The effect of pronuclear transfer on human preimplantation development

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Abstract

Mutations in maternally inherited mitochondrial DNA (mtDNA) can cause a range of complex diseases for which there are currently no curative treatments. Using IVF based techniques involving nuclear genome transplantation, it may be possible enable women who carry mtDNA mutations to have a genetically related child without the risk of transmitting disease. The central aim of this project is to perform preclinical studies testing the safety and efficiency of pronuclear transfer (PNT).

Surprisingly, the PNT technique developed using abnormally fertilised zygotes was detrimental to survival of normally fertilised zygotes. We tested the possibility that this might be due to the relatively accelerated development of normally fertilised zygotes allowing insufficient time for recovery following transplantation of the pronuclei. Switching the timing of PNT to shortly after pronuclei appearance (ePNT) rather than shortly before disappearance resulted in increased survival. Further modification of the enucleation and embryo culture media resulted in improved blastocyst quality. As part of the optimisation process, I tested the effect and reversibility of drugs that are used to inhibit the cytoskeleton of oocytes and zygotes in preparation for manipulations. Comparison of two compounds, which directly inhibit actin polymerisation, revealed marked differences in the reversibility. However, latrunculin B, which is rapidly reversed, has a detrimental effect on blastocyst development compared with latrunculin A, which is more potent and less readily reversible. Finally, I analysed single-cell RNA-sequencing data to determine whether gene expression in human blastocysts is altered by ePNT. This work was done in collaboration with Dr Kathy Niakan at the Francis Crick Institute. The findings indicate no detectable differences in global or lineage-associated gene expression between control and good quality ePNT blastocysts. Analysis of mitochondrial gene expression revealed high variability in the level of expression both within and between blastocysts. However, this variability was observed in ePNT and control blastocysts, and there was no detectable difference between them.

In conclusion, this study has tested PNT in normally fertilised human zygotes for the first time; results indicate no detectable harmful effects of the ePNT procedure. We therefore conclude that it is likely to give rise to normal pregnancies.

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Publications and Presentations

Publications

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Presentations

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Chapter 1. Introduction

1.1 Mitochondria

The fertilised egg contains haploid maternal and paternal genomes, which are separately packaged into structures called pronuclei. It also contains many thousands of copies of mitochondrial DNA (mtDNA) (Shoubridge and Wai, 2007). The mtDNA copy number of oocytes is estimated to be ~100,000 in mouse oocytes (Piko and Taylor, 1987; Wai *et al.*, 2010), and variable in human oocytes, ranging from 200,000 to 500,000 (Barritt *et al.*, 2002; Craven *et al.*, 2010). Unlike nuclear DNA, mtDNA is exclusively maternally inherited. Pathogenic mutations in mtDNA can cause a broad range of debilitating and often fatal multi-system diseases, which can present at any age. There are currently no curative treatments for mtDNA disease, therefore this is an essential area of research.

1.1.1 Origin and evolution of mitochondria

Mitochondria are unique organelles as they contain their own DNA, which originates from their proteobacterium origins. There are two theories regarding the origin of mitochondria. Firstly, the endosymbiotic theory proposes that alpha-proteobacteria were engulfed by an anaerobic eukaryotic host cell through phagocytosis (Martin, 2010). The second theory suggests that the host cell was not a eukaryote but prokaryote, and eukaryotes evolved following the fusion of an archaebacterium host with a proteobacterium symbiont (Gray *et al.*, 1999). Both scenarios involved a symbiotic relationship between the host and proteobacterium, which provided a survival advantage. The evolutionary advantage resulted in progressive loss of independence and the transformation of mitochondria to intracellular organelles.

The mtDNA of lower eukaryotes encodes many more genes than human mtDNA. In humans, the majority of mitochondrial genes were transferred to the nuclear genome during evolution, 37 genes were retained within mitochondria (Roger, 1999; Embley and Martin, 2006). Numerous hypotheses have been proposed as to why 37 genes have not been transferred to the nuclear genome and instead are retained within mitochondria. Firstly, it is possible that the use of a different genetic code within mitochondria (Barrell *et al.*, 1979) prevents complete gene transfer to the nuclear genome, as it could prevent accurate transcription and translation of gene products. However, it has been argued that this cannot explain incomplete gene transfer in creatures that use the standard genetic code in the mitochondria (Ridley, 2001). It has been proposed that the genes retained in the mitochondrial genome encode proteins that

are too hydrophobic for import into the mitochondria (von Heijne, 1986; Popot and de Vitry, 1990). Others have suggested that these gene products may be toxic to the cell if present in the cytoplasm (Martin and Schnarrenberger, 1997), although there is limited evidence to support this hypothesis.

1.1.2 Mitochondrial functions and dynamics

Mitochondria are often described as the 'powerhouses' or 'battery packs' of the cell, as they are the organelles responsible for oxidative phosphorylation (OXPHOS) which produces over 90% of cellular energy in the form of ATP. In addition to their involvement in energy production, during evolution mitochondria have acquired a number of other important functions, including; execution of apoptosis (Wang and Youle, 2009), calcium handling (Babcock *et al.*, 1997) and formation of iron sulphur clusters (Veatch *et al.*, 2009).

The respiratory chain located on the inner mitochondrial membrane is involved in energy production in the form of ATP (Figure 1.1). There are 5 complexes which make up the respiratory chain; NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome C reductase (complex III), cytochrome C oxidase (complex IV) and ATP synthase (complex V). The respiratory chain complexes are composed of subunits encoded by both the nuclear and mitochondrial genomes, although complex II is entirely encoded by nuclear DNA. Electrons are produced during the tricarboxylic acid (TCA) cycle by NADH and $FADH_2$, which flow along the electron transport chain and are used to establish a proton gradient for ATP production. The electron transport chain is responsible for ~90% of cellular oxygen consumption and a small percentage of this is converted to superoxide (Boveris *et al.*, 1972), however in certain conditions superoxide production can increase and cause oxidative stress. To avoid the production of hydroxyl radicals that are damaging to biomolecules including DNA, superoxide is converted to H_2O_2 by superoxide dismutase and subsequently broken down to H_2O.

Mitochondria are involved in the intrinsic apoptotic pathway, which is triggered in response to apoptotic stimuli such as DNA damage. Following pathway activation, Smac/DIABLO (second mitochondria derived activator of caspase) and Omi/HTRA2 are released from mitochondria and bind to IAPs (inhibitors of apoptosis), preventing their inhibitory effect on caspase activity (Srinivasula *et al.*, 2000; van Loo *et al.*, 2002). Cytochrome C is an essential component of the electron transport chain, which has also been identified as an apoptotic protease activating factor (Apaf) (Liu *et al.*, 1996). Following IAP inactivation, cytochrome C is released from mitochondria and initiates caspase activation (Wang and Youle, 2009). The

release of cytotoxic proteins from mitochondria is regulated by Bcl-2 family proteins, which consist of both proapoptotic and antiapoptotic proteins (Wang and Youle, 2009). Activation of proapoptotic Bax/Bak results in the induction of mitochondrial outer membrane permeability (MOMP) and subsequent protein release leading to caspase activation and apoptosis (Wang and Youle, 2009). The restriction of proapoptotic proteins to the mitochondria and regulated release in response to apoptotic stimuli prevents activation of apoptosis in healthy cells (Wang and Youle, 2009).

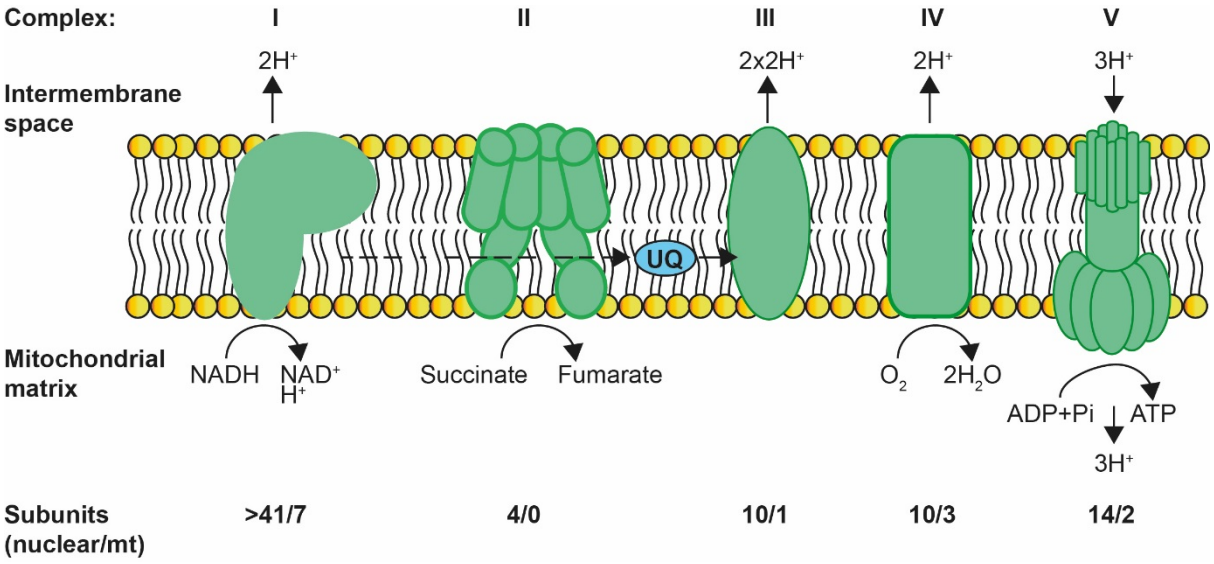


Figure 1.1: The mitochondrial respiratory chain. Schematic representation of complexes I to V of the electron transport chain, located on the inner mitochondria membrane. Number of subunits encoded by nuclear and mitochondrial genomes is also shown at the bottom of the image. Images is adapted from Mandavilli *et al.* (2002).

