluripotency but also for triggering differentiation and that mtDNA is strongly associated with these processes.

That oxidative phosphorylation may not be the main mechanism for ATP synthesis in iPSCs, was also shown by Kelly et al. [103]. Although mitochondrial

levels dropped significantly in cells treated with the mitochondrial toxin R6G, the decrease in ATP levels was not proportional. This was despite steady state levels of the electron chain complexes CI-CIV being detected.

Upon differentiation, iPSCs can activate mitochondrial biogenesis leading to the development of their mitochondrial network and acquisition of metabolically active mitochondria [35, 36, 53, 103]. Differentiation of iPSCs results in mitochondrial maturation into elongated organelles with extensive cristae, increase of the intracellular levels of ATP and decrease of lactate production [36, 53]. Interestingly, derivation of hiPSCs from adult fibroblasts with high number of population doublings and containing considerably shortened telomeres and aged mitochondrial complement showed that reprogramming to pluripotency resulted in mitochondrial rejuvenation [53]. Differentiation of these iPSCs resulted in improvement of the quality and function of the mitochondrial complement with higher membrane potential and more developed mitochondria and increased levels of ATP of the re-derived fibroblasts in comparison to the maternal adult fibroblasts [53]. Taken together, these results show that the generation of iPSCs from adult somatic cells can result in reprogramming of the mtDNA, mitochondria and overall metabolic pathways leading to the acquisition of the less well-developed and more quiescent mitochondria as found in mouse and human ESCs. Nevertheless, relevant differences have been found in two iPSC clones at the level of expression of genes involved in mitochondrial biogenesis, including NRF1, TFAM, TFB2 M and MTERF and the mitochondrial uncoupling protein 2 and 4 (UCP2 and UCP4) [35]. Moreover, higher levels of expression of these mitochondrial-related genes were associated with less efficient reprogramming of one of these two iPSC clones [35].

In addition, recent reports have also detected differences between undifferentiated miPSCs and mESCs at the level of expression of the mitochondrial transcription and replication factors, PolgA and Peo1. As a result, these miPSCs were unable to accumulate the numbers of mtDNA copies/cell characteristic of differentiating mESCs and regulate ATP content in a manner similar [103]. Results obtained after seven days of differentiation indicate a relative increase in OXPHOS complex formation in iPSCs, but this was accompanied by low levels of ATP. This apparent decrease in ATP levels due to the inability to organize functional complexes and subsequent increase in complex synthesis is similar to compensatory mechanisms commonly observed with mtDNA-depletion like syndromes, and may also be the result of incomplete reprogramming. The Day 14 differentiation data showed insignificant differences in the OXPHOS complex levels among the various reprogrammed cell types and ESCs, which is not reflective of the lower mtDNA copy numbers in iPSCs. This phenomenon seems to resemble mitochondrial diseases at the stage where the threshold for mutant mtDNA (with respect to wild-type) remains to be surpassed and, hence, suppresses the disease phenotype.

Most interestingly, treatment with an inhibitor of de novo DNA methylation, 5-Azacytidine prior to differentiation enabled miPSCs to accumulate mtDNA copies per cell in a manner similar to mESCs [103]. It was hypothesised that iPS cells behave more like their counterpart ESCs after treatment with 5-Azacytidine,

either through epigenetic regulation of nuclear-encoded mitochondrial replication factors or through the modulation of other genes involved in the switch to pluripotency. It would be interesting to see if the differential gene expression patterns for pluripotency markers and replication factors observed in iPS cells aligned more with expression patterns of ESCs after epigenetic modifications. Similar treatment with 5-Azacytidine however, was not able to regulate copy numbers in ESC–ESC or somatic cell-ESC fusions as for iPSCs, again, suggesting that the tetraploid nature of these cells may be an impediment to complete reprogramming.

These data certainly suggest that further analysis of the reprogramming of mtDNA and overall mitochondrial properties are required to better understand and control the mechanisms involved in acquisition of mitochondria with "stemness" properties. The differences in mitochondrial properties detected between iPSC clones and ESCs suggest that analysis at the level of mitochondrial reprogramming should be conducted when assessing the pluripotency of iPSCs. It is possible that this discrepancy, detected in expression of mitochondrial transcription and replication factors, and in the levels of mtDNA copies per cell, might arise from incomplete reprogramming and subsequently disrupted regulation of mitochondria and mtDNA replication. Incomplete reprogramming is likely to compromise differentiation or result in fully differentiated cells having aberrant numbers of mitochondria and mtDNA which may lead to compromised cellular function. This could possibly have profound consequences for the application of iPSCs as an alternative source of clinically useful cell types in regenerative medicine.

Reversion to true pluripotency, which is crucial for the establishment of the mtDNA copy number "set point", depends on the reestablishment of the OCT4–SOX2–NANOG pathways. The mtDNA set point ensures that all pluripotent cells have low number of mtDNA copy that can be expanded during differentiation so that specialized cells acquire the appropriate numbers of mtDNA copy to meet their requirements for ATP generated though OXPHOS [111]. Reprogrammed cells are not truly pluripotent because of incomplete reprogramming, wherein despite expression of similar levels of pluripotency factors as ESCs, mtDNA copy number is not strictly regulated. Induced pluripotency, only after the addition of epigenetic modifiers, closely resembles this form of complete pluripotency. The exact mechanisms of epigenetic modifications that are required post-reprogramming to mimic the effect that regulators such as 5-Azacytidine exert on mtDNA copy number remain to be explained. These modifications are most probably established in the oocyte preceding fertilisation, which would then conceivably explain why none of the reprogramming methods so far produce cells that can truly match the gene expression and copy number profile of ESCs.

Due to the decreased number of CpG sites in mtDNA [112], epigenetic modifications at that level are thought to have a limiting influence on reprogramming of mitochondria. Nevertheless, DNA methylation at the level of the promoters of the mtDNA-specific transcription and replication factors during reprogramming should be assessed as this might contribute to the suppression of mtDNA transcription and replication. This would result in reduced oxidative capacity and most likely drive the cells to activate anaerobic metabolism as the major source of ATP production. It has

been previously hypothesized that acquisition of "stemness" cellular morphology and nuclear DNA reprogramming can be achieved within the relatively short timeframe, whereas reprogramming of mtDNA and the mitochondrial complement requires a higher number of population doublings to be achieved [53].

5.5 Multipotent ASCs Maintain Quiescent Mitochondrial Activity

The term adult stem cells refers to the multipotent cells present in the newborn and adult body that differentiate at least into the cell types that constitute the tissue in which they reside. However, adult stem cells can have very heterogeneous properties reflecting their pre-lineage committed nature, distinct origin and possibly their diverse "niche" conditions [113]. It would be expected that the mitochondrial properties of the adult stem cells would correlate with their ability to differentiate into different cell types with variable energy requirements. Remarkably, undifferentiated human (h)MSCs derived from bone marrow biopsies showed reduced expression of the mitochondrial biogenesis-associated gene PGC1α and the mitochondrial transcription and replication factors, PolgA and Tfam as well as limited numbers of mtDNA copies [114]. Moreover, undifferentiated hMSCs showed elevated levels of the glycolytic enzymes, low expression of mtDNA- and nuclear DNA-encoded subunits, low oxygen consumption and a high rate of lactate production suggesting that, most likely, undifferentiated ESCs, iPSCs and hMSCs rely on glycolysis as the major source of energy [114]. This is consistent with reports that suggest that the oxygen tension in the stromal niche is extremely low leading cells to rely mostly on anaerobic metabolism [115, 116]. Similar results have also been reported for human hematopoietic stem cells (hHSCs) isolated from peripheral blood. Undifferentiated hHSCs have low levels of expression of the ETC subunits, and reduced number of mitochondria and mitochondrial oxygen consumption comprises only about 10 % of that measured in mature cell types [117]. Endothelial precursor cells differentiated from mESCs have also been shown to possess reduced mtDNA levels and decreased expression of the mtDNA-encoded cytochrome c oxidase B gene. Moreover, mitochondrial metabolism is rapidly activated to acquire a mitochondrial profile similar to endothelial cells upon transplantation [118]. Further evidence of the importance of the state of maturation of mitochondria in maintenance of both pluripotency and multipotency are the perinuclear arrangement of elementary mitochondria in undifferentiated hHSCs [117] and monkey [119] and human MSCs [114].

Moreover, upon induction of osteogenic differentiation, expression of the mtDNA transcription and replication factors was shown to increase with a concomitant increase in the expression of the mtDNA- and nuclear DNA-encoded ETC subunits [114]. This resulted in activation of mitochondrial aerobic metabolism as confirmed by the increase in mitochondrial mass, oxygen consumption

rate and intracellular ATP and decrease in expression of glycolitic enzymes and lactate levels [114]. Interestingly, exposure to the mitochondrial respiratory inhibitors antimycin A (complex III inhibitor) and oligomycin (complex V inhibitor) had no significant effect on undifferentiated hMSCs, but resulted in a visible decrease in viability of fully differentiated osteoblasts [114].

5.6 ROS and Mitochondrial "Stemness"

The aforementioned reports strongly link reduced mtDNA content and low mitochondrial activity with the undifferentiated stem cell state and the increase in the number of mtDNA copies/cell and activation of mitochondrial aerobic metabolism with cellular differentiation. Recently, the potential role of mitochondria regulating the maintenance of pluripotency and cell fate has been linked to the levels of ROS within a cell [35, 88, 120]. ROS are generated as a by-product of mitochondrial respiration when O_2, normally the final electron acceptor of the ETC, is prematurely and incompletely reduced to superoxide radical ($\cdot O_2^-$). Production of ROS tends to increase when mitochondria are not producing enough ATP and therefore have high membrane potentials and an elevated pool of reduced coenzyme Q (CoQ) or when there is a high NADH/NAD+ ratio in the mitochondrial matrix. It has been hypothesized that the ROS generated by the ETC leaves the mitochondria and acts as key signaling molecules regulating a variety of cellular functions [121, 122]. In endothelial cells, for example, ROS have been shown to regulate vascular tone, oxygen sensing, cell growth, proliferation, apoptosis and inflammatory responses [33].

In undifferentiated ESCs and iPSCs, maintenance of pluripotency and self-renewal seems to require low intracellular levels of ROS. Undifferentiated mouse and human ESCs generate lower levels of ROS than most somatic cell types, this being primarily due to reduced mtDNA content, immature mitochondrial network and concomitantly reduced OXPHOS [29, 121, 122]. Similarly, low levels of ROS have also been measured in undifferentiated hiPSCs [35]. Interestingly, analysis of the expression of antioxidant enzymes during differentiation of hESCs revealed apparently conflicting results. While increases in the expression of the antioxidant enzymes GPx1 (glutathione peroxidase 1), Cu/Zn SOD (superoxide dismutase), Prx1 (peroxiredoxin 1) and Prx2 have been described [29], decreases in the expression of catalase, SOD1-2-3, SOD2 and GPX2 have also been reported [36, 122]. Although analysis of the expression of different antioxidant enzymes may explain the different results, it is also possible that these two reports conducted their analyses using hESCs at different stages of mitochondrial differentiation, which in turn resulted in inconsistent levels of ROS and levels of expression of antioxidant enzymes.

Expression of antioxidants during ESC differentiation is likely to be regulated in a lineage and stage-specific manner and correlate with the stage of mitochondrial

maturation, leading to the upregulation of some antioxidants enzymes in some lineages but not others. Indeed, hiPSC analysis showed that expression of the antioxidant enzymes GSR (glutathione reductase), SOD2, MGST1 (microsomal glutathione S-transferase 1), GPX2 and MAPK26 (mitogen-activated kinase 26) was decreased during differentiation whereas expression of GSTA3 (glutathione S transferase), and HSPA1B (heat shock protein 1B) was increased [35]. Similarly, analysis of the expression of antioxidant enzymes during osteogenic differentiation of hMSCs revealed a dramatic increase for catalase or MnSOD whereas glutathione reductase, GPx, Prx-I, Prx-III, thioredoxin-I (Trx-I), and thioredoxin reductase (TrxR) showed no upregulation [114]. Nonetheless, consensual data from a number of reports indicate the presence of low levels of ROS in undifferentiated ESCs, iPSCs and adult stem cells and an increase in intracellular levels of ROS upon cellular differentiation [35, 36, 88, 114]. Further supporting this concept, it has been shown that supplementation of media with the antioxidant vitamin C resulted in enhanced generation of mouse and human iPSCs [123]. Vitamin C was proposed to improve reprogramming of adult mouse and human somatic cells by reducing cell senescence and promoting upregulation of important genes associated with reprogramming [123].

Moreover, experiments conducted in hypoxic conditions showed improved expression of the pluripotency genes Oct4, Nanog and SSEA4 along with decreased spontaneous differentiation of hESCs cultured for long periods of time in undifferentiated conditions [50, 124]. Under low oxygen tension, the activity of the mitochondrial ETC is substantially reduced leading the cells to rely on glycolytic metabolism, which in turn results in decreased levels of ROS within the cell. However, hypoxia not only suppresses OXPHOS but also inhibits the proteolyses of hypoxia inducible factors (HIFs; [125–127]. The increased activity of these transcription factors results in downregulation of nuclear DNA-encoded OXPHOS enzymes and an increase in expression of glucose transporters and glycolytic enzymes, which further reinforces the shift to glycolytic metabolism [126, 127]. Also possibly related to the importance of low levels of ROS in the maintenance of pluripotency, hypoxic culture conditions have been shown to enhance reprogramming of mouse and human fibroblasts to iPSCs [128]. Maintenance of low levels of ROS in pluripotent cells is likely to be important for the preservation of non-mutated mtDNA and nDNA. The introduction of mutations to ESCs, iPSCs or pluripotent embryonic cells in vivo could have catastrophic consequences as these genomic alterations would be propagated throughout consecutive cell divisions, leading to a high proportion of mutated mature cells and subsequent dysfunctional tissues and organs. In addition, oxygen tension is also likely to be relevant for stem cells in vivo. ESCs and adult stem cells reside in specific “niches” characterized by particular temperature, oxygen levels, extracellular matrixes and specific interactions with neighboring cells that are likely to support maintenance of pluripotency and a relatively quiescent metabolism [50, 124]. Environmental changes in these “niches” can certainly alter stem cell state and induce proliferation and/or differentiation.

Despite the maintenance of pluripotency and self-renewal being associated with low levels of ROS, moderate increases in ROS production have been shown to take place during differentiation, leading to the hypothesis that ROS could act as a signaling molecule regulating the state of maturation and activity of mitochondria upon commitment into a specific cell fate [35, 88, 120]. Differentiation of mESCs in the presence of the cellular antioxidants polyethylene glycol-catalase, N-acetyl cysteine and the mitochondrial-specific antioxidant mitoubiquinone showed that the low levels of mitochondrial-produced ROS resulted in compromised cardiomyocyte differentiation [88]. Similarly, low levels of ROS induced by a low concentration of glucose (5 mM), also resulted in compromised cardiac differentiation in mouse ESCs and iPSCs. The effect was reverted by supplementation with ascorbic acid, which increased the levels of intracellular ROS leading to an increased percentage of beating foci and increased expression of cardiomycyte marker-genes [88].

Other authors have reported that exposure to moderate levels of ROS enhances ESC differentiation towards the cardiomyogenic and vascular lineages whereas continuous exposure to high levels of ROS results in inhibition of differentiation [120]. Although the intracellular levels of ROS increase during differentiation due to the increase in OXPHOS metabolism, the activities of antioxidant enzymes control the levels of ROS within a range of concentrations to avoid severe outcomes for the cells [120]. Maintenance of moderate levels of ROS during differentiation are likely to be vital for preservation of efficient aerobic production of ATP and consequently for appropriate function of the mature cell phenotype. MtDNA has been postulated to be 10–100 times more vulnerable to mutations than nDNA due to the high levels of ROS and reduced antioxidant defenses in mitochondria [129–132]. In addition, repair of mtDNA mutations is restricted to base excision carried out by POLG and is therefore less efficient than the mechanisms of nDNA repair [133, 134]. Hence, this may explain why tissues with large ATP requirements such as the brain, heart, skeletal muscle, eye, ear and liver tend to be the most seriously affected by mitochondrial diseases [135].

5.7 Stem Cell Models of Mitochondrial Disease

Most of the known mitochondrial disorders are caused primarily by a dysfunctional respiratory chain resulting from mutations in nuclear DNA or mtDNA genes coding for the ETC subunits, mtDNA transcription and replication factors or proteins involved in the assembly of the ETC complexes [136, 137]. In addition, mitochondrial-related diseases may arise from mutations in nuclear DNA genes required for other energy-related mitochondrial functions including synthesis of iron–sulphur clusters, amino acids, steroid hormones and neurotransmitters and regulation of cytoplasmic calcium levels and key events in apoptosis [138, 139]. As mentioned above, undifferentiated ESCs and iPSCs have efficient antioxidant defense mechanisms and low levels of ROS production due to the quiescence of

mitochondrial OXPHOS. However, the onset of ESC and iPSC differentiation results in an increase in replication and transcriptional activities of mtDNA and maturation of mitochondria leading cells to activate aerobic production of ATP and therefore increase the cellular levels of ROS. These changes have profound consequences on differentiating ESCs or iPSCs, which tend to accumulate DNA damage with the increased levels of ROS [121, 122]. Mutations at the level of the mtDNA also tend to accumulate over time [135], causing multiple cellular dysfunction, including defective protein degradation or cellular secretion [140]. Accumulation of mtDNA mutations in differentiating ESCs or iPSCs is likely to result in incompetent, mature cells that are unable to carry out their specific functions due to impaired ATP production. In this context, it remains to be investigated whether mutations at the level of mtDNA during cellular differentiation and the efficiency of the mature cells to produce enough ATP through OXPHOS can regulate and activate signaling pathways that would prevent these cells from persisting in the mature tissues. As previously mentioned, high levels of ROS are detrimental for differentiating ESCs and can possibly activate apoptosis preventing the cells from surviving in culture [120].

Disease modeling based on animal models, particularly rodents, has been extensively used in translational scientific research including drug testing. However, genetic and anatomical differences have led to inadequate phenotypic correlations between these genetic models and the human diseases that they attempt to recapitulate [101, 102]. A potential alternative is the generation of stem cell disease models that can be used to differentiate mature cells with the phenotypic characteristics of a specific disease, especially mitochondrial diseases.

5.7.1 Human ESC Models of Mitochondrial Disease

Initial studies with stem cell lines derived from embryos carrying monogenic disorders have provided substantial evidence that stem cell models of diseases are a valuable approach for studying developmentally regulated events involved in the pathogenesis of a specific disorder [141–143]. Human ESC lines have been generated from embryos discarded from in vitro fertilization (IVF) clinics due to diagnosis of mutations associated with Fragile X syndrome [141], myotonic dystrophy type 1, cystic fibrosis and Huntington disease [142, 143]. Out of these, Huntington disease, Fragile X syndrome and cystic fibrosis involve mitochondrial dysfunction related to increased oxidative stress, mtDNA mutations and impaired oxidative phosphorylation. However, alterations in mitochondrial function arise from disease-specific nuclear DNA mutations in non-mitochondrial-related genes, which primarily lead to abnormal cellular function followed by dysfunctional alterations at the mitochondria and mtDNA levels [144, 145]. Alternatively, stem cell models of human disease can be generated through directed mutagenesis using homologous recombination as shown by mutation of the hypoxanthine phosphoribosyltransferase gene (HPRT1) that reproduces a Lesch-Nyhan disease

phenotype [146]. Here, mutation of the HPRT1 gene results in the inability to convert hypoxanthine into inosine 5′-monophosphate leading to increased levels of uric acid causing gout-like symptoms, urinary stones and some neurological disorders. Unfortunately, embryos harboring diagnosed mutations are scarcely provided for research proposes and producing disease-specific ESC lines by homologous recombination is highly inefficient. As such, no ESCs models of diseases caused by mutations of mtDNA or mitochondrial-related genes or diseases having mitochondrial dysfunction as a primary consequence have been described to date. Another approach for using hESC lines for disease modeling is to genetically modify the cells to express a disease-causing transgene using cell-type-specific promoters [147]. This has been demonstrated by expressing a mutant form of superoxide dismutase 1 (*SOD1*) associated with familial amyotrophic lateral sclerosis (ALS) in hESC-derived motor neurons. Neurons transfected with mutant *SOD1* exhibited reduced cell survival and shortened axons [147]. Although the biology of ALS is not clearly understood, it has been reported that mutation of *SOD1* results in higher susceptibility of the molecule to lose its metal ions (demetallation) and form large and stable amyloid-like protein oligomers that tend to accumulate in the mitochondria leading to mitochondrial dysfunction and activation of apoptosis [148]. However, this approach would again only be useful for modeling monogenic diseases caused by highly penetrant mutations and not for modeling complex disorders for which genetic determinants are either unknown or poorly understood.

5.7.2 Human iPSC Models of Mitochondrial Disease

Reprogramming biology is now providing a novel route for the generation of disease models. Apart from the immense potential of regenerative medicine, the generation of iPSCs from adult cells of human patients offers an unprecedented opportunity to generate valuable disease models to study developmental progression in vitro. These iPSC models can then be induced to differentiate into cell type(s) affected by the disease and have the great advantage of carrying the precise genetic mutations, both known and unknown, and the full patient genetic background that has led to the development of the syndrome. Several patient-specific iPSC lines have already been generated for a number of complex diseases including Parkinson disease (PD; [149]), Friedrich's ataxia (FRDA; [150, 151]), Huntington disease, type 1 diabetes mellitus, Down syndrome, Becker muscular dystrophy, and Lesch-Nyhan syndrome [14]. Nevertheless, a clear understanding of the reprogramming phenomenon and tight quality control of the established iPSCs is still required in order to clearly distinguish the disease phenotype from epigenetic adjustments [152].

To date, pluripotent cell models of mitochondrial disease are limited to the generation of iPSCs from patients of genetic PD and FRDA. Despite idiopathic PD being the most common form of the disease, 2–3 % of all PD cases can currently

be linked to a single genetic factor [153, 154]. iPSCs have been generated from skin fibroblasts of patients suffering from inherited PD carrying a mutation in the PTEN-*induced putative kinase 1* (*PINK1*) gene [149]. *PINK1* encodes a mitochondrial kinase localized on the outer mitochondrial membrane, which is involved in cellular defense mechanisms including mitochondrial degradation upon increased levels of mitochondrial oxidative stress [155, 156]). *PINK1*-mutant iPSCs were shown to differentiate into dopaminergic neurons with no significant differences in comparison to the WT-iPSCs [149]. However, upon mitochondrial depolarization induced by the potassium ionophore valinomycin, the *PINK1*-mutant iPSC derived dopaminergic neurons showed impaired recruitment of Parkin to the mitochondria [149]. Parkin is an E3 ubiquitin ligase acting downstream of PINK1 that is translocated to dysfunctional mitochondria to promote mitophagic degradation in cells of PD patients [157, 158]. Consistent with the decreased recruitment of Parkin to the mitochondria, the *PINK1*-mutant iPSCs also showed increased mtDNA copy number and upregulation of PGC1α suggesting that impaired degradation of depolarized mitochondria and activation of mitochondrial biogenesis take place in *PINK1*-mutant neurons as a compensatory response to restore deficient mitochondrial function [149].

Another iPSC model of mitochondrial disease has been generated from skin fibroblasts of FRDA patients. FRDA is the most common inherited ataxia disorder caused by degeneration of the central and peripheral nervous systems, resulting in progressive gait and limb ataxia, a lack of tendon reflexes, leg weakness and cardiomyopathy [159, 160]. The phenotypic onset of the disease relates to the abnormal increase in the number of GAA triple-repeats in intron 1 of the nuclear-encoded *Frataxin* (FXN), which leads to heterochromatin formation and transcriptional silencing of the gene [159, 161]. FXN protein localizes to the inner mitochondrial membrane and its function is thought to be related to the biogenesis of iron-sulphur cluster synthesis and therefore, to the assembly of the ETC [160]. Indeed, Frataxin deficiency has been shown to result in defective oxidative phosphorylation, increased levels of ROS [160] and accumulation of iron in the mitochondria of the most affected tissues such as cardiomyocytes and neurons [162]. iPSC lines established from FRDA patients showed unstable expansion of the GAA trinucleotide repeats in intron 1 suggesting that FRDA-iPSCs can recapitulate the disease phenotype [150, 151]. Moreover, analysis of two different FRDA-iPSC clones (from different patients) and wild-type iPSC and ESC lines have shown relevant differences in the expression of genes related to mitochondrial function and DNA repair [150]. Given that FRDA-iPSCs can be directed to differentiate into sensory neurons and cardiomyocytes [151], these cells can now be used as models to study the cellular pathology of FRDA, including mitochondrial dysfunction that seems to be intrinsically involved in the disease phenotype. Defective synthesis of iron-sulfur clusters in the mitochondrial matrix certainly accounts for defective OXPHOS and the reported increase in ROS levels, which in turn, is likely to cause mtDNA mutations, especially during differentiation of cell types with elevated OXPHOS requirements such as neurons and cardiomyocytes.

5.8 Conclusion

The data reviewed here clearly demonstrate that the level of mtDNA activity is intimately related to the stage of pluripotency of a cell. ESCs, iPSCs and adult stem cells are characterized by quiescent mitochondrial metabolism, reduced expression of the ETC subunits, low oxygen consumption and considerable reliance on anaerobic production of ATP. Conversely, cellular differentiation is characterized by the onset of mtDNA biogenesis in order to activate the more efficient form of mitochondrial metabolism and fulfill the higher energy demands of differentiated cells. As such, the generation of iPSCs from adult cell types containing a mature mitochondrial complement requires the complete reprogramming of the cytoplasm to reestablish a "pluripotent" mitochondrial network. Although nuclear reprogramming has been shown to regulate the expression of the mitochondrial-related genes and revert the mature mitochondrial network and mtDNA copy number of the adult cell towards the pluripotent state, some specific differences at the level of the expression of mitochondrial transcription and replication factors and in the number of mtDNA copies/cell have been described in different iPSC lines. Moreover, these specific differences in iPSC lines, which relate to incomplete reprogramming of the mitochondrial network and mtDNA copy number, reinforce the concept that analysis of the state of maturation of the mitochondria should be conducted when assessing pluripotency of newly established cell lines. Generation of human pluripotent stem cells having a fully reprogrammed nucleus and cytoplasm can then be used as human models for specific diseases allowing easy assessment of new therapeutic drugs. Specifically, the generation of iPSCs using cells from human patients constitutes a unique tool as they can be induced to differentiate into cell type(s) affected by the disease and have the great advantage of carrying the precise genetic mutations that led to the development of the syndrome. To date, the pluripotent stem cell models of mitochondrial diseases are restricted to the generation of iPSCs from patients suffering from genetic PD and FRDA. Differentiation of PD-iPSCs and FRDA-iPSCs into disease-specific cell types appears to recapitulate the cellular pathology and with time, may be used to study mitochondrial dysfunctions associated with these diseases. However, iPSCs harbouring nuclear- and mtDNA-encoded mutations are desperately required to fully characterise the role of these mutations in the progression of their respective diseases.

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Chapter 6
The Role of Mitochondrial DNA in Tumorigenesis

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Abstract Mitochondria are widely accepted as an important organelle responsible for numerous processes ranging from ATP production, fatty acid oxidation, as well as the control of cellular apoptosis and steroidogenesis. The mitochondrial genome (mtDNA) exists exclusively within the mitochondrion and in multiple copies within the mitochondrial matrix. In tumorigenesis, modifications are known to arise during the transcription and replication of mtDNA, which often lead to changes in mtDNA copy number. Moreover, different cancers tend to be associated with base changes at various locations within the mitochondrial genome. This chapter discusses current knowledge of the relationship between mtDNA and cancer. The influence of various tumors on mtDNA copy number, and key mtDNA variants associated with disease and cancer will also be discussed, together with the role of mitochondria in tumor cell energy metabolism.

6.1 Introduction

Within eukaryotic cells, mitochondria are found to exist in the cytoplasmic compartment. They are responsible for carrying out a number of functions including cellular apoptosis and proliferation, steroidogenesis as well as the production of energy. Mitochondria contain a genome that functions separately from nuclear

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DNA. The processes that assist in the maintenance of the mitochondrial genome may undergo changes for a number of reasons. It is important to understand the mechanisms that lead to these defects, the impact that these may have on the function of the mitochondrion and possible approaches that can be used to control these events. This chapter will provide background to the role of mtDNA in energy production, the various theories surrounding the mechanism of mtDNA replication and the factors involved in this process, as well as those participating in mtDNA transcription. Furthermore, factors influencing mtDNA copy number will be discussed in the context of cancer, and a discussion of the Warburg hypothesis and other theories will be presented thereafter.

6.2 Mitochondrial DNA

The mitochondrial genome encodes 13 polypeptides that contribute directly to the electron transfer chain, along with 22 tRNAs, and 12S and 16S rRNAs (Fig. 6.1) that assist in the translation of the mitochondrial genome. The electron transfer chain comprises four multimeric protein complexes, together with the ATP synthase, which is responsible for synthesis of ATP (Fig. 6.2). Coupling between activity from the electron transfer chain, otherwise known as the respiratory chain, and that from the ATP synthase is referred to as oxidative phosphorylation [1]. The sequential flow of electrons through the protein complexes drives the production of energy as oxygen becomes reduced to water within the matrix, during the process of which an electrochemical gradient is formed between the matrix and the intermembrane space. This proton gradient is used to generate ATP as hydrogen ions translocate back into the matrix via the ATP synthase complex.

MtDNA exists as a closed circular molecule (Fig. 6.1), and with multiple copies of the genome residing within a single mitochondrion, there arises the possibility of the coexistence of wild type and mutated copies of mtDNA, in a state referred to as heteroplasmy. Heteroplasmy can be detrimental to cells if the proportion of mutant mtDNA copies exceeds the threshold level above which changes in cellular phenotype may be induced. Generally, it has been estimated that this ranges from 70 to 90 % [2]. However, different tissues exhibit variation in their mutant threshold, with germ cells, for example, having minimal tolerance for the accumulation of mtDNA mutations [3]. Although tumors tend to display heteroplasmy, few studies have reported the existence of homoplasmic mutations in tumors [4, 5], whereby all copies of mtDNA within a tumor cell possess the same specific mutation. Whilst these represent rare cases, investigations have unraveled that the chances for this to occur are numerous, for example a single mutated copy of mtDNA might have a replicative advantage over others [6]. Alternatively, it has been suggested through mathematical modeling that, instead of selection mechanisms for the mutant copies, accumulation of mutants may occur by chance just through random segregation during cellular division, an event which happens at a faster rate in tumor cells when compared to their normal counterparts [7].

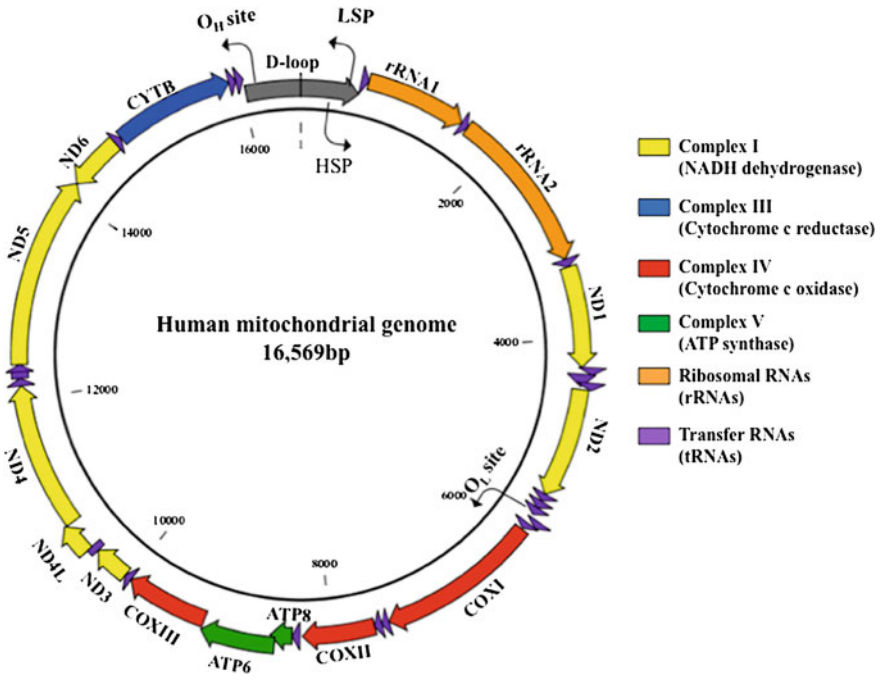


Fig. 6.1 Diagrammatic representation of the mitochondrial genome: The mitochondrial genome encodes 13 polypeptides involved in the electron transport chain. Specifically, it encodes 7 subunits of complex I, 1 subunit of complex III, 3 subunits of complex IV and 2 subunits of complex V. There are 2 origins of replication, one on the heavy strand (O_H), and another on the light strand (O_L), which replicate the genome in opposite directions. Replication of the heavy and light strands of mtDNA are stimulated by the actions of their respective promoters HSP and LSP, both of which reside within the D-loop region, which represents the only triple-stranded, non-coding region of the mitochondrial genome

Aside from mutations, which will be discussed in detail later, depletion of mtDNA may also occur. A critical level of depletion is defined as having a 30 % reduction in mtDNA copy number [8]. This phenomenon is known to severely affect oxidative phosphorylation [9]. Currently, the defective regulation of nine nuclear-encoded genes has been identified as the possible cause for mtDNA depletion syndromes [10]. These include polymerase gamma (*POLG*), succinate-CoA ligase alpha (*SUCLG1*) and beta (*SUCLA2*), deoxyguanosine kinase (*DGUOK*), *MPV17* a mitochondrial inner membrane protein, chromosome 10 open reading frame 2 (*C10orf2*), ribonucleotide reductase M2B (*RRM2B*), mitochondrial thymidine kinase 2 (*TK2*), and thymidine phosphorylase (*TYMP*). All of these are believed to be primarily involved with replication as well as maintaining the integrity of the mtDNA genome [10]. Syndromes associated with these defects include progressive external ophthalmoplegia (PEO) [11, 12] and Kearns-Sayre syndrome (KSS) [13]. The resulting phenotypes generally belong to one of three groups, which are encephalomyopathic, myopathic or hepatocerebral in origin

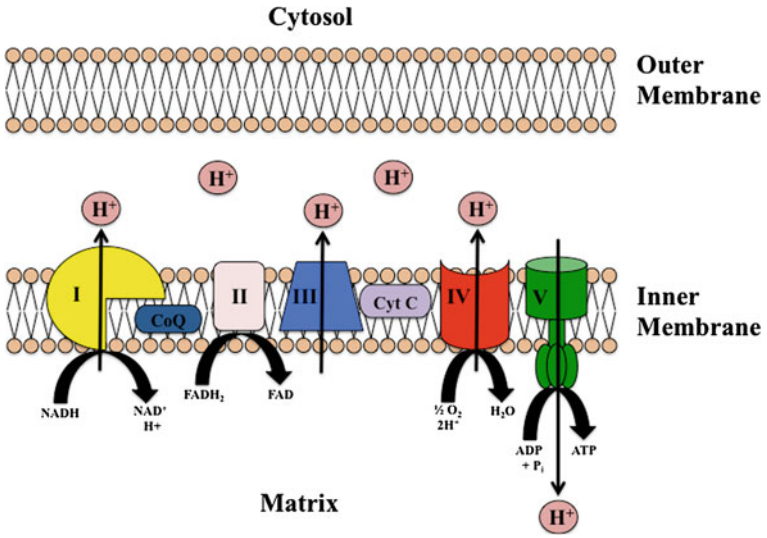


Fig. 6.2 Schematic diagram of the electron transport chain: The electron transport chain is situated at the mitochondrial inner membrane and functions under aerobic conditions. It comprises 5 protein complexes in total. Only complex II is exclusively encoded by the nucleus. Hydrophobic carriers, coenzyme Q (CoQ) and cytochrome C (Cyt C) exist in between the complexes, which enables the sequential transfer of electrons from one complex to another, until electrons reach molecular oxygen, which accepts electrons thereby becoming reduced in the process to form the by-product water. During this time, the build up of a proton electrochemical gradient promotes the formation of ATP generated from complex V, which can be used to provide energy for various metabolic processes, in particular for those cells of high energy demanding tissues, such as the heart and liver

[14]. Whilst various therapies have been explored as possible treatments for mtDNA depletion syndrome, including the use of pyruvate [15], it is important to understand how the factors that control mtDNA copy number are regulated, both under normal conditions and in a tumorigenic state.