Mitochondria also have an important role in the storage and regulation of intracellular calcium (Babcock *et al.*, 1997). Uptake of calcium ions is regulated by the electrochemical potential gradient and facilitated by the mitochondrial calcium uniporter (MCU) present on the inner mitochondrial membrane (Baughman *et al.*, 2011). Conversely, the Na^+/Ca^{2+} exchangers (mNCX) and H^+/Ca^{2+} exchangers (mHCX) are involved in the efflux of calcium ions (Carafoli *et al.*, 1974; Jiang *et al.*, 2009). The endoplasmic reticulum (ER) is the organelle responsible for storage of the majority of Ca^{2+} within the cell, and is connected to the mitochondria by contact sites known as mitochondria-associated ER membranes (MAMs) (Rizzuto *et al.*, 1998). The importance of mitochondria in the storage and handling of calcium implicates them in the regulation of calcium signalling. The role of mitochondria in calcium

signalling regulation has previously been reviewed in detail (Rizzuto *et al.*, 2012); this review discusses the role of mitochondria and Ca^{2+} in; metabolism regulation, autophagy regulation and the involvement of calcium signalling in release of proapoptotic proteins from mitochondria.

Mitochondria are dynamic organelles, which can exist as a tubular network or discrete organelles. To achieve this, mitochondria undergo fission and fusion, which regulates the shape and size of mitochondria in addition to biological functions. Mitochondrial fission is regulated by dynamin-related protein (Drp1), which associates with the outer mitochondrial membrane to form an oligomer wrapped around the membrane, able to constrict mitochondria following hydrolysis of GTP and a conformational change, causing mitochondrial fission (Smirnova *et al.*, 2001). The ER also plays a role in mitochondrial fission; it has been found that association of ER tubules with mitochondria is a key initial event during mitochondrial fission (Friedman *et al.*, 2011). In mammals, the membrane-anchored dynamic family members mitofusins 1 and 2 (Mfn1 and Mfn2) regulate outer mitochondrial membrane fusion and Opa1 assists fusion of inner membranes (Youle and van der Bliek, 2012). The fission and fusion cycle is regulated by metabolism, with a fused, tubular network of mitochondria observed when oxidative phosphorylation increases (Rossignol *et al.*, 2004). Fission and fusion are important for response to stress and maintenance of mitochondrial health and activity; defects can be compensated for during fusion events and fission can result in the segregation of damaged mitochondria from an otherwise healthy population of fused mitochondria (reviewed in Youle and van der Bliek (2012)).

Mitophagy is the selective degradation of damaged mitochondria by autophagy. The best characterised mitophagy pathway is the PINK1-Parkin mediated pathway, in which initiation of mitophagy is triggered by the activation and stabilisation of PINK1 kinase on the outer mitochondrial membrane (reviewed in Youle and Narendra (2011)). PINK1 is able to activate and recruit Parkin, an E3 ubiquitin ligase, which enables assembly of ubiquitin chains that PINK1 can phosphorylate (Youle and Narendra, 2011). Binding of ubiquitin chains by the autophagy receptors NDP52 and OPTN is required for successful mitophagy (Lazarou *et al.*, 2015). Mitophagy ultimately functions as a quality control procedure to remove damaged mitochondria, in addition to regulating mitochondrial number in accordance with metabolic demand (Youle and Narendra, 2011).

1.1.3 Mitochondrial DNA

Mitochondrial DNA (mtDNA) is circular, double-stranded DNA that is 16.5kb in length and encodes only 37 genes (Figure 1.2); 13 genes encode protein subunits of respiratory chain complexes, 22 encode tRNAs and 2 encode rRNAs, which allows translation of mtDNA encoded subunits within the mitochondrial matrix. Copies of mtDNA are packaged into nucleoids by mitochondrial transcription factor A (TFAM) (Kukat *et al.*, 2011). Nucleoids support the organisation and expression of the mitochondrial genome and have an average diameter of 100nm and usually contain a single copy of mtDNA (Kukat *et al.*, 2011). The nuclear genome encodes >1000 other proteins required for mitochondrial function. Therefore, it is essential that the nuclear and mitochondrial genomes are co-ordinately regulated.

Replication of mtDNA involves factors that are encoded by the nucleus. Key components include mitochondrial polymerase γ (POLG), which synthesises mtDNA by the action of a catalytic subunit (POLGA) and a small processivity subunit (POLGB). Also required are Twinkle, a mtDNA helicase, the activity of which is regulated by mtSSB (mitochondrial single-stranded binding protein), which also inhibits reannealing (Korhonen *et al.*, 2004).

Two models for the replication of mtDNA have been proposed; these are the strand-displacement (asynchronous) model (Clayton, 1982) and strand-coupled (symmetric) model (Holt *et al.*, 2000). Both models state that replication is initiated at the origin of replication (O_H) on the heavy strand of mtDNA. However, the strand-displacement model suggests that clockwise replication displaces the light strand, which exposes the light strand origin of replication (O_L) allowing replication of the entire DNA in an anticlockwise direction (Clayton, 1982). Alternatively, the strand-coupled model suggests replication of the light strand is initiated soon after the heavy strand, resulting in the formation of Okazaki fragments that are converted to DNA (Yasukawa *et al.*, 2006); replication occurs bidirectionally until complete (Holt *et al.*, 2000).

A number of proteins encoded by nuclear DNA are required for mitochondrial transcription (reviewed in Falkenberg *et al.* (2007)). These include mitochondrial RNA polymerase (POLRMT) and mitochondrial transcription factors TFAM and TFB2M. Transcription is initiated by the binding of TFAM to regulatory elements upstream of promotors of the heavy (HSP1 and HSP2) and light (LSP) strand, which causes a conformational change allowing binding of POLRMT. This results in the production of long polycistronic transcripts which are processed in order to produce mRNA, tRNA and rRNA. Transcription termination is modulated by mitochondrial transcription termination factor (mTERF).

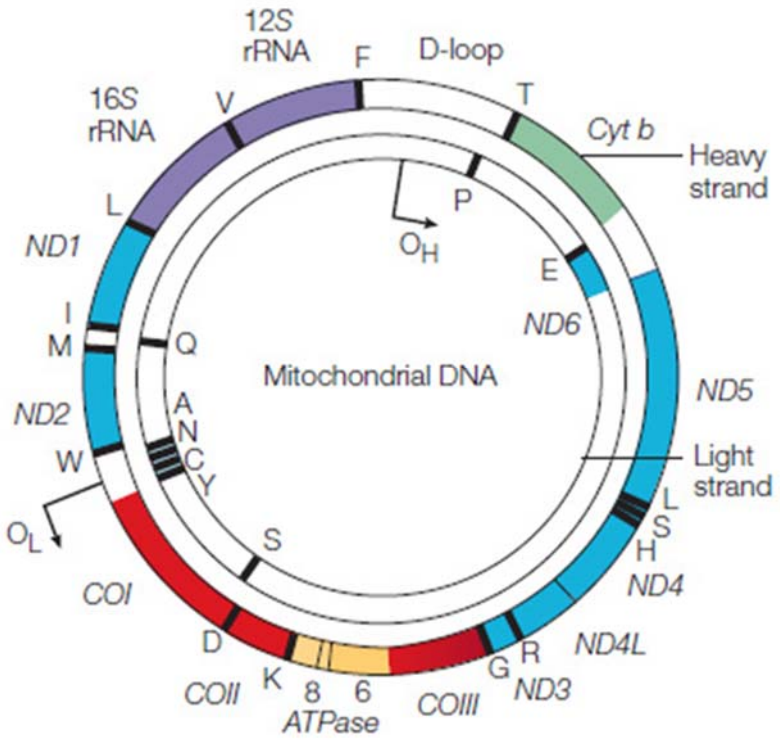


Figure 1.2: The human mitochondrial genome. Map showing the circular, double-stranded 16.5kb mitochondrial genome, which encodes 13 proteins (complex I: blue, complex III: green, complex IV: red, complex V: yellow), 22 tRNAs (black lines and single letter code) and 2 rRNAs (purple). The D-loop contains sequences required for replication and transcription initiation. The origins of replication are also shown (heavy strand: O_H, light strand O_L). This image is taken from Taylor and Turnbull (2005).