6.3 Mitochondrial DNA Replication and Transcription

Due to the limited capacity of the mitochondrial genome to encode factors participating in the mtDNA replication and transcriptional machinery, the proteins that are required for maintenance of the mitochondrial genome are nuclear-encoded and undergo translation in the cytosolic compartment prior to their import into the mitochondrion [16]. Transcription and replication of the mitochondrial genome involve interaction between several interacting factors. They are jointly initiated by mitochondrial transcription factor A (TFAM) and mitochondrial transcription factor B2 (TFB2M), which generate a DNA/RNA hybrid primer in

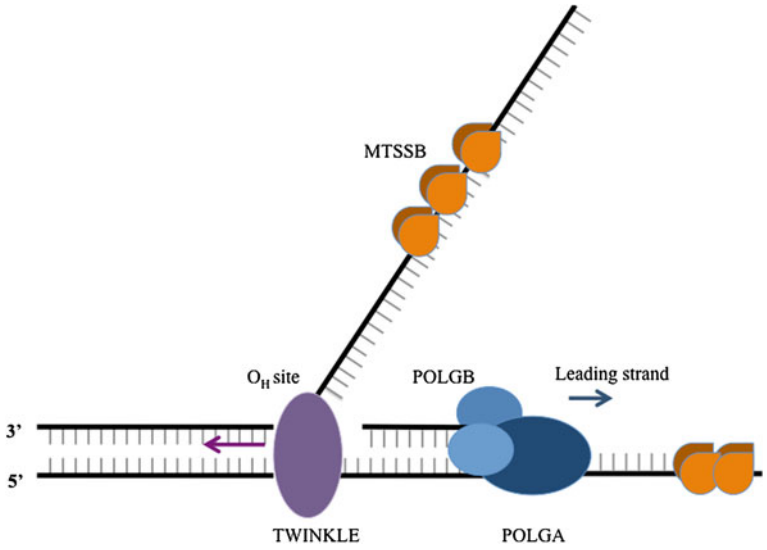


Fig. 6.3 Strand asynchronous model for the replication of mtDNA: Replication of the mitochondrial genome begins at the origin of replication located on the heavy strand (O_H site). Unwinding of the duplex DNA occurs in this region, through the actions of TWINKLE, the mtDNA-specific helicase. The newly separated strands are bound by mitochondrial single stranded binding proteins (mtSSB) to ensure the two strands do not reanneal to one another prematurely, before replication is able to take place. The mtDNA polymerase complex comprises the catalytic subunit (POLGA), together with two supporting processivity subunits (POLGB). Misincorporation of nucleotides is minimized via the actions of an internal $3' \rightarrow 5'$ exonuclease activity within POLGA.

the presence of mitochondrial RNA polymerase [17]. TFB2M represents one of two mitochondrial coactivator transcription factors, with TFB2M being more active relative to its counterpart mitochondrial transcription factor B1 (TFB1M) [18]. The DNA/RNA hybrid that is produced is utilized by the catalytic subunit of the polymerase γ A (POLGA) to replicate the mtDNA template [19], an enzyme that possesses $3' \rightarrow 5'$ exonuclease activity. POLGA is assisted in this process by its accessory subunits, POLGB that promotes DNA binding and processivity of the enzyme complex [20]. Other factors include the mtDNA helicase, TWINKLE [21], and the mtDNA specific single stranded binding protein (mtSSB) [22, 23], which together allow for efficient replication of mtDNA (Fig. 6.3). As such, activity of the POLG multisubunit complex alone is not capable of inducing changes to mtDNA copy number [24]. Whilst TWINKLE has largely been associated as the main factor involved with unwinding of the mtDNA template, structural analyses of TFAM have determined that the protein exhibits the capability to modify DNA structure, leading to suggestions that TFAM may assist in the unwinding of the mitochondrial promoter regions, necessary for efficient transcription to occur [25].

It has been long understood that there is asynchronous replication of mtDNA, originating initially from the heavy strand promoter, followed by switching to the light strand promoter, which synthesizes mtDNA in the opposite direction after two-thirds of replication has been completed on the heavy strand (Fig. 6.3). This mechanism was first proposed in 1972, and was later challenged by other groups upon identification of double stranded regions during replication [26]. This led others to hypothesize that there is a strand-coupled mechanism that occurs, similar to conventional nuclear DNA replication [27]. Recent studies have elaborated on the strand-synchronous theory, revealing that the double stranded replication intermediates are comprised of RNA–DNA hybrid regions. This led to the RITOLS theory, which suggests that RNA intermediates are incorporated into the lagging strand of the mtDNA replicant [28]. It is likely that both replication mechanisms occur concurrently, with the strand-synchronous method predominating during periods of mtDNA replenishment, following exposure to induced stress [29].

6.4 Mitochondrial DNA Copy Number During Development

During development, mitochondria have to undergo maturation events before they are capable of carrying out their functions [30, 31]. Changes involve modifications to the structure of the organelle, with earlier appearances being defined by the presence of a less electron-dense matrix, together with immaturely developed cristae on the inner mitochondrial membrane [32]. Indeed, changes seen during differentiation have been reported to correlate with enhanced metabolic capacity, which is reflective of the changes in metabolic demands during embryonic development.

Various attempts have been made to explain the relationship between mtDNA copy number and its changes during development. One such suggestion has been that perhaps mtDNA copy number is influenced by the expression of pluripotency genes, based on results demonstrating that induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) share similar levels of copy number [33], estimated to range from 1,200 to 1,500 per cell [34]. Others have published work contradicting this theory, presenting evidence to suggest the reverse is in fact true—that mitochondria have an active role in the influence of pluripotency in human embryonic stem cells [35, 36]. For example, increases in the expression of pluripotency factors, such as octamer-binding transcription factor 4 (Oct4), have been thought to be caused by defective mitochondrial function [36]. It is interesting that, although copy number in human ESCs (hESCs) has been reported to significantly increase over 30 % during the early stages of differentiation [37], one contrasting study was able to show a fall in mtDNA copies per cell to 600–1,000 in hESCs that have undergone induced differentiation to the neural lineage [34].

Nonetheless, it is generally accepted that cells, such as embryonic stem cells, contain few copies of mtDNA [38, 39] and produce ATP predominantly through

glycolysis [40]. However, during the differentiation of embryonic stem cells into high-energy cell types, such as neurons, mtDNA copy number is expanded accordingly to increase the capacity of the electron transfer chain to allow sufficient generation of ATP through oxidative phosphorylation [38, 39]. However, it should be appreciated that mtDNA copy number in each cell tends to be dependent on the individual cell's specific function and thus its specific requirements for energy generated through oxidative phosphorylation [17].

6.5 Mitochondrial DNA in Tumor Cells

Although mutations to the mitochondrial respiratory chain complexes are frequently observed in tumors, it is uncertain whether these mutations are induced following the onset of tumorigenesis, or they are the cause of tumorigenesis. Mutations may arise as a result of the enhanced leakage of electrons from the respiratory chain, leading to production of superoxides [1]. Aside from changes to mitochondrial respiratory activity as occurs during the Warburg effect, which will be discussed in further detail later in the chapter, any changes in the individual subunits of the respiratory chain may also impact on the metabolic dysregulation observed in tumor cells [1].

6.5.1 Mitochondrial DNA Copy Number in Tumor Cells

It has been hypothesized that precise regulation of mtDNA copy number is vital as it is involved with tumor initiation, can cause genomic instability, and also has an influence on therapeutic response to treatment [41]. MtDNA copy number in tumor cells may be influenced by parameters such as the precise location of the mutation. For example, mutations in the D-loop region, where the nuclear-encoded transcription and replication factors interact with mtDNA, may impact directly on the replicative ability of mitochondrial genome [42]. However, it is intriguing that recipients of chemotherapy or radiotherapy for treatment of various cancers have displayed further mutations within their mtDNA when compared to their pretreatment state [43]. Moreover, it was postulated that if these mutations persist in the mitochondrial genome, their self-replicating copies might be detrimental to the outcome of the treatment [44, 45]. Perhaps with future research, it may be beneficial for pharmaceutical industries to develop drugs that assist in the protection of the mitochondrial genome during radiation therapy, thus minimizing or eliminating any side effects arising from mtDNA mutations [43].

While there is no definitive screening method for the detection of the onset of pancreatic cancer, a significant positive relationship has been observed between the risk factors involved in developing this cancer and in its mtDNA copy number [46]. In addition, a potential correlation exists between mtDNA copy number and

Table 6.1 Changes in mtDNA copy number in tumors

Tumor origin	mtDNA Copy number	Reference
Breast	Decreased	[53–56]
Gastric	Decreased	[57]
Colon	Decreased	[223]
Liver	Decreased	[49, 50, 59, 62]
Brain	Increased	[67]
Head & Neck	Increased	[68–70]
Lung	Decreased	[60]
Prostrate	Increased	[71]
Acute lymphoblastic leukemia	Increased	[72]
Endometrial	Increased	[73]
Esophageal	Increased	[74]
Non-Hodgkin lymphoma	Increased	[75]
Ovarian	Increased	[76]
Thyroid	Increased	[53]
Ewings sarcoma	Decreased	[61]
Fibrolamellar	Decreased	[62]
Renal cell carcinoma	Decreased	[63–66]

the risk of developing lung cancer, although more work is required to validate this preliminary finding [47]. Despite this, increasing evidence emerges to suggest that mtDNA copy number could participate in the development of tumorigenesis.

Nevertheless, different types of tumors appear to regulate mtDNA copy number differently. For example, endometrial adenocarcinoma tends to be associated with increased copy number ranging from approximately 770 mtDNA copies per cell in normal endometrium to 2,000 mtDNA copies in cases of endometrial cancer [48]. Hepatocarcinoma appears to decrease copy number [49] from approximately 7,000 in non-tumorigenic hepatocytes to 5,800 in tumor hepatocarcinoma [50]. The reason why different cancers have characteristically high or low mtDNA copy numbers is uncertain. Nonetheless, these observations have been used as clinical diagnostic markers for the progression of cancer [51]. One might anticipate that in tumorigenic states, there would be up-regulation of activity in the replicative machinery, together with increases in mitochondrial biogenesis and up-regulation of components involved in oxidative phosphorylation [52], which would lead to enhanced numbers of wild-type mtDNA to compensate for mutated copies. However, it could equally be expected that in tumorigenesis, there should be low mtDNA copy numbers, as this situation would be reflective of cells in their less-differentiated state, similar to the early stages of cellular development.

Currently, it appears that mtDNA copy number is decreased in breast cancer [53–57], gastric cancer [57, 58], hepatocellular carcinoma [49, 50, 58, 59], lung cancer [60] Ewings sarcoma [61], fibromellar cancer [62], and renal cell carcinoma [63–66]. On the contrary, increases in mtDNA copy numbers have been reported in brain cancer [67], head and neck cancer [68–70], prostate cancer [71], acute lymphoblastic leukemia [72], endometrial cancer [73], esophageal cancer [74],

non-Hodgkin lymphoma [75], ovarian cancer [76] and thyroid cancer [53]. Each of the above reports can be found in Table 6.1, with deviations in mtDNA copy number observed between the cancerous tissue and its neighboring healthy tissue.

6.5.1.1 Decreases in mtDNA Copy Number in Tumor Cells

Of the 16 known DNA polymerases in eukaryotic cells, POLGA is the only DNA polymerase known to function within the mitochondria [77]. Loss of function of POLGA in tumorigenic cells has not only demonstrated changes to mtDNA copy number, but also shown decreases in mitochondrial function. Additionally, up-regulation in the synthesis of reactive oxygen species (ROS) has also been reported [78], a feature which potentially manifests into the disease state [79]. A potential role for POLGA defects in tumorigenesis was outlined by Singh et al. [80], who ectopically expressed a proofreading deficient POLGA in a breast cancer cell line. The transformed cell line exhibited mtDNA depletion in comparison to controls and showed increased tumorigenic properties when using in vitro based cell invasion assays [80]. This study highlighted how defects to POLGA may contribute to mtDNA depletion and potentially promote tumorigenic properties.

Mutations in TFAM have also been associated with respiratory chain defects [81] and more recently in tumor cells [82]. Many reports have suggested that high TFAM levels correspond to increases in mitochondrial transcription. However, others have proposed an optimal concentration of TFAM, above which it is believed that it is inhibitory to transcription [83]. A study by Guo et al. investigated the frequency of truncated TFAM mutations in colorectal cancers with microsatellite instability and microsatellite stability. TFAM mutations were found in 74 % of colorectal cancers with microsatellite instability, which was also associated with reduced TFAM protein production and mitochondrial mass in these cancers. Furthermore, no mutations in TFAM were observed in colorectal cancers with microsatellite stability, demonstrating that TFAM mutations were unique to those cancers with microsatellite instability [82]. When the colorectal cancer cell line, RKO, was transfected with either the truncated TFAM gene or wild-type TFAM, RKO cells harboring the mutant TFAM gene exhibited mtDNA depletion compared to the wild type. Furthermore, RKO cells containing the TFAM mutation also grew at an accelerated rate compared to control cells. These observations were confirmed in vivo, in which cells containing the TFAM mutant generated larger tumors in severe combined immunodeficient (SCID) mice than cells containing wild-type TFAM. This suggests that mutant TFAM promotes cell proliferation whilst the wild-type restores normal proliferation rates. Reductions in mtDNA gene expression were also observed in mutant TFAM cells compared to wild-type. Furthermore, cytochrome B, which has previously been shown to be associated with apoptosis [84], was reduced and resistance to the chemoagent cisplatin was increased. The authors speculated that mutant TFAM is able to enhance chemo-resistance in a cytochrome B dependent manner [82].

The interaction of TFAM with the mitochondrial heavy strand promoter has also been analyzed in vitro and showed reduced binding affinities in mutant cell extracts compared to wild type [82]. Such observations suggest that mutant TFAM reduces interaction at the heavy strand promoter, which inhibits both mtDNA replication and gene expression, resulting in mtDNA depletion, enhanced proliferation, and chemo-resistance [82]. Interestingly, replenishment of mtDNA copy number following induced depletion with agents such as ethidium bromide, is not enhanced by elevation of TFAM levels [85]. Aside from this, it is equally important to consider that more upstream proteins may regulate TFAM levels, for example, peroxisome proliferator activated receptor γ coactivator 1α (PGC-1α), which is often overlooked as a potential regulator of mtDNA copy number [86].

Mutations within the D-loop region have also been frequently reported in various cancers [55, 59, 61]. The D-loop is a non-coding region of mtDNA and functions as a regulatory site for mtDNA transcription and replication. It can therefore be postulated that mutations within this region can disrupt the binding affinity of TFAM during the initiation of mtDNA transcription, and subsequently partially inhibit mtDNA replication leading to reduced mtDNA turnover and depletion over time. In support of this hypothesis, mtDNA depletion has been associated with D-loop mutations in hepatocellular carcinoma [59], breast cancer [55] and Ewings sarcoma [61]. Furthermore, as mtDNA depletion has been linked with tumorigenic and chemo-resistance properties in some cancers [80, 82], it is plausible that D-loop mutations may instigate and/or maintain these processes.

Although the electron transport chain functions as the primary generator of ATP, it is also a generator of ROS that include hydrogen peroxide and superoxide [87]. ROS are potentially harmful to DNA and increase the likelihood of DNA damage, genomic instability and potentially neoplasmic transformation. MtDNA resides in close proximity to the electron transfer chain and, although mtDNA is packaged by TFAM [88], which provides some protective properties, mtDNA has reduced DNA repair mechanisms compared to nuclear DNA and is more susceptible to damage and, thus, the presence of mutations are not uncommon. Achanta et al. showed that the tumor suppressor gene, *p53*, which is implicated in DNA repair, cell cycle regulation, and apoptosis, interacts directly with POLGA to maintain mtDNA stability in response to DNA damage induced by ROS and other insults [89]. p53 was found to enhance the DNA replication properties of POLGA whilst knockdown of p53 was shown to increase the susceptibility of mtDNA to damage and increased the frequency of in vivo mutations, which were reversible following transfection with wild-type p53. In support of this, other studies have found that lower levels of p53 have correlated with reductions in mtDNA copy number [90, 91], due to the additional role of p53 as a checkpoint protein involved in mitochondrial biogenesis [92]. Furthermore, p53 may act as an external repair protein that enhances accuracy of mtDNA replication [93].