Mitochondrial translation resulting in protein synthesis involves initiation, elongation and termination. These events are not yet fully characterised, but have been extensively reviewed (Smits *et al.*, 2010; Mai *et al.*, 2016). Assembly of the initiation complex requires the mitochondrial translation factor mtIF3 and mitoribosome dissociation. Binding of mRNA to the small subunit of the ribosome is facilitated by mtIF3, ensuring accurate start codon positioning. When this is complete, the small and large ribosomal subunits can recombine, displacing mtIF3 and allowing progression to the elongation stage. This requires mtEFTU which regulates accuracy of translation and the elongation factors mtEGF1 and mtEFG2. Finally, termination of translation occurs when a stop codon is encountered and mitochondrial release factors (mtRF1, mtRF1a) prompt protein release. Ribosome recycling is facilitated by ribosomal recycling factor mtRRF.

The mutation rate of mtDNA is up to 10 times higher than nuclear DNA; it is likely that repair mechanisms have been sacrificed due to the small size of the mitochondrial genome. Furthermore, the close proximity to the OXPHOS system may increase vulnerability of the mitochondrial genome to mutation through damage from reactive oxygen species (ROS). The high mutation rate of mtDNA has caused high incidence of polymorphic variants within mtDNA between individuals. Mutations in mtDNA have also divided the human population into haplogroups, representing the occurrence of neutral variants between geographically separated populations (reviewed in Wallace *et al.* (1999)).

1.1.4 Pathogenic mtDNA mutations

Although the majority of variants occurring in mtDNA due to mutations are neutral, sometimes pathogenic mutations arise, which can cause mitochondrial dysfunction and disease if present above a certain threshold. Presentation of disease symptoms is complicated by heteroplasmy; each cell contains many mitochondria and each mitochondrion contains multiple copies of mtDNA packaged into nucleoids. Therefore, individuals carrying mtDNA mutations may be homoplasmic or heteroplasmic for the causative mutation. Homoplasmy is the presence of a mutation in all copies of mtDNA, whereas heteroplasmy is the presence of both mutated and non-mutated mtDNA molecules. The threshold for disease presentation is variable depending on the type of mutation; deletions in mtDNA typically have a threshold of around 60% (Moraes *et al.*, 1992; Shoubridge, 1994; Rossignol *et al.*, 2003) whereas a point mutation in tRNA may have a high threshold of over 90% (Boulet *et al.*, 1992). The variability in symptoms and differences in severity between tissues or cells within a tissue is caused by random segregation of mtDNA and clonal expansion. Clonal expansion refers to the ability of mutations to accumulate within cells, however this occurs over a long period of time, which contributes to the late presentation of some mitochondrial diseases (reviewed in Greaves *et al.* (2012)).

Mitochondrial disease is not only caused by mutations in mtDNA; as the nuclear genome also encodes proteins essential for mitochondrial function, mutation in nuclear encoded mitochondrial genes can also result in disease. This in turn complicates disease inheritance, as mutations in mtDNA are maternally inherited whereas mutations in nuclear encoded mitochondrial genes can be inherited from either the mother or father. It is also possible for mutations to occur *de novo* (Lebon *et al.*, 2003). Mitochondrial disease is most commonly caused by mutations in nuclear encoded mitochondrial genes.

Due to the variable mutations and the essential function of mitochondria in energy production in all tissues, mitochondrial diseases present with a broad range of symptoms that can affect multiple organ systems and present at any age. Most commonly affected are tissues that require high levels of energy, including the heart, muscle and brain. Treatment of mitochondrial disease involves management of symptoms, as there is currently no available cure.

Over 300 disease causing mutations have been identified in mtDNA since the first pathogenic mutations were described in 1988 (Holt *et al.*, 1988; Wallace *et al.*, 1988a). Mutations causing mitochondrial and OXPHOS dysfunction can result in inability of the cell to produce sufficient energy in the form of ATP, causing lactic acidosis and multi-systemic disorders generally affecting tissues that require high levels of energy (Greaves *et al.*, 2012). A number of clinical syndromes have been defined, which describe the symptoms and affected tissues. However, the high phenotypic variability between patients and a general lack of awareness presents a challenge for the diagnosis of mitochondrial disease. The incidence of disease presentation caused by mutation in mtDNA is estimated to be 1 in 10,000 in the North East of England, but it is suggested that this may be a large underestimate as the frequency of occurrence of pathogenic mtDNA mutations is much higher (Chinnery *et al.*, 2012; Greaves *et al.*, 2012). Thus, it is possible that a number of patients may be incorrectly diagnosed.

One example of disease caused by mtDNA mutations is Leigh syndrome, a severe and often fatal neurological disorder, which usually presents within the first year of life. This syndrome is characterised by developmental delay, seizures, progressive loss of movement, and death may be caused by respiratory failure. Leigh syndrome can be caused by a range of mutations in either nuclear or mtDNA. Mutations affecting over 60 genes have been identified to date (Gerards *et al.*, 2016), the first identified mtDNA mutation causing Leigh syndrome was identified in 1992 (Tatuch *et al.*, 1992) and occurred in the MT-ATP6 gene (m.8993T>G).

Other syndromes that can be caused by pathogenic mtDNA mutations include, myoclonic epilepsy with ragged-red fibers (MERRF) and mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS). MERRF is a progressive disease, which usually presents in children or young adults and generally affects the muscles and nervous system (Greaves *et al.*, 2012). MELAS patients usually present in early childhood, before 10 years of age, with seizures and stroke-like episodes (Greaves *et al.*, 2012). Both syndromes are caused by mutations in mtDNA; the most common MELAS causing mutation is m.3243A>G in the MT-TL1 (mitochondrial tRNA) gene (Goto *et al.*, 1990). Similarly, MERRF is also commonly

caused by pathogenic mutation in a tRNA gene, specifically the MT-TK gene (m.8344A>G) (Wallace *et al.*, 1988b). However, both syndromes may be caused by mutations in other mtDNA genes, commonly the MT-ND5 gene which encodes a subunit of OXPHOS complex I may be affected in MELAS (Dimauro and Davidzon, 2005), and a number of mutated tRNA genes have been identified in MERRF patients (Lorenzoni *et al.*, 2014). Interestingly, the same mutation does not always cause the same syndrome. For example, m.3243A>G in the MT-TL1 gene does not always cause MELAS, but frequently presents as maternally inherited diabetes and deafness (MIDD) (van den Ouweland *et al.*, 1992); due to differences in heteroplasmy levels. This highlights the variability in mitochondrial disease between patients.

In summary, the mitochondrial genome encodes only 13 of the ~1,500 proteins required for mitochondrial function (Calvo and Mootha, 2010). Thus, normal mitochondrial functioning requires interaction between proteins that are encoded by the nuclear and mitochondrial genomes. Mitochondrial disease can also be caused by pathogenic mutation in nuclear encoded mitochondrial genes, which disrupts mitochondrial function. Generally, the disease causing nuclear DNA mutation will disrupt mtDNA maintenance and expression, nucleoside transport/synthesis or mitochondrial dynamics (Greaves *et al.*, 2012).

1.2 Inheritance of nuclear and mtDNA

1.2.1 Oogenesis, meiosis and fertilisation

During foetal development, oogenesis starts by the proliferation of primordial germ cells (PGCs), which migrate to the gonadal ridge and undergo numerous rounds of mitotic cell divisions resulting in the production of oogonia. These oogonia enter meiosis to ultimately become oocytes. Females are born with a stock of approximately 1 million oocytes, each of which is surrounded by a small number of flattened pre-granulosa cells, to form a primordial follicle. Primordial follicles are recruited for growth on an ongoing basis throughout life. Puberty triggers activation of the ovarian/pituitary axis, enabling follicles to grow to the preovulatory stage. From puberty to menopause, mature oocytes capable of undergoing fertilisation are released, in a cyclical manner, into the oviduct during ovulation.

Meiosis is the process by which male and female gametes are able to transmit one copy of each chromosome to the fertilised egg. Meiosis is a reductive cell division that results in the production of haploid gametes from diploid progenitors, and involves a single period of DNA replication followed by two successive rounds of chromosome segregation. The product of

each round of male meiosis is four viable sperm, whereas a single oocyte is the only viable product of female meiosis.

Replicated maternal and paternal homologues undergo reciprocal exchange of DNA to form crossovers which act as physical linkages (chiasmata) to form bivalent chromosomes (Moore and Orr-Weaver, 1998; Petronczki *et al.*, 2003; Kleckner, 2006; Herbert *et al.*, 2015). This produces oocytes that remain in a non-growing state, arrested at meiotic prophase until shortly before ovulation, which corresponds to decades in the case of humans. The pool of primordial follicles established during foetal development becomes depleted throughout life, primarily due to cell death (Herbert *et al.*, 2015). Between puberty and menopause, a tiny minority (~400) are selected for ovulation. Ovulation is triggered by a surge in Luteinizing hormone (LH), which also triggers exit from arrest in prophase of meiosis I. The nucleus of the prophase I arrested oocyte is known as the germinal vesicle (GV). Fully-grown oocytes, undergo GV breakdown (GVBD), which marks entry into M phase of meiosis I and completes the 1st meiotic division, forming the 1st polar body shortly before ovulation. In mouse oocytes, formation of the 1st polar body is preceded by migration of the spindle from its central position in the oocyte to the cortex by a microfilament-mediated process, which can be disrupted by drugs that disrupt actin dynamics (Longo and Chen, 1985; Verlhac *et al.*, 2000; Liu *et al.*, 2002; Calarco, 2005). Conversely, treatment with inhibitors to disrupt microtubule organisation prevents spindle formation, but chromosomes are able to localise to the cortex (Longo and Chen, 1985; Verlhac *et al.*, 2000). Furthermore, live imaging of mouse oocytes has revealed that spindle migration requires interaction of a reorganising cytoplasmic actin network that is nucleated by Formin-2 with myosin enriched spindle poles (Schuh and Ellenberg, 2008). The authors demonstrate that inhibition of myosin activation prevents spindle movement (Schuh and Ellenberg, 2008). These studies show that interaction between actin and microtubules is essential during meiosis for accurate spindle positioning. Similar findings haves also been reported from studies using human oocytes (Kim *et al.*, 1998).

During anaphase of the first meiotic division bivalents are converted to two dyad chromosomes, consisting of sister chromatids linked by cohesion at the centromere (Moore and Orr-Weaver, 1998; Petronczki *et al.*, 2003). Because anaphase I occurs at the oocyte cortex, a highly asymmetric cell division ensues, and one set of dyads is lost to the first polar body, a small structure which is destined for degeneration. The dyads that remain in the oocyte align on the metaphase II (MII) spindle, which is formed at the cortex of the oocyte. The oocyte then enters a second period of arrest known as MII arrest, where it remains until the fertilising sperm triggers the second meiotic division.

In mouse oocytes, the cortical layer of actin is produced from free ends of actin filaments (F-actin) stemming from the oocyte plasma membrane, which intermesh to form a dense cortical layer (Sun and Schatten, 2006). During oocyte maturation, a thick cortical layer of actin known as the actin cap is formed at the oocyte cortex adjacent to the spindle, which contributes to maintenance of spindle position and plays an essential role in polar body extrusion, determining the site at which this will occur (Longo and Chen, 1985). The MII spindle is maintained at the oocyte cortex by an actin-dependent mechanism involving Rac1 (Halet and Carroll, 2007) and Arp2/3 (Yi *et al.*, 2011). Arp2/3 is active at the oocyte cortex adjacent to the spindle, where it nucleates the actin cap, a thick cortical layer of actin (Deng *et al.*, 2007).

The oocyte is surrounded by a protective glycoprotein layer called the zona pellucida that the sperm must penetrate (Wassarman and Litscher, 2008). Fertilisation triggers exit from MII arrest (Clift and Schuh, 2013). At fertilisation, phospholipase C zeta (PLCζ) is released into the oocyte cytoplasm from the sperm. This triggers calcium release from endoplasmic reticulum (ER) stores (Saunders *et al.*, 2002), resulting in the generation of calcium oscillations that continue for a number of hours and leading to a series of events termed 'oocyte activation' (Miyazaki *et al.*, 1986), including anaphase of meiosis II when dyad chromosomes are resolved to their constituent chromatids, which either remain in the oocyte or are expelled into the second polar body.

Fertilisation also induces remodelling of the oocyte cytoskeleton, reflecting changes occurring within the oocyte when meiosis is completed and the first mitotic cycle begins. These include disassembly of the MII spindle and microtubule-dependent positioning of the pronuclei at the centre of the zygote to facilitate symmetric cell division during the 1st mitosis. The centrosome present in the zygote originates from the sperm, and is therefore associated with the male pronucleus (Reinsch and Gonczy, 1998). A large microtubule aster from the centrosome associates with the female pronucleus, allowing it to move towards the male pronucleus by a dynein dependent mechanism (Reinsch and Karsenti, 1997; Deng *et al.*, 2007; Wuhr *et al.*, 2009). The first mitotic spindle forms centrally in the zygote following nuclear envelope breakdown. Actin filaments in the zygote are enriched at the cortex, providing mechanical strength and prepared for the first mitotic division.

Oocyte activation also triggers a polyspermy block, for which three mechanisms have been discovered in mouse oocytes. The first two mechanisms to block polyspermy are not well characterised, but are thought to occur very quickly after fertilisation to prevent more than one

sperm fusing with the oocyte membrane. The receptor for sperm cell surface protein Izumo1 has been identified as folate receptor 4 (Juno); the interaction between Izumo1 and Juno is shown to be conserved in many mammalian species (Bianchi *et al.*, 2014). It has been suggested Juno is involved in the polyspermy block, as it is highly expressed on the membrane of unfertilised eggs but not detected approximately 30 minutes after fertilisation (Bianchi and Wright, 2014). The authors found that Juno was present within vesicles in the peri-vitelline space, and suggest that these vesicles act as rapid sperm-blocking agents (Bianchi and Wright, 2014). The third mechanism prevents sperm binding the zona pellucida surface, and occurs over a number of hours involving cortical granule exocytosis (Barros and Yanagimachi, 1971). Ovastacin is released from cortical granules after fertilisation and cleaves ZP2, which subsequently prevents sperm binding to the zona pellucida (Burkart *et al.*, 2012). The incidence of polyspermy is quite high following IVF techniques (Ho *et al.*, 1994). Polyspermy is generally defined by the presence of >2 pronuclei and precludes the use of embryos from fertility treatments, as there is a high risk of triploidy which may cause severe birth defects and miscarriage (Feenan and Herbert, 2006). On the other hand, zygotes with only a single pronucleus are also observed following IVF, this could arise due to parthenogenetic activation, or due to the maternal and paternal genomes becoming surrounded by a single pronuclear membrane. Alternatively, the second pronucleus may have failed to form (Feenan and Herbert, 2006).

1.2.2 Female age-related segregation errors

Chromosome segregation errors occurring during female meiosis contribute to the majority of meiotic errors that are detected in human pregnancies (Hassold and Hunt, 2001). Although most of these errors are not compatible with embryonic development and result in failed implantation, some can progress to later stages. This includes trisomy 21, the most common trisomy, which results in Down's syndrome. It is known that the risk of trisomy 21 increases in pregnancies to females over ~35 year of age (Nagaoka *et al.*, 2012). As the number of women delaying motherhood is increasing in recent years, incidence of trisomy 21 has also increased (Morris and Alberman, 2009; Loane *et al.*, 2013). The increased incidence of chromosomal segregation errors in older women contributes to the age-related decline in fertility. Oocytes produced during foetal life are arrested at prophase I until they are recruited for growth and the hormonal surge triggers meiotic progression and ovulation. Thus, oocytes ovulated by perimenopausal females have been arrested for a number of decades, and work in mice indicates that the cohesin complexes which stabilise bivalent chromosomes, become depleted during this time (Ballesteros-Meija, unpublished). Age-related loss of cohesin is

associated with disruption of the bivalent structure and an increased incidence of chromosome segregation errors, notably premature separation of chromatids, in mouse oocytes (Chiang *et al.*, 2010; Lister *et al.*, 2010). Consistent with this, numerous studies have reported a strong positive correlation between female age and premature loss of centromeric cohesion leading to single chromatids in MII-arrested human oocytes (Herbert *et al.*, 2015).

1.2.3 Inheritance of mitochondrial DNA (mtDNA)

Mitochondria are unique as they contain their own DNA remaining from their bacterial origins. However, traditional Mendelian inheritance is not followed. Unlike nuclear DNA, which is inherited from both parents, mtDNA is strictly maternally inherited. The occurrence and mechanism of paternal mtDNA destruction has been debated, but a recent study performed extreme-high depth mtDNA re-sequencing and results excluded the possibility of a dilution effect and suggests that an active mechanism at the molecular level ensures the elimination of paternal mtDNA (Pyle *et al.*, 2015). Destruction of paternal mitochondria has been shown to occur by ubiquitination and autophagy (Al Rawi *et al.*, 2011; Sato and Sato, 2011). However, further research is required in order to characterise the molecular mechanisms underlying elimination of paternal mtDNA.