Loss of p53 function is a common characteristic of tumorigenesis and occurs at a frequency of 50 % [94, 95]. These data suggest that a loss of p53 function will be detrimental for the function of POLG and the maintenance and replication of mtDNA,

and therefore may contribute to mtDNA depletion, the emergence of multiple variants and large-scale deletions observed in disease and tumors [89, 91, 96].

6.5.1.2 Increases in mtDNA Copy Number in Tumors

MtDNA copy number has been shown to increase in various tumor types (see Table 6.1). Increases in mtDNA copy number have been linked with the aging process [97], and in support of this, one of the greatest risks for the development of cancer is increased age [98]. It has been suggested that during the aging process, accumulation of mutations and deletions in mtDNA occur [97, 99, 100]. Investigators suggest that this increase in mtDNA content over time functions as a feedback loop to compensate for defective oxidative phosphorylation [101], which exposes mtDNA to increasing amounts of oxidative stress.

Experiments by Lee et al. showed that primary lung cells increased their mitochondrial mass and mtDNA copy number in response to hydrogen peroxide exposure and suggested that these alterations in mtDNA are early molecular events to adapt to endogenous or exogenous oxidative stress [102]. Increases in mtDNA copy number have also been reported in aged rhesus monkey brain tissue [103] and murine neural stem cells compared to non-aged controls [104]. Although aged neural stem cells increased their mtDNA copy number, their oxygen consumption was reduced and they adopted an aerobic glycolytic metabolic profile [104]. This suggests that oxidative phosphorylation becomes increasingly defective during aging, presumably due to increased frequency of mtDNA mutations, which was also observed in aged neural tissue [103].

Changes to mtDNA copy number with aging appear to be tissue specific. Whilst changes in mtDNA copy number were shown to be increased in brain tissue, mtDNA copy number was reduced in liver tissue and unchanged in cardiomyocytes [105]. These tissue specific changes may correlate with the differences in mtDNA copy number between tumor types. Finally, there is some evidence to suggest that alterations in mtDNA copy number, through mutation, deletion and as a consequence of aging, can result in defective oxidative phosphorylation [104]. In these cases, aging cells adopt an aerobic glycolytic metabolism, also known as the Warburg effect (discussed in more detail later), which is a “metabolic hallmark” of multiple tumors [106]. This suggests that loss of mtDNA integrity may play a direct role in the initiation of altered metabolism and tumorigenesis.

Furthermore, the mitochondrial replication factors are associated with increased copy number. TWINKLE has interestingly been argued to possess a predominate role in the control of mtDNA copy number [24]. Work conducted in mice involving forced overexpression of Twinkle in energetically demanding tissues, exhibited threefold increases in mtDNA copy number. Knockdown experiments on TWINKLE conducted in human cells further supported its contribution in mediating mtDNA copy number [24].

Together, there is reportedly a collaborative effect of the joint overexpression of TWINKLE and TFAM [107]. While transgenic mice overexpressing either of

these factors exhibited an increase in mtDNA copy number, overexpression of both proteins simultaneously revealed much higher increases in mtDNA copy number [107]. However, it was discovered that generating bitransgenic mice overexpressing both Twinkle and Tfam led to increases in the size of the mtDNA-protein nucleoid complexes, relative to wild-type untreated mouse controls. A single nucleoid is able to package several mtDNA molecules, and their segregation within the mitochondrion is believed to influence inheritance of mutant or wild-type copies of mtDNA [108]. The effect of large mtDNA-protein nucleoid complexes is detrimental to mtDNA function, as the lower the mtDNA copy number per nucleoid, the more compromised the ability of the respiratory chain is to function effectively [107]. Perhaps, this phenomenon is also true for the human system where up-regulation of both factors in tumors may negatively impact on proper functioning of mtDNA, compromising energy production and thereby an ability to maintain the metabolic demands of tumor-affected tissues.

6.5.1.3 Mitochondrial DNA Depleted Tumor Cells

Cells lines devoid of mtDNA, termed ρ^0, have been generated using multiple tumor cells derived from lung [109], bone [110], and cervical cancers [111], amongst others. Ethidium bromide has been used extensively as an mtDNA depletion agent. Low concentrations of ethidium bromide intercalate into mtDNA and inhibit the function of POLGA resulting in stalled mtDNA replication. Subsequent cell divisions result in a progressive dilution of mtDNA until a final population remains devoid of mtDNA. ρ^0 cells cannot utilize oxidative phosphorylation and rely exclusively on glycolysis for the generation of ATP. However, ρ^0 tumor cells retain functional mitochondria and maintain mitochondrial membrane potential. Consequently, ρ^0 tumor cells are a useful experimental tool and provide the opportunity to study how mtDNA impacts upon tumor cell properties. Reports of the effects of ethidium bromide induced mtDNA depletion in tumor cell lines varies greatly. Increased tumorigenecity was observed in ρ^0 tumor cells derived from breast [112], lung [113], osteosarcoma [110], melanoma [114], and prostrate tumor cells [115]. However, reduced tumorigenic properties were also observed in other cell lines of the same tumor origin [109, 116, 117]. Ethidium bromide not only intercalates into mtDNA but also nuclear DNA and the non-specific effects of ethidium bromide may account for the contrasting reports of altered tumorigenic properties in ρ^0 tumor cells, especially if used at higher concentrations.

Studies have shown that mtDNA depletion results in altered chromosomal gene expression and epigenetic modification, which can be reversed following replenishment with donor mtDNA [110, 118]. These reports provide evidence that cross-talk occurs between both nuclear and mitochondrial genomes and that the mtDNA status has the power to induce genomic DNA alterations. In light of these observations, it is plausible that the mtDNA variants present in cancer cells may

influence chromosomal stability and initiate tumorigenesis and the mechanisms by which this process occurs require further investigation.

6.5.2 *Mitochondrial DNA Variants in Disease and Tumorigenesis*

Mutations in mtDNA can be somatically-acquired, inherited through the maternal lineage or acquired via genetic mechanisms [119]. These tend to be characterized by single-base nucleotide substitutions and large- or small-scale deletions or insertions. Normally, pathogenic diseases are heteroplasmic, with severity correlating with the proportion at which the variants are present [120]. If the proportion of non-mutated wild-type mtDNA copies is capable of supporting cellular function, the phenotype will likely be normal. It is not until the numbers of these wild-type mtDNA copies are insufficient to support cellular function that the cell begins to display pathogenic phenotypes. However, mtDNA mutations can also be presented in a homoplasmic state. In 1998, Vogelstein and colleagues were able to demonstrate the presence of somatic mtDNA mutations, the majority of which reached homoplasmic levels, in human colorectal cancer [6].

While over 2,000 complete human mitochondrial sequences have been screened for analysis of genome variability inclusive of natural polymorphisms and haplogroups [121–125], over 200 pathogenic mutations have been detected throughout the mitochondrial genome [119]. The presence of these may compromise functionality of various mitochondrially-encoded proteins, influence the ability of the mitochondrial genome to undergo transcription and replication, or the processing of the numerous mitochondrially-encoded tRNAs and rRNAs [119]. However, substantial evidence is still lacking to support the possibility that mutations in mtDNA are a causative factor of the development of a tumor [126].

6.5.2.1 Key Polymorphic Variants Associated with Mitochondria in the Context of Disease

A characteristic point mutation at position 3243 of the mitochondrial genome, involving an A → G nucleotide transition, has been associated with 80 % of all mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) cases [127], a maternally inherited mitochondrial disorder [128]. Its discovery in 1990 [129] stimulated discussion that the presence of this variant could interrupt tertiary interactions required for the folding of the leucine tRNA ($tRNA^{Leu(UUR)}$), which functions in the decoding of codons reading UUR. Moreover, within the $tRNA^{Leu(UUR)}$ gene, there is thought to reside a binding site for the mitochondrial termination factor (mTERF), responsible for termination of mitochondrial transcription [130]. Given that the A3243G nucleotide substitution

is located in the middle of the mTERF binding site, it has been shown that binding of mTERF is compromised, therefore decreasing efficiency of transcription termination within the region of the termination site [131]. However, other mutations primarily involving the genes participating in complex I of the mitochondrial respiratory chain, have been identified as potential causes of MELAS. Such candidates include G3946A and T3949C both residing within the NADH dehydrogenase 1 (ND1) gene [132].

Mutations to complex I subunits NADH dehydrogenase 1, 4 and 6 (ND1, ND4, and ND6) have also been linked to Leber's hereditary optic neuropathy (LHON) syndrome, which presents as degeneration of vision in both eyes among young adults [133]. Since identification of the G → A nucleotide transition at position 11778 of the mitochondrial genome as the first variant present in LHON, over 30 additional variants have been discovered [134, 135].

Myoclonic epilepsy and ragged red fiber syndrome (MERRF) is another mitochondrial encephalomyopathy disorder [129]. MERRF involves a nucleotide transition from an A → G in $tRNA^{Lys}$ at position 8344, thought to cause a reduction in its specific aminoacylation [136], as well as premature translation termination at certain codons for lysine [136]. Following this discovery, a second characteristic mutation was uncovered, involving a T → C transition at position 8356 of the same gene [137], capable of causing defective protein synthesis [138]. Additional nucleotide substitutions thought to be associated with MERRF include A8296G [139], G15967A [140] and G8363A. The G8363A mutation is also found in Leigh syndrome [141], another mitochondrial specific neurodegenerative disease. Leigh syndrome is conventionally understood to be induced by nucleotide base changes at positions 8993 and 9176, located within the *ATP6* gene [142], position 14487 within the ND6 gene [143] and more recently at position 4296 in the tRNA isoleucine gene [144]. An additional candidate for the onset of Leigh syndrome was proposed to include the G13513A mutation residing within the *NADH dehydrogenase 5* (*ND5)* gene region, which is shared with the MELAS or LHON/MELAS phenotype [145, 146]. The T8993G nucleotide substitution is one that is also shared by neuropathy, ataxia, and retinitis pigmentosa (NARP), which converts amino acid residue leucine to an arginine at this position [147]. Whilst it is normal for the NARP phenotype to be expressed in the presence of 70–90 % of mutant mtDNA copies, Leigh syndrome tends to predominate in cases where mutational load has accumulated in excess of 90 % [148]. However, this is not always the case, as one study has suggested that there is no association between the T8993G mutation and development of the NARP or Leigh syndrome phenotypes [149]. The molecular impact of the T8993G substitution has been proposed to prevent the ATP synthase complex structure from forming correctly, thereby compromising ability of the cell to produce ATP [150].

It is important to appreciate that the presence of the characteristic variants for the above-mentioned diseases is not exclusive to one disease. Reports have indicated that patients carrying the T→C nucleotide transition at position 8356 of the mitochondrial genome can be found to have pathogenicity for mitochondrial

Table 6.2 Summary of the key mutations associated with mitochondrial disease: For a more comprehensive list of mitochondrial mutations refer to the MITOMAP database: (http://www.mitomap.org/MITOMAP/ClinicalPhenotypesPolypeptide)

Mitochondrial disease	Characteristic mutation	Gene affected	Mutation type	Reference
MELAS	A3243G	$tRNA^{Leu}$	Heteroplasmic	[127]
	G3946A	ND1	Heteroplasmic or homoplasmic	[132]
	T3949C	ND1	Heteroplasmic	[132]
LHON	G11778A	ND4	Heteroplasmic or homoplasmic	[134, 135]
MERRF	A8344G	$tRNA^{Lys}$	Heteroplasmic	[136]
	T8356C	$tRNA^{Lys}$	Heteroplasmic	[137]
	A8296G	$tRNA^{Lys}$	Heteroplasmic or homoplasmic	[139, 221]
	G15967A	$tRNA^{Pro}$	Heteroplasmic	[140]
	G8363A	$tRNA^{Lys}$	Heteroplasmic	[141, 221]
	G611A	$tRNA^{Phe}$	Heteroplasmic	[222]
	G3255A	$tRNA^{Leu}$	Heteroplasmic	[132]
	G12147A	$tRNA^{His}$	Heteroplasmic	[152]
Leigh syndrome	G8363A	$tRNA^{Lys}$	Heteroplasmic	[141]
	T8993G	ATP6	Heteroplasmic	[142]
	T9176C	ATP6	Heteroplasmic or homoplasmic	[142]
	T14487C	ND6	Heteroplasmic	[143]
	G4296A	$tRNA^{Ile}$	Heteroplasmic	[144]
NARP	T8993G	ATP6	Heteroplasmic	[147]

encephalomyopathies of both MERRF syndrome and MELAS [151]. Other variants also exist that are potential candidates for the MELAS/MERRF phenotype including the heteroplasmic G12147A nucleotide transition, which impacts on the $tRNA^{His}$ gene [152], and the G5521A transition affecting $tRNA^{Trp}$ [24]. Further cases include the overlap of MERRF with PEO [153], MERRF-NARP syndrome [154] and MERRF with KSS [132].

Although the conventional method for detecting the presence of these variants includes DNA Sanger sequencing, other methods have included the use of restriction fragment length polymorphism analysis (RFLP) and real time PCR. More sophisticated technology has since been developed for analysis of these base substitutions, including the use of biochips to enhance throughput for detection [155]. Table 6.2 summarizes the mitochondrial variants present in the above-mentioned diseases.

Table 6.3 Summary of the key mtDNA mutations associated with the risk of developing several cancer types

Cancer type	Nucleotide change	Gene affected	Amino acid change[a]	Reference
Breast	G9055A	ATP6	A -> T	[159]
	A10398G	ND3	T -> A	[159]
	T16519C	D-loop	S -> P	[159]
Head and neck	A11812G	ND4	Syn (L)	[164]
	G11719A	ND4	Syn (G)	[164]
Bladder	G2056A	16S rRNA	–	[165]
	T19971C	ND3	Syn (L)	[165]
	G11518A	ND4	Syn (L)	[165]
	T12519C	ND5	Syn (V)	[165]
	A16532T	D-loop	–	[165]
Cervical	C150T	D-loop	–	[166]

[a] Syn = synonymous amino acid change; single letter amino acid codes follow the IUPAC standard for vertebrate mitochondria; only mutations within a coding region are included.

6.5.2.2 Key Polymorphic Variants Associated with Mitochondrial DNA in Tumorigenesis

A comprehensive analysis of the mitochondrial mutations as observed in cancer has been covered in a review published by Carew and Huang [156]. Likewise, another has been provided by Brandon et al., which includes tRNA and rRNA mutations, those that are synonymous or non-synonymous as well as those present in the control region of the mitochondrial genome [157]. In brief, it is thought that the risk of developing cancer is partly influenced by the efficiency of the mitochondrial electron transfer chain, as ROS are believed to primarily be produced via this process [158]. A study that focused on how mtDNA sequence variations can contribute to development of breast cancer, revealed the presence of three mutations, G9055A, A10398G, and T16519C (Table 6.3), thought to enhance the risk of developing the tumor [159]. Similarly, it was also discovered that nucleotide transitions T3197C and G13708A were potential candidate markers for reduced risk of developing breast cancer [159]. The analysis of patients originating from different haplotype groups, again determined differences in the susceptibility to developing this cancer type. For other types of cancers, such as prostate cancer, studies have also shown an association between haplogroups and the risk of developing a tumor [160]. It is interesting that one study has reported the absence of such association between polymorphisms within the mitochondrial genome and the risk of developing prostate cancer [161]. The role of mtDNA haplogroups also extends to the risk of developing esophageal cancer, as one controlled case study has determined that selective Chinese populations belonging to subhaplogroups D, D4a and D5 may be at a higher risk of developing this particular type of cancer [162].

Whilst mutations in mtDNA are not a definitive cause of cancer, alterations to mtDNA remain to have a significant role, with studies reporting up to a 70 %

association between mtDNA mutation and colon cancer [6], and another unraveling a 5–37 % association with gastric cancers [163]. A study looking at mtDNA variants in samples representative of head and neck cancer, found the presence of nine nucleotide transitions, and one insertion across the whole of the mitochondrial genome [164]. The observation that the mutations within saliva samples obtained from the same patients diagnosed with head and neck cancer led to suggestions that perhaps development of this type of cancer can be predetermined in salivary samples [165]. A similar association was also made between bladder cancers and urinary samples obtained from the same patient [165].

Further case controlled studies have found that a D-loop polymorphism at C150T is positively correlated with the risk of developing human papillomavirus (HPV) and cervical cancer regardless of mtDNA copy numbers, in a population of Chinese women [166]. The presence of this C150T variant was proposed to contribute toward accelerating rates of mitochondrial replication or enhance ATP production, and therefore supporting the survival of tumorigenic cells [167]. Additionally, outside the discussion for nucleotide base substitutions, a 4977 bp deletion found in the region between nucleotide 8470 and nucleotide 13477 has been thought to disrupt activity of key genes participating in oxidative phosphorylation. Genes affected included ATP synthase subunits 6 and 8 (*ATP6*, *ATP8)*, as well as *cytochrome c oxidase subunit III* (*COXIII)* and several of the complex I subunits [168]. This defect has been found in several types of cancers, which include thyroid [169], esophageal [170] and gastric cancer [57].

Overall, there is evidence to suggest a correlation exists between various cancer types and mtDNA mutations. It is intriguing that one study attempted to determine the timing at which mtDNA variants develop, during the progression of head and neck squamous cell cancer. MtDNA variants that occur in this cancer type appear at a frequency of between 21 and 51 %, mostly found within the D-loop region [171]. Whilst those mutations arising within the coding regions of the mitochondrial genome were observed to reside within the genes of *ATP6*, *ND2*, *ND5*, *cytochrome B* (*CYTB*) and *COXIII* [172], they were indicative of mtDNA mutations arising later during or following establishment of the cancer. This suggests that, in some cases, cellular dysregulation is a cause for the acquisition of abnormal mitochondrial activity [171]. Furthermore, a separate study has identified the possibility that mitochondrial mutations and mtDNA copy number may be regulated independently of one another in human tumor cells [173]. These observations add to the complexity of the role of mitochondria in tumorigenesis, and emphasize the need to further examine this field.

6.6 Epigenetic Regulation of Mitochondrial DNA

Nuclear DNA commonly undergoes epigenetic modifications to regulate expression of its encoded genes. These changes may take the form of chromatin remodeling or DNA and histone modifications [174]. The limitation in the size of

the mitochondrial genome, and therefore the number of genes encoded by the mitochondrial genome, means that mtDNA is not subjected to the same modifications as genetic material from the nucleus. Early investigations of the mitochondrial genome in various species have reported the absence of epigenetic regulation of mtDNA [175, 176]. However, there is some evidence to support the existence of very low DNA methylation activity on CCGG sites (CpG islands) in as few as 2–5 % of mtDNA molecules, with the percentage of epigenetic modifications decreasing with the duration of culture in vitro [177–179].

Although little is known about the epigenetic regulation of the mtDNA-specific transcription and replication factors, there are reports suggesting that TFB1M and TFB2M function as methyltransferases. This is based on evidence that both are capable of binding to s-adenosyl methionine, a chemically reactive methyl donor of the methyltransferase process [180, 181]. It was later discovered that dimethylation of adenine residues at the 3′ end of 12S rRNA, mediated by TFB1M, was necessary for the biogenesis of ribosomal subunits in mitochondria, as stability and functionality of the protein complex during mitochondrial translation were compromised in the absence of TFB1M [182].

The functioning of mitochondrial tRNAs is also dependent on methylation, without which tRNAs fail to form the classic cloverleaf secondary structure [183]. Interestingly, with advances in technology, this concept has since been challenged with single molecule fluorescence resonance energy transfer (smFRET) detecting the collaborative influence of two methylation changes being required for the proper structuring of human mitochondrial tRNA [184]. However, not all modifications function to stabilize the tRNA structure, as changes, such as the addition of dihydrouridine, enhance the flexibility of the tRNA, thereby losing rigidity and structure of the molecule [185]. Similarly, other changes can influence translation fidelity via the specific actions of the aminoacyl tRNA synthetases, which are responsible for the attachment of the amino acid group to the 3′ end of an uncharged tRNA molecule [186, 187].

There is a mitochondrial-specific isoform of DNA methyltransferase 1 (DNMT1), which possesses a mitochondrial targeting sequence (mtDNMT1) [188]. MtDNMT1 is capable of interacting with Sirt1 [174] and its expression appears to be mediated by PGC-1α. Furthermore, it has been proposed that it is responsible for methylation of the cytosine residues at specific CpG dinucleotide regions on the mitochondrial genome [188]. Assessment of mtDNMT1 binding showed that protein interaction in the D-loop region, containing promoters for the light and heavy strands of mtDNA, negatively influenced expression of ND6 located on the light strand. At the same time expression ND1 was found to be stimulated on the heavy strand [188]. These results revealed a possible mechanism whereby epigenetic control of specific sites within the triple-stranded D-loop region may impact on expression of certain mitochondrial genes. This could then modulate epigenetic changes within the nucleus, as some studies have identified close interactions between the mitochondrion and the nucleus [110]. More specifically, loss of the integrity of the mitochondrial genome has been found to cause

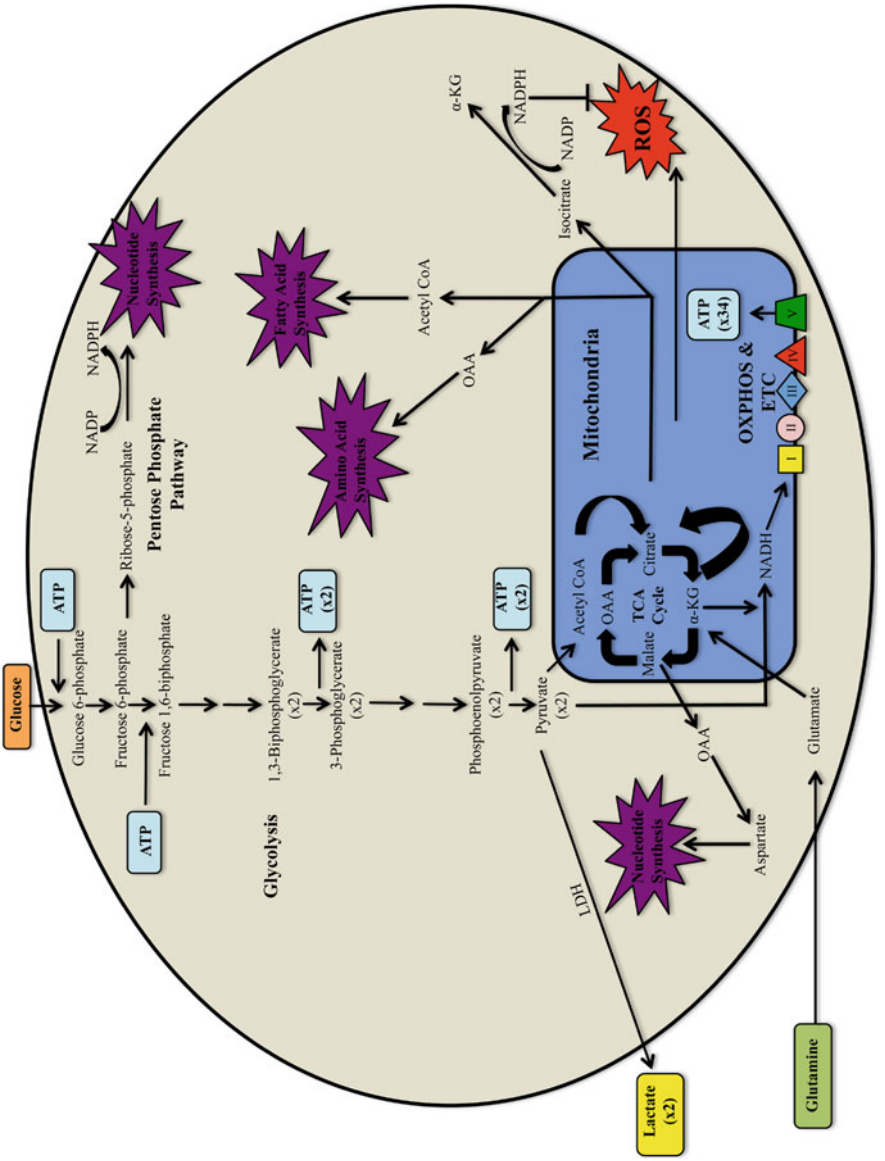


Fig. 6.4 ATP production via glycolysis and oxidative phosphorylation (OXPHOS) and biosynthetic substrate production pathways. Terminally differentiated cell types primarily catabolize glucose through glycolysis and oxidative phosphorylation to obtain a maximal yield of ATP. In tumor cells, the glycolytic rate is enhanced and oxidative phosphorylation metabolism is reduced. The rate of glucose and glutamine uptake is increased and utilized for the generation of biosynthetic intermediates to fuel proliferation and growth. Abbreviations: Alpha-ketoglutarate (*α-KG*), Lactate dehydrogenase (*LDH*), Nicotinamide adenine dinucleotide phosphate (NADP), *NADP* reduced (*NADPH*), Oxaloacetate (*OAA*)

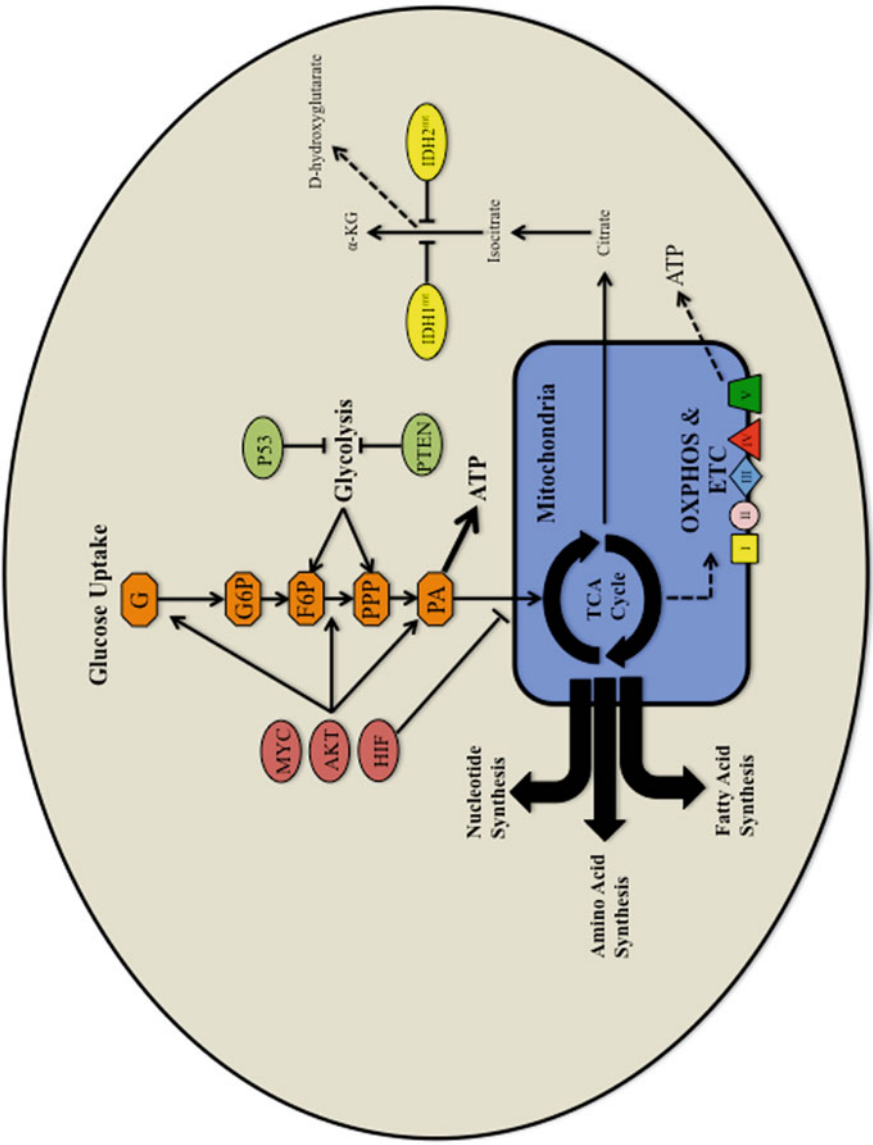
modifications to the nuclear genome in the form of DNA methylation, believed to be the result of the inability to repair oxidative damage originating from the mitochondria [118].

In the context of tumorigenesis and mtDNA copy number, one might speculate that the control of transcription as modulated by methylation of cytosine residues in mtDNA may down-regulate copy number, as an imbalance in the expression of particular subunits of the electron transfer chain would consequently result in the formation of dysfunctional protein complexes following translation and assembly of the subunits. Alternatively, dependent on the tumor type, failure of mtDNMT1 to be properly regulated could lead to fewer epigenetic events taking place in the mtDNA D-loop. This may result in higher copy numbers, as the mitochondrial genome attempts to compensate for the proportion of non-functional copies, with the up-regulation in the activity of nuclear-encoded factors involved in the mitochondrial replication machinery, for example POLGA.

6.7 The Role of Mitochondria in Tumor Energy Metabolism and the Warburg Effect

In 1956, Otto Warburg demonstrated that tumor cells catabolized glucose at an accelerated rate compared to non-transformed cells [106]. Furthermore, tumor cells selectively utilized glycolytic metabolism, even in the presence of sufficient quantities of oxygen (a phenomenon known as aerobic glycolysis), which would normally activate oxidative phosphorylation, a more energy efficient pathway. From an energy production perspective, glycolytic metabolism under aerobic conditions appears wasteful and inefficient. Glycolysis generates only 2 molecules of ATP per glucose molecule and pyruvate and lactate are produced as by-products [189]. In contrast, during oxidative phosphorylation, pyruvate enters the citric acid cycle and donates electrons to the electron transfer chain to yield 34 molecules of ATP per glucose molecule [190] (see Fig. 6.4).

It is not yet fully understood why the majority of tumor cells so far studied adopt glycolytic metabolism. However, ATP production may not be a limiting factor as ATP yield from an enhanced glycolytic rate can exceed that of oxidative phosphorylation and may be advantageous to proliferative cell types [191]. A number of potential mechanisms have been proposed as to why tumor cell types adopt glycolytic metabolism and include the requirement for glycolytic intermediates for cell growth and proliferation, aberrant oncogene activation and tumor microenvironment stimuli.


Glucose Uptake
G
G6P
F6P
PPP
PA
Glycolysis
P53
PTEN
MYC
AKT
HIF
ATP
Mitochondria
TCA Cycle
OXPHOS & ETC
I
II
III
IV
V
ATP
Citrate
Isocitrate
α-KG
D-hydroxyglutarate
IDH1mt
IDH2mt
Nucleotide Synthesis
Amino Acid Synthesis
Fatty Acid Synthesis

◀ **Fig. 6.5** Oncogenic and tumor suppressor signaling can induce the Warburg Effect. Aberrant oncogenic signaling, c-Myc (MYC), and activation of the Akt pathway enhance glucose uptake and glycolysis, providing the cell with ATP and biosynthetic intermediates for cell proliferation. Activation of HIF increases glucose uptake and utilization during periods of hypoxia, promotes survival, and reduces tumor cell mitochondrial metabolism. Loss of function of the tumor suppressors P53 and PTEN can lead to upregulated Akt signaling and increased glycolytic rates. Mutations to the citric acid cycle proteins SDH, FH1, IDH1 and IDH2 enhance glycolysis and synthesis of D-hydroxyglutarate, respectively. Abbreviations: Alpha-ketoglutarate (*α-KG*), Electron transfer chain (*ETC*), Fructose-6-phosphate (*F6P*), Furmarate hydratase 1 (*FH1*), Glucose (*G*), Glucose-6-phosphate (*G6P*), Hypoxia inducible factor (*HIF*), Isocitrate dehydrogenase 1 (*IDH1*), Isocitrate dehydrogenase 2 (*IDH2*), Oxidative phosphorylation (*OXPHOS*), Phosphatase and tensin analog (*PTEN*), Phosphoenolpyruvate (*PPP*), Pyruvate acid (*PA*), Succinate dehydrogenase (*SDH*), the citric acid cycle (TCA)

6.7.1 Proliferating Cells Require Biosynthetic Intermediates to Support Growth

Terminally differentiated cell types generally exhibit very low proliferation rates or are post-mitotic [192]. For these cell types, there is reduced demand for the production of nucleic acids, amino acids and fatty acids required to support cell division and increase cellular mass [192]. Therefore, cellular energy requirements are generated predominantly through the complete metabolism of glucose via glycolysis and oxidative phosphorylation to yield ATP, CO_2 and H_2O [192]. However, events such as embryonic development and tissue regeneration require periods of rapid cell proliferation and the majority of tumor cells also exhibit high proliferation rates [192]. In order for proliferation to occur, a cell must satisfy its energy requirements while generating sufficient quantities of nucleotides, amino acids and fatty acids to support growth. To achieve this, proliferating cells direct glycolytic intermediates to pathways designated for synthesis of substrates to support cell division [193]. Glucose and glutamine are essential substrates for energy production and biosynthetic intermediates for both normal and tumor cells. However, tumor cells take up glucose and glutamine at an accelerated rate and exploit the availability of glucose and glutamine to satisfy their needs for ATP and to generate substrates to support rapid proliferation [193]. The metabolic processing of glucose and glutamine to generate ATP and biosynthetic substrates are summarized in Fig. 6.4.

During periods of proliferation, entry of pyruvate into the mitochondria is reduced [194]. Excess pyruvate is converted to lactate via lactate dehydrogenase and secreted from the cell, allowing continued uptake of glucose and maintaining the activity of glycolysis [190]. However, pyruvate that is not converted to lactate enters the mitochondria and is converted to acetyl coenzyme-A in the citric acid cycle by pyruvate dehydrogenase, which is essential for lipid synthesis [195]. Acetyl coenzyme-A is also converted to oxaloacetate and utilized for amino acid synthesis [195]. Further processing of oxaloacetate to aspartate by transamination provides substrates for nucleotide synthesis [195]. Generation of nucleotides is

also achieved via the by shunting of glucose into the pentose phosphate pathway [192] (see Fig. 6.4).

Glutamine also provides the necessary substrates for energy production and cell proliferation [195]. Glutamine is imported into the cell and converted to glutamate, which enters the citric acid cycle. Within the mitochondria, glutamate can be converted to aspartate, acetyl coenzyme-A and oxaloacetate to aid in nucleotide, lipid and amino acid synthesis, respectively [195]. Pyruvate can also be resynthesized from glutamate and used to generate glucose-6-phosphate by reverse glycolysis [192]. The newly formed glucose derivative can either be remetabolized to generate ATP or enter the pentose phosphate pathway to assist in nucleotide synthesis [192]. In addition to substrate production, conversion of glutamine into pyruvate and alpha-ketoglutarate and its entry into the pentose phosphate pathway results in the conversion of nicotinamide adenine dinucleotide phosphate (NADP) into its reduced form, NADPH [192].