During oogenesis, mtDNA is transmitted from PGCs to oogonia during multiple rounds of mitotic cell division and subsequently to the primordial-stage oocyte. Oocyte growth and maturation is associated with rapid replication of mtDNA to form the founder population for the developing embryo and transmission to the next generation (Figure 1.4).

The mtDNA genetic bottleneck hypothesis explains the occurrence of differing levels of heteroplasmy between a mother and her offspring, which makes it impossible to predict the risk of disease in children born to women heteroplasmic for a pathogenic mtDNA mutation. The hypothesis was proposed following early research that found *de novo* heteroplasmic mutation in Holstein cows could persist and become homoplasmic within three generations (Upholt and Dawid, 1977; Olivo *et al.*, 1983). Further research in the mouse has provided support for the bottleneck hypothesis, and although it is poorly understood, three models for the bottleneck have been proposed. Firstly, it is suggested that mtDNA copy is dramatically reduced before PGC expansion, which is followed by unequal segregation of mutated and wildtype genotypes causing shifts in oocyte heteroplasmy (Cree *et al.*, 2008). Thus, due to sampling effects, some oocytes will have a higher level of mutated mtDNA than others (Figure 1.3), which results in variable levels of mutated mtDNA between the mother and offspring. Secondly, nucleoids may be homoplasmic therefore reducing the number of

segregating units and accelerating genetic drift (Cao *et al.*, 2007; Cao *et al.*, 2009). Finally, there may be replication of a subpopulation of mitochondrial genomes during oocyte maturation (Wai *et al.*, 2008). In any case, the bottleneck hypothesis suggest that the heteroplasmy level within individual oocytes that will transmit the mitochondrial genome to the next generation is determined before birth.

1.2.4 Summary of inheritance

To summarise, meiosis is a reductive cell division that enables offspring to inherit nuclear DNA from both parents. Conversely, mtDNA is strictly maternally inherited as mtDNA from the sperm is eliminated. Thus, the mitochondria present in the oocyte represent the founder population for mitochondria in the developing embryo.

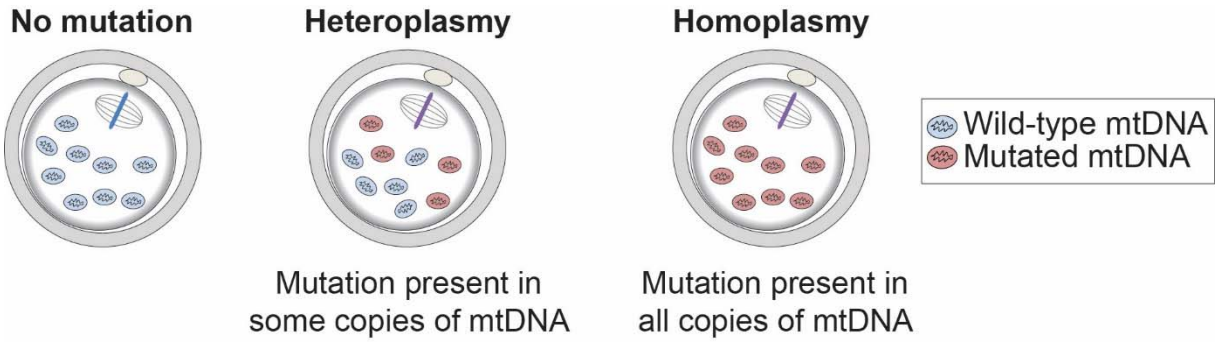


Figure 1.3: Heteroplasmy in MII oocytes. The mtDNA genetic bottleneck results in females carrying pathogenic mtDNA mutations producing oocytes containing variable levels of mtDNA mutations.

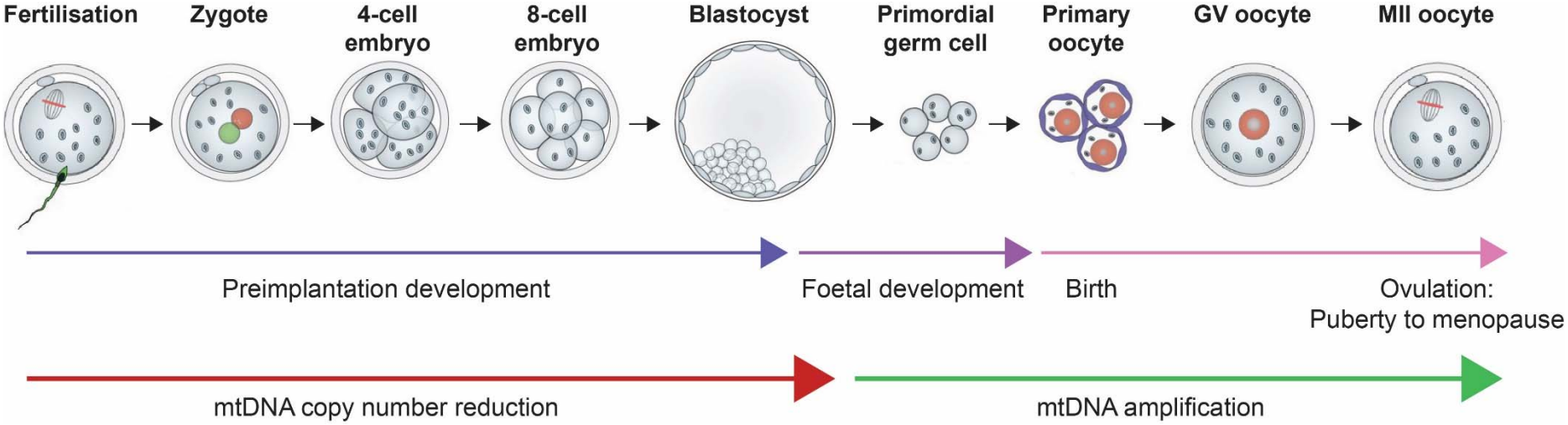


Figure 1.4: Human preimplantation development and oogenesis. Human preimplantation development is associated with a reduction in mtDNA copy number, as the oocyte contains the founder population of mitochondria for the developing embryo, and mtDNA is segregated between blastomeres during each embryonic division. Oocyte maturation is associated with rapid replication of mtDNA, producing oocytes containing more than 100,000 copies of mtDNA at the time of ovulation. This figure is adapted from an image by Mahdi Lamb.

1.3 Preimplantation development

Following fertilisation, the oocyte transitions from meiosis to mitosis. Paternal chromatin is rearranged; the protamines around which sperm DNA is packaged are exchanged for histones, and haploid maternal and paternal DNA becomes enclosed in pronuclear membranes (Clift and Schuh, 2013). This 1-cell embryo is commonly known as the zygote. It is within the separate pronuclei that maternal and paternal DNA is replicated before the pronuclear membranes break down and maternal and paternal chromosomes align on the first mitotic spindle, primed for the first mitotic division to produce a 2-cell embryo (Clift and Schuh, 2013).

The zygote is not transcriptionally active, so relies on maternal mRNA transcripts and proteins present in the oocyte cytoplasm for initial development. The transition from maternal to embryonic control of gene expression is known as embryonic genome activation (EGA). During this transition, maternal transcripts must be degraded; it has been discovered that at the time of fertilisation, transcripts required for meiotic processes are quickly degraded while those important for embryonic development are not, however regulation of transcript degradation is unknown (Alizadeh *et al.*, 2005). EGA occurs at different times in mouse and human embryos. In mouse embryos, EGA is thought to occur from the 1- to 2-cell stage (Aoki *et al.*, 1997; Hamatani *et al.*, 2004). Whereas in human embryos, EGA occurs at the 4- to 8-cell stage (Braude *et al.*, 1988). Further characterisation of the EGA in human embryos revealed that the EGA is independent of cell number, but always occurs on day 3 of development (Dobson *et al.*, 2004). Additionally, although the majority of mRNAs were targeted for destruction before day 3, a number of mRNAs appeared to increase in quantity; it is suggested that this represents preferentially stable mRNAs within the pool of maternal mRNAs targeted for degradation, or minor transcriptional activity (Dobson *et al.*, 2004; Niakan *et al.*, 2012). It has been suggested that the different timings of EGA in mouse and human embryos may indicate different roles of maternal and zygotic transcripts in the mouse and human (Cockburn and Rossant, 2010).

During early human development, successive cell divisions occur and usually at the 16-cell stage, cells begin to compact forming a solid ball of cells known as the morula. Compaction is associated with membrane flattening, adherens and tight-junction formation (Hardy and Handyside, 1996; Fleming *et al.*, 2001; Bloor *et al.*, 2002), and blastomeres begin to show polarity involving intracellular reorganisation (Cockburn and Rossant, 2010). When the morula is formed, the embryo is capable of metabolic homeostasis as gap junctions permit

passage of ions and small non-electrolytes including glucose and signalling molecules (Brison *et al.*, 2014).

The blastocoel fluid filled cavity begins to form in the 32-cell morula (Cockburn and Rossant, 2010; Clift and Schuh, 2013), which involves transport of Na^+ and subsequent osmotic fluid accumulation through the action of Na^+/K^+ ATPases and aquaporins (Watson and Barcroft, 2001). Blastulation of human embryos *in vitro* normally occurs at 5 days post fertilisation (Cockburn and Rossant, 2010), blastocyst development is characterised by expansion of the blastocoel cavity. Cells are allocated to the trophectoderm (TE) or inner cell mass (ICM) depending on their location within the embryo. The outer layer of embryonic cells is allocated to the TE, which will become the extra-embryonic tissue whereas ICM cells will become the foetus and the yolk sac. Cavity expansion and embryo growth produces the late blastocyst approximately 6 days post fertilisation (Cockburn and Rossant, 2010). When the blastocyst is ready to implant, it hatches out of the zona pellucida by a process that is not well understood.

The events of preimplantation development are similar across mammalian species. Therefore, animal models are a useful tool in the study of human preimplantation development. However, there are differences between species in the expression of lineage specific genes (Niakan and Eggan, 2013). Expression of lineage specific genes has been well characterised in the mouse. In the mouse, the Hippo signalling pathway results in Cdx2 upregulation in the outer morula cells which form the TE of the blastocyst (Nishioka *et al.*, 2009; Sasaki, 2010). Tead proteins control Hippo signalling by transcription mediation to regulate cell proliferation (Ota and Sasaki, 2008). The inner cells form the ICM, with upregulation of the transcription factors OCT4, NANOG and SOX2 (Palmieri *et al.*, 1994; Avilion *et al.*, 2003; Mitsui *et al.*, 2003). Subsequently the ICM undergoes further specification into the epiblast (EPI) and primitive endoderm (PE) lineages. This process is less well understood; it has been shown to be driven by differential FGF signalling in the mouse (Guo *et al.*, 2010) although this is not required for ICM segregation in human (Roode *et al.*, 2012). It is also known that the transcription factors GATA4, GATA6 and SOX17 are involved in the specification of the PE lineage (Morrisey *et al.*, 1998; Koutsourakis *et al.*, 1999; Niakan *et al.*, 2010), whereas NANOG is expressed in EPI cells (Niakan and Eggan, 2013).

More recently, single-cell RNA-sequencing (scRNA-seq) technology has been utilised to perform detailed studies on gene expression and lineage-specification in mammalian blastocysts (Xue *et al.*, 2013; Yan *et al.*, 2013; Piras *et al.*, 2014; Petropoulos *et al.*, 2016). It has been shown that developmental stages from the oocyte to morula are characterised by

distinct gene expression profiles, suggesting stepwise transcriptional changes in numerous pathways including the gene regulation and metabolism, which are largely conserved between mouse and human embryos (Xue *et al.*, 2013). Consistent with previous reports (Chazaud *et al.*, 2006; Plusa *et al.*, 2008; Roode *et al.*, 2012; Niakan and Eggan, 2013), a recent scRNA-seq study using 88 human blastocysts identified a period of co-expression of lineage-associated genes prior to lineage establishment (Petropoulos *et al.*, 2016). The similarities and differences between lineage-specification in the mouse and human have been highlighted in a recent publication using scRNA-seq (Blakeley *et al.*, 2015). For example, the expression of NANOG, FOXA2 and CDX2 was found to be restricted to the EPI, PE and TE lineages respectively in both mouse and human blastocysts (Blakeley *et al.*, 2015).

Mitochondria play an important role in preimplantation development, with mitochondrial dysfunction compromising developmental success (reviewed in (Dumollard *et al.* (2007)). The 'quiet embryo hypothesis' (Leese, 2002) and a 'Goldilocks zone' (Leese *et al.*, 2016) of optimal metabolic activity compatible with embryo viability have been described. This suggests that embryos with an overly 'active' metabolism may have increased metabolic processes linked to stress and DNA damage, which could cause increased levels of Reactive Oxygen Species (ROS) and have a negative effect on embryonic development (Leese *et al.*, 2008). Despite a brief period of mtDNA replication that has been suggested to occur immediately post-fertilisation (McConnell and Petrie, 2004), it is generally thought that there is no replication of mtDNA during preimplantation development. According to current knowledge, mtDNA is maternally inherited from the oocyte and subsequently segregated between daughter cells during each embryonic division. It has been proposed that an increased number of mitochondria are present in the TE compared to the ICM, as the TE is responsible for high energy consumption and ATP production and increased MitoTracker staining was observed in the TE (Houghton, 2006). A specific aim of my project is to investigate gene expression patterns in unmanipulated control and pronuclear transfer (PNT) blastocysts, including mitochondrial gene expression, using scRNA-seq.

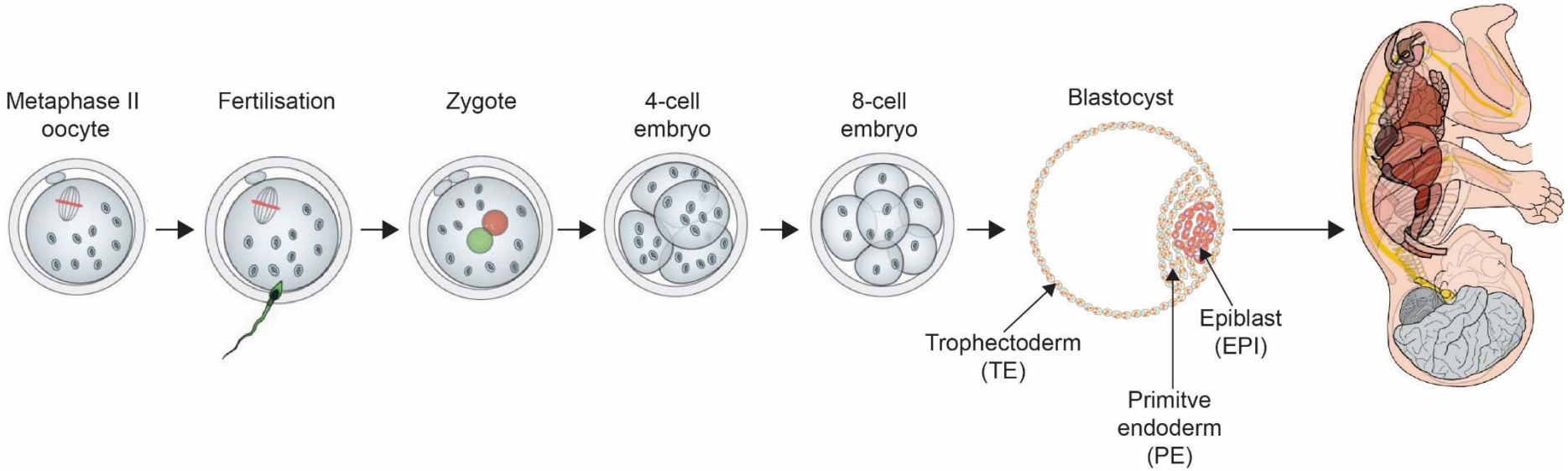


Figure 1.5: Human preimplantation development. Schematic showing human preimplantation development from fertilisation of the MII oocyte to blastocyst formation and foetal development. The trophectoderm (TE) forms the placenta, whereas the inner cell mass is composed of the epiblast (EPI) and primitive endoderm (PE) lineages, which become the foetus and yolk sac, respectively. This figure is adapted from an image by Mahdi Lamb.

1.4 Current reproductive options for females carrying mtDNA mutations

The mtDNA genetic bottleneck results in the production of oocytes with varying levels of heteroplasmy (Figure 1.4). This means that it is not possible to predict the level of pathogenic mtDNA mutation that may be transmitted from mother to child. Furthermore, even if the mutation is transmitted, the threshold effect makes it difficult to predict whether the child would develop symptoms of mitochondrial disease. Therefore, genetic counselling for patients is essential. There are several reproductive options currently available to women carrying mtDNA mutations. However, there are a number of limitations and disadvantages associated with these options.

1.4.1 Oocyte donation

Females producing oocytes that contain high levels of mutated mtDNA or that are homoplasmic for the mtDNA mutation may consider using donated oocytes containing healthy mtDNA. This approach would involve fertilising donated oocytes using the partner's sperm by IVF; embryos developing following IVF can be selected for transfer to the uterus of the affected female. Although this would ensure that the baby would not be affected by mtDNA disease, it of course means that the nuclear DNA would come from the donor oocyte so the baby would not be genetically related to the mother. Therefore, some potential mothers may not consider this as an option.