ROS are by-products of glucose and glutamine metabolism. However, NADPH is required to generate glutathione, which is a cellular anti-oxidant [195]. Increased NADPH production, therefore, functions to control redox potential [195]. Although high levels of ROS can promote cell proliferation and genomic instability, both of which can promote a tumorigenic transformation, excessive amounts of ROS can induce cell death [192]. Hence, by increasing the production of NADPH via various pathways, the tumor cell negates the negative effects of ROS, avoiding apoptosis, and thus continues to proliferate.

One of the earliest conclusions that was drawn regarding the origins of the Warburg effect was impaired mitochondrial respiration [106]. However, tumor cells have been shown to retain functional mitochondrial metabolism [190]. In addition, functional mitochondria are required to generate substrates to support tumor cell proliferation and this strongly suggests that the factors contributing to the Warburg effect are complex and not solely dependent on the cells mitochondrial status.

6.7.2 *Mutations in Oncogenes and Tumor Suppressors Drive Aerobic Glycolysis*

Over the last decade, our knowledge on regulatory pathways that induce metabolic changes in cells has increased. The metabolic alterations observed under the Warburg effect have been shown to be directly influenced by a number of oncogenes and tumor suppressor genes expressed (Fig. 6.5). The proto-oncogene *c-Myc* is overexpressed in $\sim$70 % of tumors [196] and activates the transcription of multiple factors, many of which are associated with cell metabolism, reviewed in [197]. c-Myc activation increases glucose uptake, recruitment of glucose transporters and glucose utilization [197]. In addition, c-Myc activates the expression of

the citric acid cycle associated genes, leading to increased mitochondrial mass and activity [198], and providing support for cell proliferation.

p53 is a tumor suppressor gene that plays an essential role in the regulation of glucose metabolism [199]. It induces the transcription of TP53-induced glycolysis and apoptosis regulator (TIGAR), which reduces the activity of fructose 2,6 biphosphatase and diverts glucose into the pentose phosphate pathway and slowing of the overall rate of glycolysis [199]. p53 also increases the utilization of the citric acid cycle for oxidative phosphorylation by increasing the transcription of cytochrome c oxidase 1 and 2 [200]. p53 regulates the expression of phosphatase and tensin homolog (PTEN), both of which are often silenced in tumors and leads to increased activation of the phosphoinositide 3-kinase (PI3K)-AKT pathway [192]. The PI3K-AKT pathway is associated with cell proliferation and glucose metabolism and activation of this pathway enhances glucose transporter recruitment and glucose uptake [193, 201, 202]. The aberrant activation of this pathway through PTEN loss results in cells becoming dependent on increased glucose influx, which is a characteristic associated with the Warburg effect.

In addition to oncogene and tumor suppressor mutations, mutations in the citric acid cycle enzymes may also induce the Warburg effect. Mutations in succinate dehydrogenase, reviewed in [203], and fumarate hydratase [204] have been identified in multiple cancers and were associated with increased glucose utilization via the hypoxia inducible factor 1 alpha (HIF1α) pathway. Furthermore, mutations in isocitrate dehydrogenase 1 (IDH1) and 2 (IDH2) have been reported in gliomas [205]. Functional IDH1 and IDH2 convert isocitrate to alpha-ketoglutarate, reducing NADP to NADPH, while mutant IDH1 and IDH2 isoforms convert alpha-ketoglutarate to D-hydroxyglutarate [206]. It is not clear how these mutations affect cell metabolism or the role they play in tumorigenesis. However, due to the role that NADPH plays in the generation of biosynthetic intermediates and redox control, altered generation of NADPH and D-hydroxyglutarate may yet prove to be beneficial to tumor cells [207].

6.7.3 The Tumor Microenvironment Selects for Glycolytic Cell Types

As tumors develop and grow over time, they eventually outstrip their blood supply and reach or exceed diffusion limits, resulting in restricted oxygen and nutrient availability [208]. During this scenario, a hypoxic environment can develop, inducing gene expression changes in tumor cells [197]. Under these conditions, a key hypoxia associated transcription factor, HIF1α, becomes stabilized and translocates to the nucleus, where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) to activate oxygen regulated gene expression and glycolytic metabolism, reviewed in [197].

The expression and stabilization of HIF1α is associated with a number of metabolic adjustments in tumor cells. During periods of hypoxia, HIF1α inhibits the transport of glycolytic substrates into the mitochondria through targeting of pyruvate dehydrogenase kinase 1 [197], which functions to inhibit the conversion of pyruvate to acetyl coenzyme-A in the early stages of the citric acid cycle and leads to a decrease in mitochondrial respiration [209]. In addition, pyruvate dehydrogenase kinase activation leads to a block in the utilization of glycolysis substrates for anabolic purposes and reduces the activity of biosynthetic pathways [209]. HIF1α also elicits effects on the mitochondria, reducing mitochondrial mass and enhancing the efficiency of ATP production by improving electron transport chain coupling efficiency and reducing the production of reactive oxygen species [210]. Collectively, however, HIF1α slows cell metabolism, reduces anabolism and promotes cell survival.

Hypoxic regions in the heart following an ischemic insult can be alleviated by growth of new vasculature, a process mediated in part by vascular endothelial growth factor [208], which is a target of HIF1α. Hypoxic tumors are able to utilize this pathway and encourage blood vessel growth into the tumor mass [208]. However, blood vessel formation is often disorganized and provides limited and variable amounts of oxygen and nutrients. An advancing tumor is therefore likely to experience spatial fluctuations of normoxia, hypoxia and nutrient availability [208], leading to sporadic activation of HIF1α and periods of tumor cell proliferation and quiescence. It is also plausible that the everchanging tumor microenvironment may select for cells with metabolic characteristics that promote survival under harsh environments and rapid proliferation under nutrient rich conditions. Thus, tumor cells with augmented glycolytic capacity may prosper under such conditions.

6.8 Oxidative Phosphorylation in Tumor Cells

Recently, there has been some evidence to suggest that the Warburg effect is not entirely indicative of the metabolism of all tumor types, and that oxidative metabolism may play an essential role in the formation and progression of some tumors, reviewed in [211]. As previously discussed, aging is one of the single most important risks for the development of cancer [98] and is associated with increased mtDNA mutation rate and mitochondrial dysfunction [99], which may prompt a reduction in oxidative phosphorylation in favor of glycolysis in aged cells. Furthermore, a new hypothesis has emerged, that morbidity and mortality may be directly related to a loss of oxidative phosphorylation and that longevity may be associated with conservation of this process [211]. During tumorigenesis, there may be a selective growth and survival advantage for tumor cells that are able to reestablish oxidative phosphorylation, while the rest of the body adopts aerobic glycolysis [211]. This hypothesis has been described as the “two compartment metabolic system” by Ertel and colleagues and resembles a parasite–host

relationship between tumor cells and the surrounding tumor stroma. The aged cells of the stroma are primarily glycolytic and secrete energy rich nutrients into the extracellular matrix. The tumor cells, with active oxidative phosphorylation, are able to maximize their nutrient supply, feeding off both nutrients from the blood supply and also those secreted by the stromal cells.

Experimentally, this has been supported by a study using a co-culture system model of human fibroblasts and MCF7 breast cancer cells [212, 213]. Breast cancer cells were found to secrete hydrogen peroxide into the media, inducing oxidative stress and accelerated aging in the fibroblast cells, resulting in activation of HIF1α and Nuclear Factor-Kappa B (NFkB) pathways and increased rates of glycolysis. As a consequence of increased glycolysis, the neighboring fibroblasts secreted lactate and glutamine into the media, which were taken up by the breast cancer cells and induced mitochondrial biogenesis. The authors also reported that, in breast cancer cells with increased mitochondrial biogenesis, resistance against chemotherapy agents was enhanced [212]. These outcomes suggest that mitochondria may play a role in chemotherapy resistance and in support of this, a recent study showed increased electron transport chain coupling was associated with chemo-resistance [214].

In another study, cytochrome c oxidase staining of breast cancer samples was utilized as an indictor of oxidative phosphorylation activity [215]. Concentrated cytochrome c oxidase staining was observed in the tumor cells, with the cells of the surrounding tumor showing negative staining, suggesting the tumor cells were oxidative while the stromal cells were glycolytic. Furthermore, cytochrome c oxidase staining was found to be more intense in the tumor cells compared to healthy adjacent epithelial cells, suggesting enhanced oxidative phosphorylation in the tumor cells. Tumor cells isolated using laser dissection were also shown to have increased transcriptional activity of genes associated with oxidative phosphorylation [215].

PGC-1α and PGC-1β are key regulators of mitochondrial biogenesis and regulate the expression of the nuclear respiratory factor genes [86]. The nuclear respiratory factors bind to the promoter region of TFAM and induce mtDNA replication, transcription and replication, increasing mtDNA copy number and enhancing oxidative phosphorylation potential [86]. A recent report suggests that PGC-1α/β and the nuclear respiratory factors may play a key role in the maintenance and reestablishment of oxidative phosphorylation capacity in tumor cells [216]. In support of enhanced PGC-1α/β activity in tumorigenesis, knockdown of PGC-1α prevented carcinogen-induced tumorigenesis in the liver and colon in mice, whilst overexpression of PGC-1α enhanced tumor xenograft growth [216]. The tumor suppressor gene *p53* has also been reported to negatively regulate PGC-1α/β expression, resulting in reduced mitochondrial function and oxidative phosphorylation [217]. Loss of p53 function frequently occurs in tumorigenesis and it has been proposed that p53 loss may enhance mitochondrial biogenesis, boosting the oxidative capacity of tumor cells [211]. However, this concept remains controversial, as p53 loss has also been associated with mtDNA depletion [91]. These findings support the concept that there is a compensatory mechanism to increase

mtDNA copy number if mtDNA mutations are present and accounts for the variability in mtDNA copy number between different tumor types, as described in Sects. 6.5 and 6.6.

In addition, p32, a cancer cell surface marker also commonly found in the mitochondrial matrix, has been demonstrated to participate in the modulation between glycolytic and oxidative phosphorylation states [218]. Suppression of p32 was observed to convert respiratory activity from oxidative phosphorylation to glycolysis, a consequence that is reflected in cancer stem cells by reduced tumorigenicity [218]. Reversion to normal p32 levels from the suppressed state was found to switch respiration from its glycolytic state back to dependency on oxidative phosphorylation. Such findings provide further evidence of competent oxidative phosphorylation in tumor cell metabolism and tumorigenesis.

If increased oxidative phosphorylation capacity were indeed a "hallmark" of various tumor cell subgroups, inhibition of oxidative phosphorylation would be an attractive therapeutic strategy. In support of this concept, Metformin, which disrupts mitochondrial function, was shown to be beneficial in the treatment of p53 null tumor xenografts [219]. However, its effectiveness was limited in p53 positive tumor xenografts, supporting the notion that p53 null tumors have increased oxidative phosphorylation metabolism [219]. Furthermore, a study by Škrtic et al. showed that inhibition of mitochondrial translation was an effective strategy for inhibiting the growth of human acute myeloid leukemic tumors [220].

6.9 Conclusion

The emerging evidence suggests that increased oxidative phosphorylation in breast cancers plays a key role in tumor survival and progression, which adds increased complexity to the understanding of tumor metabolism [211]. It is increasingly likely that tumor subgroups are present, expressing metabolic characteristics of the classical Warburg effect and others showing enhanced oxidative phosphorylation. The initiation of these metabolic adjustments is multifaceted, with aging, genomic instability, and oncogene activation likely to all play a role in some form. However, studies over the last decade now suggest that one metabolic profile does not fit all tumor types and that the development of successful therapies to target tumor cell metabolism will need to be multimodal. It has become increasingly evident that mtDNA and mitochondria are essential for multiple aspects of tumor cell metabolism. In the Warburg model, the citric acid cycle is utilized to generate the required substrates to support rapid proliferation, whilst other tumor cell types enhance their oxidative phosphorylation capacity to survive and evade the metabolic decline of the aging body. Thus, targeting mtDNA and the mitochondria remain very attractive therapeutic strategies for the treatment of cancer. Critically, we will still need to determine whether mtDNA rearrangements are the 'chicken or the egg' of tumorigenesis.

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Chapter 7
Assisted Reproductive Technologies: The Potential to Prevent the Transmission of Mutant mtDNA from One Generation to the Next

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Abstract Mammalian cells contain multiple identical copies of mitochondrial DNA (mtDNA) that encode genes involved in the production of ATP through the process of oxidative phosphorylation (OXPHOS). Mutations and deletions to mtDNA produce novel sequence variants, resulting in heteroplasmic mixing of mutant and wild-type molecules, which may culminate in a variety of severely debilitating and lethal multi-systemic diseases. The maternal inheritance of mtDNA is a strictly regulated process and presents a complex reproductive situation, as there are currently no proven clinical strategies available to prevent the transmission of mutant mtDNA from the mother to her offspring and to subsequent generations. Furthermore, the segregation of mtDNA during development randomly alters the mutant loading within embryonic tissues, limiting the possibility to safely predict the probability of disease manifestation. Despite these limitations, a patient may undergo an assisted reproductive program, consisting of genetic counseling and tissue sampling for biochemical and genetic screening. Encouraging studies in non-human models have developed micromanipulation approaches to reduce the transmission of mutant mtDNA between generations. However, these methodologies require further experimental validation to determine whether assisted reproductive technologies can prevent the transmission of mutant mtDNA.

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7.1 Introduction

The maternal inheritance of mitochondrial DNA (mtDNA) presents a difficult reproductive scenario for females carrying mtDNA mutations within their gametes, as these mutant molecules will be transmitted to their offspring [1, 2]. Depending upon the type of mutation, the mutant:wild-type loading, and the segregation of mtDNA at cytokinesis during development, the offspring may be asymptomatic, or suffer from debilitating mitochondrial disease characterized by dysfunctional oxidative phosphorylation (OXPHOS) [3–6]. Until recently, these disorders were believed to be rare. However, experimental data have provided evidence to the contrary [7, 8]. Precise epidemiological data are difficult to obtain yet studies have suggested that 1 in 3,500–6,000 individuals [7, 9] are affected and 1 in 200 women harbor mtDNA mutations [10]. Since, the first description of pathogenetic mutations associated with the mtDNA [11, 12] over 250 additional alterations to the mitochondrial genome (e.g., point mutations, rearrangements, and large-scale deletions) have been described [13, 14], which are listed at MITOMAP [15]. As the inheritance of mtDNA mutations is a unique genetic situation, it represents an important decision to any prospective mother considering children. Currently, there are no successful therapeutic treatments or clinical practice available to prevent the transmission of mutant mtDNA from the mother to the offspring. Here, we describe the current knowledge and the options that have the potential to prevent the transmission of mtDNA between generations.

7.2 The Need to Prevent the Transmission of mtDNA: The Heteroplasmic Threshold

When the mtDNA composition of mammalian cells is identical then the cells and the individual are homoplasmic [16]. The mitochondrial genome is 10–100 times more susceptible to mutations than nuclear DNA [17–20], most likely due to the close proximity of mtDNA to mitochondrial reactive oxygen species (ROS) [19, 21] and the high intrinsic error rate of the mtDNA-specific Polymerase γ (POLG) [22]. Mutations to mtDNA may cause a wide range of clinical symptoms, such as dyslexia, liver failure, cardiomyopathy, diabetes, neuropathy, and encephalopathy, typically affecting tissues with high energy demands [23]. Mixing of mtDNA variants is known as heteroplasmy [4, 11, 12, 24]. The random segregation of heteroplasmic mtDNA during development or within individuals can result in variable heteroplasmic levels between and within tissues [25, 26]. Furthermore, prospective mothers may carry mtDNA mutations without exhibiting disease symptoms, which only become apparent upon reproductive failure or birth of children suffering from mtDNA disease.

MtDNA variants are categorized into three groups: ancient adaptations favoring environmental conditions, which define mtDNA haplotypes; age-related accumulations of somatic mtDNA mutations; and novel mutations that result in maternally transmitted diseases [27]. The percentage of heteroplasmy in cells and tissues necessary to cause disease symptoms may range between <25 and 100 %, with the severity of the phenotype usually determined by the degree of mutant loading within the affected tissue [3–6, 28]. For example, in myoclonic epilepsy with ragged-red fibres (MERRF), over 85 % mutant (8344A > G) loading is typical [5]. In the case of Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP), high mutant loads of the 8993T > G/C mutation modifies the phenotype from a mild neuromuscular disorder to a severe or even fatal encephalopathy called Leigh Syndrome [9]. Lower levels (50 %) of a point mutation in the brain in the ND5 gene, otherwise causing Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS; 12770A > G), can also lead to Leigh Syndrome [6] and, in cultured cells at levels of 30–45 %, can disrupt Complex I assembly and function. Therefore, very different levels of mutant mtDNA load can lead to multi-organ dysfunction.

Although it is generally accepted that mtDNA is replicated independently of the nuclear genome [29], some evidence exists to the contrary [30, 31, 32]. Replication of the mitochondrial genome in post-mitotic tissues, such as muscle and brain [33], occurs at a slower rate than highly proliferative tissues. These replication events may preferentially replicate mutant mtDNA molecules since not all mtDNA molecules are replicated at once. Accordingly, mutant loading and the perceived pattern of segregation are altered. How mtDNA replication is initiated and whether mtDNA molecules are replicated randomly or preferentially remain to be elucidated and this will be dependent on whether the mtDNA content is periodically reduced and increased during development or subject to arrest followed by expansion. The segregation of mtDNA is associated with random genetic drift particularly within the germline [34, 35], though some studies have implicated biological fitness, mutant loading [36, 37], severity of the mutation [38] and the type of mutation [39–41] as influencing mtDNA segregation and heteroplasmic loading in somatic tissues during development and postnatally. The underlying mechanisms that direct mtDNA inheritance remain uncertain and further genetic, epidemiological, and mathematical modeling studies are essential to decipher these mechanisms.