1.4.2 Prenatal diagnosis

Prenatal diagnosis involving chorionic villus sampling or amniocentesis is most commonly used for detection of chromosomal abnormalities, though there are also reports of the use of this method for mtDNA mutations (Harding *et al.*, 1992; White *et al.*, 1999; Leshinsky-Silver *et al.*, 2003; Jacobs *et al.*, 2005). However, there are concerns that mtDNA mutation load of the prenatal sample would provide an accurate indication of the mutation load in other foetal tissues, as it is known that mtDNA mutations can show tissue-specific differences in heteroplasmy levels. Furthermore, mtDNA mutations may segregate unequally between extra-embryonic and embryonic tissue, thus the mutation load in extra embryonic tissue may not correlate to the mutation load of the child (Harding *et al.*, 1992). Moreover, it is difficult to relate the sampled mutation load to potential clinical outcome. This can result in extremely hard decisions for the parents regarding the pregnancy. Finally, there is a risk of miscarriage associated with this procedure and this method is not useful for couples that would not consider terminating a pregnancy.

1.4.3 Preimplantation genetic diagnosis (PGD)

Preimplantation genetic diagnosis (PGD) is an established technique used to prevent transmission of mutations in nuclear DNA. The procedure involves testing embryos at the 6- to 8-cell stage by removing 1 or more cells for genetic analysis. Embryos that are found to not be affected by the mutation in nuclear DNA can then be selected for transfer to the uterus. PGD has also been applied to reduce the risk of mtDNA disease transmission. However, use of PGD for mtDNA disease is complicated by the need to define heteroplasmy thresholds, which is difficult due to the variability between mutations (Hellebrekers *et al.*, 2012a). This makes clinical decisions regarding which embryos should be transferred difficult. Furthermore, the question of whether samples are representative of the whole embryo is also important for the success of PGD for reducing the risk of mtDNA disease transmission.

A number of strategies could be applied in order to predict embryonic mtDNA mutation loads. It has been proposed that polar bodies, the by-products of female meiosis, may provide an estimation of the mutation load in the oocyte (Dean *et al.*, 2003; Sato *et al.*, 2005). However, studies in mouse (Neupane *et al.*, 2014) and human (Gigarel *et al.*, 2011; Vandewoestyne *et al.*, 2012) have shown that polar bodies do not provide a reliable approximation of mutation load. This may be caused by the highly asymmetric segregation of mitochondria during female meiosis (Dalton and Carroll, 2013). The most common approach is to remove cells from the 6- to 8-cell stage embryo for mutation load testing and the evidence to date indicates low variability in heteroplasmy levels between blastomeres from human cleavage-stage embryos (Steffann *et al.*, 2006; Tajima *et al.*, 2007; Treff *et al.*, 2012; Sallevelt *et al.*, 2013).

Sallevelt *et al.* investigated the use of PGD in mtDNA disorders by analysing mutation load in oocytes, zygotes and embryos of 4 patients using PCR (Sallevelt *et al.*, 2013). Of these patients, 3 carried m.3243A>G which causes MELAS, and 1 patient carried m.8993T>G which is known to cause Leigh's syndrome. The study found that in most cases the mutation load measured in blastomeres was representative of the mutation load in the whole embryo (Sallevelt *et al.*, 2013), suggesting that PGD is a useful option for mtDNA patients. However, the sample size in the study was small; including only 4 patients and 2 different mtDNA mutations. Nevertheless, PGD has been successfully used to reduce the risk of MELAS transmission following biopsy of trophectoderm cells from the blastocyst (Treff *et al.*, 2012), suggesting that trophectoderm biopsy may be representative of the whole blastocyst. However there are conflicting reports on the mutation load of a child following the use of this method

(Treff *et al.*, 2012; Mitalipov *et al.*, 2014), which could be caused by differences in the assays used for mtDNA measurement.

The segregation of mtDNA during embryonic development is studied experimentally using embryos produced by females with heteroplasmic mtDNA mutations or by artificially inducing heteroplasmy. Research has indicated that variant mtDNA inherited through the germ line segregates more uniformly between blastomeres than variant mtDNA introduced by karyoplast or cytoplast fusion with a fertilised oocyte (Meirelles and Smith, 1998), with wide variation occurring between blastomeres following cytoplast fusion. Consistent with this, recent work in which heteroplasmy was induced by fusing MII oocytes from different macaque strains resulted in wide variation between blastomeres of the 8-cell embryo (Lee *et al.*, 2012). The authors proposed the occurrence of an mtDNA genetic bottleneck during preimplantation development, and suggest that their findings cast doubt on the reliability of PGD in preventing inheritance of mtDNA mutations (Lee *et al.*, 2012). However, based on the earlier work from Meirelles and Smith, together with the close correlation in mtDNA mutation load between biopsied blastomeres and those remaining in the embryo (Steffann *et al.*, 2014), experimental systems involving artificial induction of heteroplasmy may be of little relevance to our understanding of how inherited mutations in mtDNA segregate during early human development (Steffann *et al.*, 2014). A response to the Steffann *et al.* publication by Mitalipov *et al.* highlights the occurrence of mtDNA disease in 1 of the 4 reported PGD cases (Mitalipov *et al.*, 2014). The authors emphasise the unpredictable nature of mtDNA segregation and heteroplasmy during development and requirement for further research (Mitalipov *et al.*, 2014).

To conclude, evidence to date suggests that inherited mtDNA mutations segregate relatively evenly between blastomeres during embryonic development. Therefore, testing of blastomeres from the 6- to 8-cell embryo is likely to provide a reliable estimation of the mutation load in the whole embryo, and PGD provides a promising risk reduction strategy for the transmission of mtDNA disease (Richardson *et al.*, 2015). However, a major drawback of this technique is that it cannot be used in cases where only oocytes homoplasmic for the mtDNA mutation are produced (Dean *et al.*, 2003). Moreover, our clinical experience of PGD indicates that women who have high levels heteroplasmy may produce few, if any embryos with mutations loads below the threshold for disease (unpublished data).

1.5 Techniques to prevent the transmission of mtDNA mutations

There is currently no cure for mitochondrial disease, and some mutations present at high mutation loads can result in severe symptoms and possibly cause death soon after birth. Due to the unpredictable nature of mtDNA transmission and mitochondrial disease development, women carrying pathogenic mtDNA mutations are faced with difficult reproductive decisions and the current options are limited. Therefore, this is an important area of research. Using IVF based techniques involving nuclear genome transplantation, it may be possible enable women who carry mtDNA mutations to have a genetically related child with a greatly reduced chance of transmitting disease. This could be achieved by transferring the nuclear genetic material from an oocyte/zygote containing mutated mtDNA to an enucleated oocyte/zygote from a donor, containing healthy mtDNA. In this section I will discuss techniques that have been proposed to achieve this aim.

1.5.1 History of nuclear genome transplantation

The origins of nuclear genome transfer technology date back to 1938, when Hans Spemann suggested that nuclei could be transferred from advanced developmental stages to an enucleated zygote in order to study the role of DNA in differentiation (Spemann, 1938). However, it was not until the 1950s that further nuclear genome transfer experiments were reported. In 1952, Briggs and King performed the first successful nuclear transfer of a nucleus from an early tadpole embryo to an enucleated frog egg (Briggs and King, 1952). This was followed by the production of adult Xenopus following transfer of the nucleus from tadpole intestinal epithelial cell to an enucleated frog egg (Gurdon et al., 1958). However, the use of nuclear transfer technology in mammals was hindered by the small size of mammalian eggs. The first mammalian embryo was created by nuclear transfer in 1975 (Bromhall, 1975), in which a glass pipette was used to transfer the nucleus of a cell from a rabbit embryo into an enucleated rabbit egg, which successfully developed to the morula stage. McGrath and Solter first demonstrated the use of microsurgery to transfer pronuclei between mouse zygotes, to achieve successful development and the birth of healthy offspring (McGrath and Solter, 1983). These experiments enabled the development of techniques to prevent the transmission of mtDNA disease, described below.

1.5.2 Ooplasmic donation

An early suggested possibility for preventing the transmission of mtDNA disease from mother to child was the use of ooplasm donation, as it may have a dilution effect or reduce the effect of mtDNA mutations (Kagawa and Hayashi, 1997). This technique has been used as a

treatment in cases of infertility in which embryo development following IVF was low (Cohen *et al.*, 1998). The concept underpinning this treatment is that a defect within the ooplasm of the patient may be preventing onward development of the embryo. Therefore inserting ooplasm from a healthy donor may increase the chance of embryo development to the blastocyst stage and consequently increase the chance of subsequent successful pregnancy. In 1998, Cohen *et al*. reported the first successful pregnancies and births following injection of a small amount of donor ooplasm into the patient oocyte (Cohen *et al.*, 1998). Following this study, Barritt *et al*. investigated whether these children inherited mitochondrial genomes from both the mother and the ooplasm donor (Barritt *et al.*, 2001); mtDNA fingerprinting of two children at 9 and 14 months of age revealed the presence of a small amount of donor mtDNA in the blood. Following the use of ooplasm donation, two children were born with Turner's syndrome, which is a chromosomal abnormality (45X0) and one child was diagnosed with pervasive development disorder. Ooplasm donation is no longer available as the FDA stopped the use of the procedure pending clinical trials. The likely efficacy of ooplasmic donation for preventing mtDNA disease transmission is doubted, as it is unlikely to result in a significant shift in heteroplasmy to prevent disease, particularly in cases of high mutations loads (Taylor and Turnbull, 2005; Brown *et al.*, 2006).