7.3 Maternal Inheritance

Analysis of mtDNA copy number in human metaphase II (MII) oocytes is difficult due to the ethical guidelines governing the use of human gametes. Studies in other mammalian species have demonstrated that mammalian mtDNA transmission during fertilization and development appears to be conserved. At ovulation, each mitochondrion of MII oocytes contains one to two copies of the mitochondrial

genome [42] with human oocytes on average containing between ~1.9 and 3 × 10^5 mtDNA copies [43–46]. It is evident that the mean mammalian oocyte copy number varies between studies. These differences may be attributed to experimental variability though some reports have correlated copy number with oocyte quality. Santos et al. [43] determined fertilizable oocytes contained higher number of mtDNA copy than unfertilized or degenerating oocytes. The authors concluded that mtDNA content is critical to fertilization outcome and serves as an important marker of oocyte quality, explaining some cases of fertilization failure. These conclusions are validated by studies in cattle [47], humans [48] and pigs [49], which also correlate mtDNA copy number with increased developmental competency. The high copy number of MII oocytes is believed to support development since little or no mtDNA replication is observed during preimplantation development. Following fertilization, mitochondria are randomly segregated between blastomeres, progressively reducing the numbers of mtDNA copies per cell. Replication of the mitochondrial genome is generally not observed until the blastocyst stage [50–53], which is hypothesized to occur within the trophectoderm cells [51, 54] since mouse inner cell mass (ICM) cells have very few mtDNA copies per cell [55] and mouse embryonic stem cells (mESCs) have even fewer copies [56] indicating continued dilution of mtDNA.

Male germ cells contain approximately ~4–75 copies of the mitochondrial genome per gamete [57–60] within mitochondria that are arranged end-to-end in a helical manner in the midpiece and account for less than 0.03 %, of the total mtDNA present at fertilization. Similar to maternal gametes, mtDNA copy number appears to be important for sperm function and poor-quality sperm possess significantly higher copies of mtDNA [57, 61, 62]. Sperm mitochondria present at fertilization are normally eliminated by a process of ubiquitin-mediated degradation [63] rendering the paternal mitochondria developmentally redundant and at an evolutionary dead end [64]. Similarly, sperm mitochondria are degraded in mtDNA-deficient cells [65] further implying that, during spermatogenesis, paternal mitochondria are specifically labeled for elimination, through a mechanism present in somatic cells and preimplantation embryos [63, 65]. In some instances, paternal mtDNA can be transmitted to the offspring. Inter-specific crosses of different mouse strains [66, 67] or subspecies of rhesus macaques [68] or breeds of sheep [69, 70] transmit mtDNA to their offspring. However, from mouse studies, there is no evidence to suggest that this is transmitted to subsequent generations [66]. Similar failure to eliminate sperm mtDNA is observed in abnormal human oocytes up to the blastocyst stage [71]. This paternal leakage has been hypothesized to be a process whereby the blastomeres containing male mitochondria are destined for extraembryonic tissue [72]. Indeed, the process of sperm mitochondrial elimination can fail, as demonstrated by the one instance of paternal transmission where the offspring developed a muscle myopathy due to a 2 bp deletion present in the NADH-ubiquinone oxidoreductase chain 2 (ND2) gene of his father's sperm mtDNA [73]. This report highlights the complicated nature of mtDNA inheritance whereby the mtDNA content contributing to less than 0.03 % at fertilization resulted in adult muscle tissue containing 90 % mutant molecule

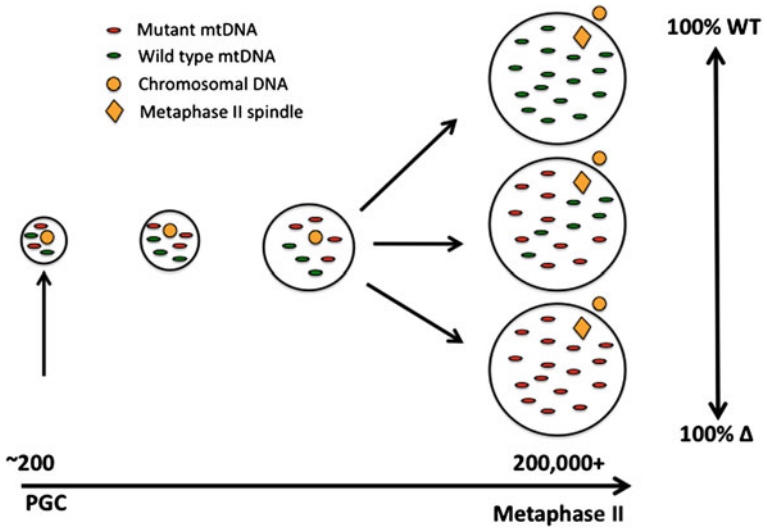


Fig. 7.1 The mitochondrial bottleneck: MtDNA randomly segregates to the primordial germ cells (PGCs) just after gastrulation. As mtDNA copy number increases during oogenesis, the variation in the degree of heteroplasmy in mature metaphase II oocytes from an individual can be considerable and thus not predictable. WT = wild type; Δ = mutant

thus representing a highly significant selective advantage. The random segregation sperm mtDNA during development concentrated the mutant DNA in one cell lineage that then exceeded the mutant-loading threshold for that tissue causing the mitochondrial disease. This scenario has serious implications when attempting to determine and/or manipulate disease progression in offspring where mutant mtDNA is present at very low levels.

7.4 Mitochondrial DNA Segregation and the Mitochondrial DNA Bottleneck

The mitochondrial bottleneck hypothesis was proposed to account for the rapid shifting of mtDNA heteroplasmy, first observed in Holstein cattle and their offspring, which then became fixed within a few generations [74]. This hypothesis accounts for the variable levels of heteroplasmy transmitted to the offspring within one generation, from a small number of founder mitochondrial genomes [75] (See Fig. 7.1). This mechanism has been proposed to favor the purification of mtDNA molecules [76, 77] so that mtDNA variants may be tested by natural selection, such as stalling or blocking oocyte maturation if the mutation is severely debilitating [38, 78, 79]. Nevertheless, mutant molecules that are either lethal or severely debilitating transit through the bottleneck, as evidence by those affected by mtDNA diseases.

There has since been multiple hypotheses to describe the specific mechanisms and timing of the bottleneck. Until recently, the bottleneck was determined to be a consequence of the considerable reduction in mtDNA copies during oogenesis [80]. Recently, evidence has been provided to the contrary, suggesting steady-state [55, 81] or increasing [82] numbers of mtDNA copies are present during early primordial germ cell differentiation. Other studies have hypothesized that the mitochondrial bottleneck occurs as a result of a reduction in mtDNA copy number during early embryogenesis [83, 84] or a subpopulation of mtDNA is preferentially replicated during early oogenesis [55] or postnatal folliculogenesis [82]. A segregation bias prior to primordial germ cell formation will feasibly produce germ cells with different mutant loading accounting for differences between an individual's germ cells and would account for the significant variability in mutant mtDNA loading in cohorts of oocytes from carriers [85]. Nevertheless, further experimental analysis is required to determine the precise timing and nature of the bottleneck within the female germline. The mtDNA bottleneck and the segregation observed between mothers and their offspring make any predictions regarding the distribution of heteroplasmy during development and the risk of disease prevalence in offspring and between siblings conceived at separate times extremely challenging.

7.5 Preventing the Transmission of Mutant mtDNA to the Next Generation through Oocyte and Embryo Sampling

Females carrying mutant mtDNA within their germline require clinical assistance to reduce the risk of transmitting these severely debilitating mutations to their offspring. The clinical staff must approach each case individually, applying multiple strategies, incorporating medical history, molecular analysis, and assisted reproductive technologies [86–88]. The lack of a clear genetic and phenotypic correlation with disease incidence makes diagnosing mitochondrial diseases complicated, as this requires molecular-genetic, histochemical, and biochemical screening of potential carriers. Females at risk of transmitting mtDNA disease, either with disease symptoms or a family history of disease, are provided with genetic counselling about their reproductive options, which will depend upon the type of mutation and the mutant loading [86, 89]. As outlined above, the clinical determination is difficult and several avenues have been investigated depending on the disease type and the levels of heteroplasmy. Many assisted reproductive technologies (ART) are extremely invasive and in vitro culture techniques have been shown to adversely affect the developing embryos [90, 91].

Depending on the individual case, the couple will undergo in vitro fertilization (IVF) or intercytoplasmic sperm injection (ICSI) so that embryonic cells are available for analysis (Fig. 7.2). In these techniques, fertilization proceeds, either by in vitro incubation of sperm and oocyte in culture media (IVF) [92] or manual

injection of a single sperm into the oocyte cytoplasm [93]. Oocytes are collected from females following hyperstimulation, which may be an extremely distressing and dangerous procedure. In addition, a number of the different techniques demand oocytes or zygotes donated from other females, creating further logistical and ethical concerns [94–96]. Destroying oocytes or embryos in favor of producing developmentally superior embryos free of disease exasperates ethical concerns. In the context of mtDNA disease, ART 'designer babies' are not produced but embryos are generated that have a significantly reduced risk of harboring disease and the resultant offspring are assured of a greater quality of life.

7.5.1 Preimplantation Genetic and Prenatal Diagnosis

In vitro techniques, such as IVF and ICSI, are limited as applications to reduce transmission of mutant molecules. They are dependent on screening approaches, such as oocyte and preimplantation genetic screening, to determine the heteroplasmic loading. Preimplantation genetic diagnosis (PGD) is a procedure whereby sampling of blastomeres is used to determine the mutant load and estimate the potential risk of transmitting a disease phenotype. To date, the UK's Human Fertilization and Embryology Authority (HFEA) has authorized the testing of over 187 diseases through PGD [97]. One or more blastomeres are removed at the 6 to 10 cell stage, which may compromise developmental outcome [98, 99]. Worldwide, successful testing has been performed for aneuploidy in developing embryos [100–102] and established the risk of transmitting mutant alleles, such as for cystic fibrosis [103, 104]. Using this approach, multiple embryos produced by IVF can be screened for the presence of the mutation and the levels of heteroplasmy. Only those embryos with low or undetectable amounts of mutant mtDNA are transferred to the uterus [94]. This approach cannot guarantee that a fetus or child will be unaffected but it dramatically improves the probability of an unaffected pregnancy. Advances in embryo handling and sensitive molecular biology techniques in recent years have greatly increased the ability to analyze the genetic composition of gametes and the preimplantation embryo prior to embryo transfer. A limitation with this technique is the mutant loading seen between analyzed blastomeres [105]. During preimplantation development there appears to be equal partitioning of mtDNA between blastomeres [106], yet the random nature of mtDNA segregation can result in variations ranging from 0 to 19 % heteroplasmy [105] or no variation at all [41].

In order to confirm low transmission of mutant molecules to the fetus after PGD, sampling may be required (or requested by the parents) directly from the fetus in utero. However, Chorionic villus sampling (CVS) also suffers from the same inadequacies as PGD. Comparison of mutational loading in somatic, embryonic, placental, and reproductive tissues for the mutations associated with NARP [105, 107] and MELAS [41] revealed that their respective mutations differentially influence random segregation, again highlighting the limitation of PGD and CVS.

7.5.2 Oocyte Sampling

By examining the first polar body of an oocyte, rather than the oocyte itself, there is a considerable reduction in the ethical implications associated with damaging or destroying oocytes. This provides an estimate of the mutant loading for a specific oocyte [108]. Heteroplasmic mice have also demonstrated equal distribution of mtDNA into the first and second polar body [106] confirming the promise of this technique. Polar body sampling has been performed on patients carrying the A3243G $tRNA^{Leu(UUR)}$ mutation and was shown to reflect the mutant loading within the corresponding oocyte [109]. Oocyte sampling has been used to determine mitochondrial heteroplasmy [105, 109] where the investigators argue that they can estimate the proportion of oocytes containing high levels of mutant molecules. In the case of low levels of mutant molecules being present (e.g., <5 %), the couple may decide to undergo natural conception. Unfortunately, there is no guarantee that subsequent ovulated oocytes will contain similar levels of heteroplasmy due to the nature of mtDNA segregation during development and the mtDNA bottleneck. Nevertheless, additional strategies would include oocyte donation, where for some individuals this might be the best course of action, as the resulting child would be genetically related to the father, but not the mother. However, this scenario carries complicated ethical and legal implications for the prospective parents [94–96].

7.5.3 Oocyte Recontructions

7.5.3.1 Metaphase II Spindle Transfer

Spindle transfer from a metaphase II oocyte (MII-ST) is similar to somatic cell nuclear transfer (SCNT), except that a haploid set of chromosomes from the carrier's oocyte is transferred to a non-affected enucleated oocyte (Fig. 7.2). The reconstructed oocyte is then fertilized through either IVF or ICSI before being transferred into the mother or a surrogate. As yet, this procedure has only been carried out in animal models and the clinical applications remain to be determined. Nevertheless, a recent study using non-human primates, namely rhesus macaque, concluded that spindle transfer might represent a reliable therapeutic approach to prevent the transmission of mtDNA in disease affected families [110]. Samples from cultured cells and blood were analyzed using real-time PCR and restriction fragment length polymorphisms (RFLP) and no accompanying mtDNA was identified in the offspring. The potential of spindle transfer as an ART in mtDNA disease is encouraging, especially for women with high mutant loading since only a small fraction of the reconstructed embryo will be heteroplasmic. Despite the conclusions drawn by Tachibana et al. [110], any clinical application requires greater stringent molecular and biochemical analysis. As previously highlighted,

mtDNA segregation is dependent upon biological fitness, mutant loading [36, 37], mutation severity [38], and the type of mutation [39–41]. Moreover, minimal levels of mtDNA variants (<0.03 %) at fertilization are known to have devastating consequences and may accumulate in a single tissue [73]. In most cases, mtDNA heteroplasmy is detectable in blood samples [111, 112] though this is not always the case [86, 112–114] and these levels may not be representative of the disease threshold levels observed in other tissues [76]. These outcomes dictate that any ART must examine a variety of tissues in a large animal model, with high levels of sensitivity and robustness before they are considered for introduction into clinical practice [115].

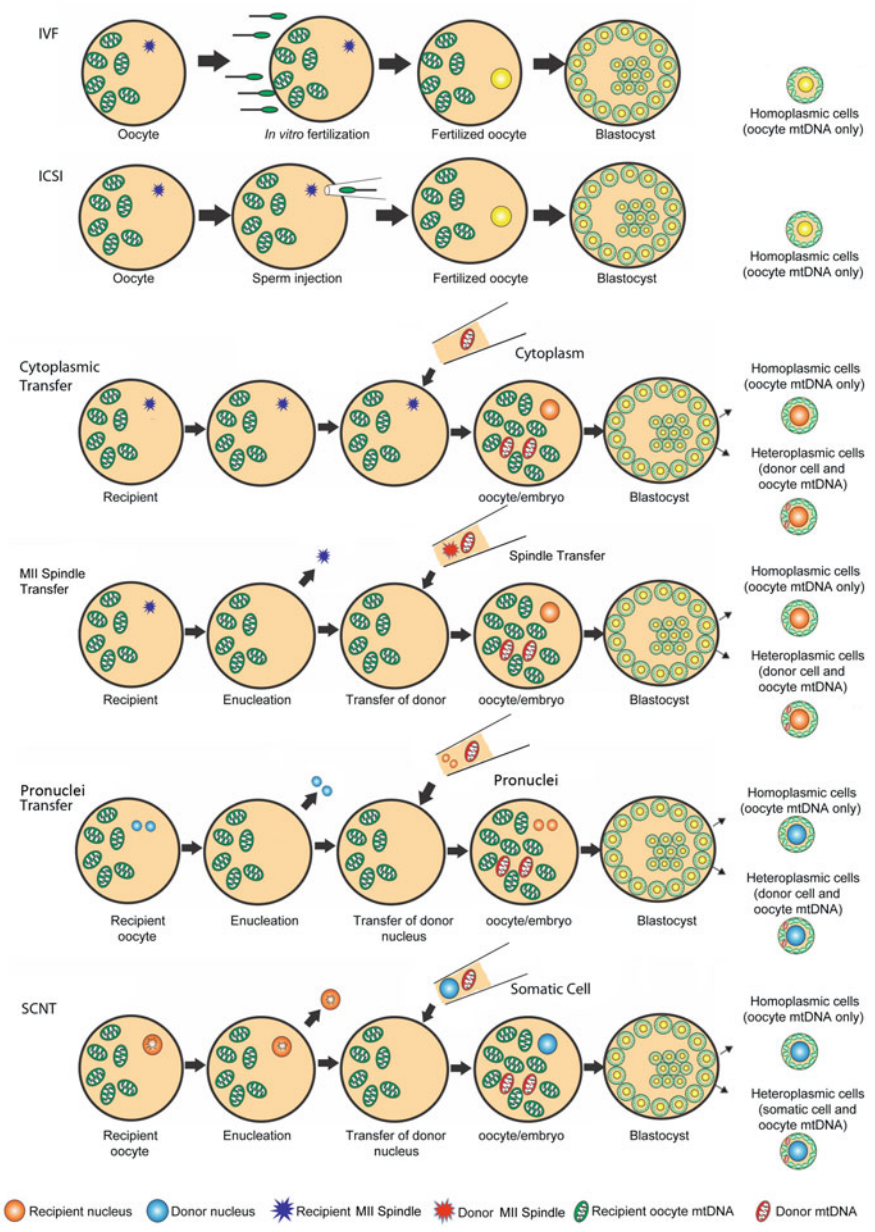
7.5.3.2 Germinal Vesicle Transfer

Many couples seeking ART will experience reproductive failure as a consequence of the aging process. Aged oocytes are more likely to suffer from aneuploidy [101, 116] and accumulate higher levels of mtDNA mutations [117]. Germinal vesicle transfer (GVT) rescues aneuploidy in aged oocytes when healthy, young oocytes are used as cytoplasts [118, 119]. It has been proposed that abnormal mitochondrial function in aged oocytes may have a role in aneuploidy [120] and the induction of mitochondrial damage in mouse oocytes prevents cytoplasmic maturation, chromosomal segregation, and spindle formation [121]. GVT has also been suggested as a technique for treating mtDNA disease [122–124] and shows promise, as the germinal vesicle is readily transferable between oocytes.

7.5.3.3 Pronuclei Transfer

Reconstruction of zygotes post-fertilization, by pronuclei transfer (PNT; Fig. 7.2), has produced live offspring in mice [125, 126] and pigs [127]. Mitochondria in close proximity to the pronuclei are carried over to the reconstructed embryo. Indeed, the transfer of mtDNA produces variable levels of heteroplasmy (0–69 %) in tissues of the progeny of mice [125] and pigs [128]. Despite the concerns with mtDNA carryover, another report demonstrated the feasibility of PNT to rescue progeny from respiratory defects, even though low levels of mutant mtDNA were transmitted [126].

Transmission of mutant mtDNA leading to disease may be exasperated if donor mtDNA were preferentially replicated over wild type molecules. The close proximity of mutant molecules in reconstructions may favor this scenario. The preimplantation and gestational lengths of mice are significantly shorter than human restricting the period when segregation and mtDNA replication events occur [115, 126]. Therefore, data from mouse studies must be viewed with caution and longitudinal studies in large animal species with closer developmental profiles and physiological attributes to human are needed before applying PNT in a clinical context.



A recent study attempted PNT in abnormal human zygotes from IVF procedures and concluded that this procedure has the potential to prevent mtDNA transmission between generations [129]. The amount of accompanying cytoplasm was shown to influence the segregation of heteroplasmic mtDNA during early cleavage stages, with donor mtDNA levels varying between 0.5 and 11.4 % per blastomere [129],

◀**Fig. 7.2** Assisted reproductive techniques used to facilitate the artificial production of developmentally competent embryos and their predicted outcomes related to mtDNA transmission. Traditionally, competent embryos may be created by in vitro fertilization (IVF) or intracytoplasmic sperm injection (*ISCI*). In these techniques, fertilization proceeds either by in vitro incubation of sperm and ooytes in culture media (*IVF*) or manual injection of a single sperm into the oocyte cytoplasm (*ISCI*). In normal circumstances, these embryos and offspring will be homoplasmic. In the context of preventing the transmission of mutant mtDNA, females with low levels of mtDNA mutations may use their oocytes in these procedures, with the application of preimplantation genetic diagnosis (*PGD*) or choronic villi sampling (*CVS*) to determine the extent of mutant mtDNA transmission. In females with high mutant mtDNA levels, donated oocytes may be required to eliminate the likelihood of disease transmission. The transfer of ooplasm containing mtDNA to disease oocytes (cytoplasmic transfer), prior to IVF or ICSI, may reduce the chance of mtDNA disease, although this technique may lead to other complications. Transferring chromosomal genetic material from disease carrying oocytes or zygotes to normal counterparts shows considerable promise in facilitating the elimination of mutant mtDNA from transmission. However, MII spindle transfer or pronucli transfer also transmit mtDNA and the extent of the resulting heteroplasmy in embryos and offspring remains a concern. Similarly, in somatic cell nuclear transfer (*SCNT*), the chromosomes from a mature oocyte are removed (enucleated) and replaced with a donor somatic cell, containing chromosomes and mtDNA. Although, the application of SCNT is very unlikely in a clinical setting, there are lessons to learn from SCNT in animal species. In particular, the production of homoplasmic offspring via chemical depletion of mtDNA in the donor cell holds great promise.

similar to segregation levels previously reported in preimplantation embryos from patients carrying mtDNA mutations [105]. This study by Craven et al. [129] was premature in its claims that sufficient research and knowledge exist to make PNT a successful ART. The use of abnormal embryos renders the data insufficient, since abnormal human embryos fail to eliminate sperm mtDNA [71] indicating mtDNA is regulated differently in abnormal embryos. The recent reports on MII-ST [110] and PNT [129] both suffer from the same limitation, namely, even a small amount of mutant mtDNA present at reconstruction can potentially cause disease in the offspring. However, these studies are a move in the right direction to limiting mtDNA transmission across generations and offer the most hope to females with low heteroplasmic levels in their oocytes.

7.5.4 What can be Learnt from Somatic Cell Nuclear Transfer?

The successful development of somatic cell nuclear transfer (SCNT) embryos was first described by Gurdon in 1962, in which a nucleus from a tadpole intestinal epithelial cell was introduced into an enucleated *Xenopus laevis* oocyte [130, 131]. This procedure was first applied to mammalian species when cloned lambs were produced from differentiated embryonic cells [132] and foetal and adult somatic tissues [133]. Following these successes, this technique has been used in a number of species, such as cattle [134], pigs [127], dogs [135] and ferrets [136], however, the rates of success have been extremely low [137]. Studies have suggested that the limited success rate may be attributed to a number of factors, such as quality of

oocytes used [138], culture and oocyte micromanipulation conditions [139–141], nuclear reprogramming [142–144], nuclear-cytoplasmic interactions, and nuclear mitochondrial incompatibility [54, 145, 146]. Despite these disadvantages, SCNT remains a source for autologous ESCs (therapeutic cloning) [138, 147, 148] and could propagate endangered species (reproductive cloning) [148–150]. However, legal and social issues restrict SCNT in humans [151–153], primarily due to the use of human tissue in experimental procedures and the implications of producing 'cloned' human embryos. Furthermore, the advent of induced pluripotent stem cells, in which autologous embryonic-like stem cells are produced in vitro without the need for human oocytes or embryo production, has further reduced public and scientific interest in human SCNT for ESC derivation.

Somatic mitochondria contain between 2 and 10 mtDNA copies per organelle [154], while there are 1–2 copies/mitochondrion in oocytes [50, 155, 156]. The mtDNA copy number in somatic cells varies depending on the cell types [56, 157, 158], yet in reconstructed embryos it represents <1 % of the total mtDNA present [145, 159, 160]. This low level of heteroplasmy may persist to the blastocyst stage [160], whereby mtDNA hetroplasmy ranges between 0 and 63 % in preimplantation embryos [161] and 0 and 59 % in live cloned offspring [68, 161–163]. Studies have also shown that the donor mtDNA may have a replicative advantage over the recipient mtDNA [163] and express increased levels of POLG and TFAM [159], which are the key mtDNA replication factors with POLGA not being expressed during preimplantation development following fertilisation [51, 159]. This suggests that there is potential for somatic cell mtDNA to be preferentially replicated, leading to deleterious effects and implications for the resultant embryo. Indeed, these observations may account for the numerous malformations characteristic of SCNT foetuses and offspring [54, 137].

The shortage of donated human oocytes has led to the suggestion that potential replacements can be obtained from animal sources [152, 153]. The use of oocytes from a species that is different to the donor cell is known as interspecies SCNT (iSCNT) [145, 164–166]. While it would never be applicable to generate human offspring, many lessons can also be learnt from its application. Numerous studies have demonstrated the production of iSCNT blastocysts, but with extremely low efficiency [145, 164–166]. Similar outcomes have been described for human-rabbit [167, 168] and human-bovine [165, 168] reconstructions. Encouragingly, Chen et al. [167] reported the derivation of human-rabbit iSCNT embryonic stem cells (ESCs), but subsequent experiments have failed to reproduce this outcome. These inefficiencies in iSCNT embryos are a consequence of incompatible nuclear-cytoplasmic factors, namely species-specific reprogramming factors and mtDNA [145]. In interspecies cybrids, increased respiratory deficiencies are characteristic of divergent mtDNA populations [169–171]. Techniques, such as depleting mtDNA [145, 159, 160, 172] with chemicals in donor cells and/or recipient oocytes have been suggested to decrease the chances of unwanted mtDNA transmission during nuclear transfer procedures and improve developmental outcomes [145]. Some have suggested the depletion process may itself decrease the developmental potential of the reconstructed oocyte [173]. However, the

production of 'true' homoplasmic clones lambs (Fig. 7.2) from depleted donor cells has, to-date, demonstrated no adverse effects [172]. Indeed, partial depletion of porcine oocyte mtDNA and the introduction of murine mitochondria and ESC extract increased development to the blastocyst stage in murine-porcine iSCNT, demonstrating compatible mtDNA and reprogramming factors to the donor nuclei are essential for developmental follow iSCNT [145].

7.6 Developmental Abnormalities Associated with Assisted Reproductive Technologies

ART aims to facilitate reproductive outcomes for couples that have underlying fertility issues or eliminate the transmission of disease phenotypes between generations. Paradoxically, these clinical interventions may inadvertently predispose embryos to epigenetic disorders [174–176], preterm birth [177], and growth anomalies [178, 175]. The frequency of these reproductive defects is extremely rare (1 in <10,000) and only slight risks of major and minor birth defects exist, when compared to natural conception [174–176]. However, numerous studies have failed to show any correlation between ART and developmental defects, citing sample size, underlying fertility, absence of age correction, and statistical assumptions, as possible experimental shortfalls [180–185].

7.6.1 Cytoplasmic Transfer

Women who suffer from recurrent reproductive failure due to substandard oocyte quality may benefit from the microinjection of 5–15 % healthy donor oocyte cytoplasm (Fig. 7.2) to improve reproductive outcomes and produce healthy babies [186, 187]. The donated ooplasm contains stored maternal mRNAs and proteins, as well as mitochondria, making the reconstructed oocyte and offspring heteroplasmic [188, 189]. Increased mtDNA copy number is associated with increased developmental competence [43, 47, 49, 51], and fertilization outcomes are increased in developmentally incompetent oocytes supplemented with developmentally competent ooplasm [49, 188]. The donor mtDNA persists through development and transmission has been detected in subsequent progeny [189], retaining the genetic material from three parents [190]. Studies in bovine [191] and mice have confirmed oocyte-fetal transmission [192, 193] and numerous physiological abnormalities, including systemic hypertension and increased body fat, have been identified in mouse offspring [194]. These physiological irregularities are attributed to the genetic distance between the mother's and donor's mtDNA resulting in a mixture of electron transport chain (ETC) complexes with slightly distinct amino acid composition [54]. Unexpected abnormalities have also been

observed in some infertile couples following human cytoplasmic transfer (CT) [195]. A single study identified foetuses with monosomy X-chromosome (45, XO: Turner syndrome), whilst another embryo developed normally, yet the child was diagnosed with pervasive development disorder (autism) at 18 months of age [195]. Therefore CT, as an ART, must be regarded with caution, as the mixing of different human mtDNA haplotypes [190] and the technique itself, potentially results in detrimental clinical consequences. Further longitudinal studies in large animal models are required where periods of gestation and longevity are more similar to human than the mouse. Nevertheless, supplementation with pure populations of mtDNA from the same genetic source might be an option. It has been demonstrated that this approach can rescue developmentally incompetent oocytes [49]. Consequently, following superovulation, one approach in clinical IVF would be to pool mitochondria from a few of a female's oocytes to recue her other oocytes.

7.6.2 Epigenetic Disorders

The most frequently described imprinting disorder associated with ART is Beckwith-Wiedemann syndrome (BWS), which is caused by the aberrant epigenetic control of a defined region on chromosome 11p15.5 [196]. Other studies have suggested that ART increases the risk of conceiving children with Angelman syndrome (AS), Prader-Willi syndrome (PWS) [197], retinoblastoma [198, 199], and Silver-Russell syndrome [200–202]. Altered imprinting and DNA methylation patterns in individuals conceived through ART have also been attributed to IVF [203–205] and ICSI [203, 206–208] procedures. Both IVF and ICSI have been linked to BWS [204–206] and SRS [200, 201], while retinoblastoma [198] is normally only associated with IVF and AS [208, 209] and PWS [200] are linked with ICSI. These studies normally do not take into account any additional factors such as superovulation, in vitro culture conditions, and cryopreservation. Indeed, cryopreservation of bovine embryos influences gene expression profiles [210, 211] and may influence developmental outcomes in children conceived after embryo thawing [212]. The manipulation of gametes and embryos under artificial conditions are the contributing factors to the increased risk of developmental anomalies, however, the primary causes differ between individual cases.

In clinical settings, female patients are normally superovulated to increase the number of available oocytes for in vitro manipulation. This hormonal stimulation has been demonstrated to alter DNA methylation patterns in mouse [213–216] and human [216, 217] embryos. Furthermore, superovulation has been implicated as the contributing factor in some cases of BWS [218] and AS [219]. Increased aneuploidy in human embryos [217] has been associated with ovarian stimulation, whilst in mice it may lead to reduced oocyte quality and preimplantation development, and retarded fetal growth [220–222]. The negative effects of superovulation in mammalian

species may be a consequence of disrupting gamete imprinting during the later stages of oogenesis prior to ovulation [223, 224].

Exposure of oocytes and embryos to in vitro culture conditions is detrimental to embryo quality [225], possibly due to imprinting defects [141], and altered gene expression [139, 226]. In some instances, bovine and ovine embryos exposed to suboptimal in vitro conditions display fetal overgrowth syndromes and placental abnormalities, termed large offspring syndrome (LOS) [141]. In cattle [227] and sheep [228, 229], this developmental phenotype has been attributed to epigenetic alteration at the *Igf2/H19* locus. This phenotype is reminiscent of BWS [227, 230], although the epigenetic loci affected in humans (KvDMR1 and ICR1 on chromosome 11p15.5) are distinct [196]. Nevertheless, in some instances, children conceived using assisted reproductive technology display aberrant methylation patterns at the *Igf2/H19* locus [231]. The effects of in vitro culture conditions may be media specific [175], with components such as serum [232, 233] providing the influential factor(s). Due to the artificial nature of the embryo culture conditions and the micro-manipulation undertaken using ART, embryos may experience environmental stress [234]. Indeed, the expression of heat shock proteins is altered in IVF embryos compared to their in vivo counterparts [226].

Any attempts to eliminate mtDNA transmission between generations are compounded by the increased probability of in vitro manipulated embryos acquiring epigenetic abnormalities. SCNT embryos display extremely reduced developmental potential and foetal abnormalities [132, 133, 235], as a consequence of aberrant reprogramming of somatic nuclei [236, 237]. Aberrant reprogramming alters the regulation of mtDNA copy number during development and differentiation [157, 159, 238]. No evidence exists as to whether MII-ST, GVT or PNT technologies affect gene imprinting, but these embryos may be heteroplasmic and mixing of genetically distant mtDNA populations in cytoplasmic transfer can lead to numerous physiological abnormalities [194]. Consequently, full and rigorous experimental investigations need to be undertaken before MII-ST, GVT, PNT and cytoplasmic transfer are introduced into clinical practice.

7.7 Concluding Remarks

The recent advances in MII-ST [110] and PNT [129] are encouraging progress in the battle to prevent the transmission of mutant mtDNA between generations. However, questions still exist as to whether these ART truly prevent mtDNA transmission [115, 238]. Lessons from inter-specific crosses [66–68], abnormal paternal inheritance [71–73] and SCNT [159, 160, 239, 240], combined with our understanding of mtDNA segregation, demonstrate that even extremely low levels of heteroplasmy during preimplantation development and the early stages of development (pre-gastrulation) can accumulate tissue-specifically during the later stages of development (organogenesis) or postnatally. In the context of human mtDNA disease, clinicians and scientists have a responsibility to ensure that

mutant mtDNA transmission is not transmitted. Depletion of mtDNA [145, 159, 160, 172] prior to ART may be one course of action. However, more investigations are required related to nuclear-mitochondrial interactions following karyoplast transfer and subsequent mtDNA transmission in ART, before these procedures can be safely applied to prevent the onset of mtDNA type-diseases in ART laboratories [241, 242].

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About the Author

Professor Justin St. John was awarded his PhD in 1999. In 2000, he was appointed as a Lecturer at the University of Birmingham, UK. His achievements as an early career scientist led to his rapid promotion to the position of Professor at the University of Warwick (2007). Whilst in the UK, he was funded by the Medical Research Council and received an Endeavour Fellowship to undertake a period of research at Monash Institute of Medical Research, Australia where he has been Director of the Centre for Reproduction and Development and a Professor in the Faculty of Nursing, Medicine and Health Sciences at Monash University since November 2009. His research focuses on developing and using specific model systems to understand how mitochondrial DNA is transmitted and replicated. He was the first to demonstrate that sperm mitochondrial DNA could persist in the late stage embryo and thus be transmitted. In novel work, he has described mitochondrial DNA replication events in undifferentiated and differentiating embryonic stem cells and defined the mitochondrial DNA set point. He has also demonstrated why donor cell mitochondrial DNA is transmitted to embryos and offspring following somatic cell nuclear transfer and developed reproductive strategies to overcome this. He is using these outcomes to develop mini-pig models of mitochondrial DNA disease and reproductive strategies to prevent the transmission of mutant mitochondrial DNA from one generation to the next. He has published widely including key papers in *The Lancet, Nature Chemical Biology, Nature Cell Biology, Stem Cells, Nucleic Acids Research, Journal of Cell Science, and Genetics.*

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