1.5.3 Germinal vesicle (GV) transfer

During oocyte growth, the oocyte is arrested at prophase I and the bivalent chromosomes formed during meiotic recombination are present within a large nucleus known as the germinal vesicle (GV). It is theoretically possible to harvest immature oocytes and transfer the GV to an enucleated oocyte. However, in conventional IVF treatment oocytes are harvested at the MII stage. Therefore, this technique would require *in vitro* maturation of oocytes from the GV to the MII stage. It would also necessitate removal of cumulus cells, which are thought to be required for normal oocyte maturation (Brown *et al.*, 2006; Barrett and Albertini, 2010). In order for this technique to be successful, strategies must be put in place that compensate for the loss of the cumulus-oocyte complex (Richardson *et al.*, 2015). To date, there are no reports of successful GV transfer using human oocytes.

1.5.4 Metaphase II spindle transfer (MST)

Currently the most promising strategy for nuclear genome transfer before fertilisation is the transfer of chromosomes between oocytes arrested at MII. This is known as metaphase II spindle transfer (MST; Figure 1.6). At this stage, chromosomes are aligned on the MII spindle, prepared for anaphase II and second polar body extrusion if fertilisation occurs. As

the chromosomes are not enclosed within a nuclear membrane at this stage, transfer of the nuclear DNA presents a number of technical challenges. The standard practice for this technique is to use liquid crystal birefringence to enable visualisation of the spindle, as this cannot be achieved using conventional light microscopy (Paull *et al.*, 2013; Tachibana *et al.*, 2013; Greggains *et al.*, 2014). However, this poses a problem in cases of chromosome misalignment or scattering, such as in oocytes from older females (Battaglia *et al.*, 1996). It is not possible to use a fluorescent DNA dye such as Hoechst for chromosome visualisation, as they intercalate into DNA and also require the use of UV light for excitation, which can cause DNA damage.

Despite the technical challenges associated with MST, this technique has been successfully performed using rhesus macaque (Tachibana *et al.*, 2009) and human (Paull *et al.*, 2013; Tachibana *et al.*, 2013) oocytes. Proof of concept studies were performed with rhesus macaque oocytes (Tachibana *et al.*, 2009), and resulted in the birth of healthy monkeys with undetectable levels of mtDNA carryover. Following these positive results, subsequent studies were performed using human oocytes. The results indicate that MST allows minimal mtDNA carryover (Paull *et al.*, 2013; Tachibana *et al.*, 2013), however the authors observed a high incidence of abnormal fertilisation following MST (Tachibana *et al.*, 2013). This was surprising as it was not observed following fertilisation of MST monkey oocytes (Tachibana *et al.*, 2009). The high incidence of abnormal fertilisation was at least partially caused by premature chromatid separation following failed extrusion of the second polar body, which causes the retention of both set of chromatids within the oocytes. As this was not observed in monkey oocytes, it indicates that human oocytes may be more sensitive to premature chromatid separation. Of the oocytes which were fertilised normally, a high proportion were able to develop to the blastocyst stage (Tachibana *et al.*, 2013).

Data were not provided regarding blastocyst morphology, however the expression of pluripotency markers (Paull *et al.*, 2013; Tachibana *et al.*, 2013), metabolic profiles (Paull *et al.*, 2013) and karyotype (Tachibana *et al.*, 2013) were found to be normal in embryonic stem cells (ESCs) derived from normally fertilised MST human blastocysts. Moreover, mtDNA heteroplasmy was maintained at low levels (<1%) in ESC lines (Paull *et al.*, 2013; Tachibana *et al.*, 2013). The results indicate that MST may provide an effective approach to prevent the transmission of mtDNA disease from mother to child. However, further research is required in order to minimise the occurrence of abnormal fertilisation following MST and determine that the technique does not disrupt preimplantation development.

1.5.5 Pronuclear transfer (PNT)

Pronuclear transfer (PNT) was first reported in 1983 using mouse oocytes (McGrath and Solter, 1983). This technique involves nuclear genome transplantation after fertilisation, when the maternal and paternal nuclear genomes are present within structures known as pronuclei. The pronuclei are removed within a small volume of membrane-enclosed cytoplasm, known as a karyoplast, which is then fused with an enucleated recipient zygote (Figure 1.6). The first PNT experiments demonstrated that healthy offspring were able to be produced following PNT between zygotes from different mouse strains (McGrath and Solter, 1983).

In subsequent studies, Lawrence Smith's lab used PNT to investigate segregation of variant mtDNA during embryonic development (Meirelles and Smith, 1997; Meirelles and Smith, 1998). Although initial PNT experiments were performed in 1983 (McGrath and Solter, 1983), the use of PNT for prevention of mtDNA disease transmission was not proposed until the 1990s (Rubenstein *et al.*, 1995). Later experiments using zygotes from a mouse carrying an mtDNA rearrangement were used for PNT and it was found that the technique could effectively reduce the presence of mutant mtDNA following transfer to an enucleated zygote containing wildtype mtDNA (Sato *et al.*, 2005). In 2010, the first proof of concept studies for the use of this technique in humans were performed in our laboratory using abnormally fertilised human zygotes (Craven *et al.*, 2010). These experiments demonstrated that PNT between human zygotes was technically feasible and compatible with subsequent blastocyst development (Craven *et al.*, 2010). Moreover, following optimisation, the carryover of mtDNA during the procedure was reduced to an average of <2% (Craven *et al.*, 2010), which is well below the expected threshold for disease (Hellebrekers *et al.*, 2012b). However, because of the limited potential for onward development of abnormally fertilised eggs, meaningful tests of safety and efficacy require the technique is tested using normally fertilised human zygotes, donated specifically for research. This was a primary aim of my research project.

1.5.6 Polar body transfer

It may be possible to use polar bodies, the by-products of female meiosis, as a source of nuclear DNA. Experiments in mice demonstrated that fusion of the first polar body with enucleated, unfertilised oocytes was compatible with efficient fertilisation and blastocyst formation, and resulted in the birth of 6 pups (Wang *et al.*, 2014). Furthermore, this technique enabled minimal carryover of mtDNA during the procedure (Wang *et al.*, 2014), which may be due to the asymmetric segregation of mitochondria during meiosis (Dalton and Carroll,

2013). The authors found that heteroplasmy was undetectable in offspring following first polar body transfer, whereas the average carryover in offspring following spindle transfer and pronuclear transfer was 5.5% and 23.7%, respectively (Wang *et al.*, 2014). If first polar body transfer is found to be compatible with efficient embryo development and reduced carryover in human oocytes, it has the potential to reduce the number of oocytes requires from females carrying pathogenic mtDNA mutations in clinical treatment.

1.5.7 Technical considerations for efficient nuclear genome transplantation

In order perform nuclear genome transplantation without the need to penetrate the plasma membrane, zygotes are treated with drugs to relax the cytoskeleton facilitating manipulations. The cytoskeleton provides mechanical support for essential functions such as cell division, and is composed of three components; actin, microtubules and intermediate filaments.

Microtubules are the largest filament of the cytoskeleton, which are composed of a heterodimer of alpha- and beta-tubulin subunits which form hollow protofilaments ~25nm in diameter. Microtubules are arranged in an array extending from microtubule organising centres (MTOC), the primary MTOC is usually located close to the nucleus and is known as the centrosome. During interphase, the slow-growing, minus ends of microtubules are associated with the anchoring structure of the MTOCs, whereas the fast-growing plus ends are positioned close to the surface of the cell (Alberts *et al.*, 2015). Microtubules are highly dynamic structures that are constantly undergoing growth and shortening. This phenomenon is known as dynamic instability and contributes to the important roles of microtubules (Kirschner and Mitchison, 1986; Desai and Mitchison, 1997; Brouhard, 2015). For example, during cell division it allows microtubule reorganisation to form the spindle, which specifies the cleavage plane during cytokinesis and segregates chromosomes into daughter cells. Furthermore, the dynamic nature of microtubules allows them to quickly reorganise to alter cell shape or transport organelles to specific locations in the cell. Microtubule associated proteins (MAPs) and molecular motors are important in regulating the function of microtubules. MAPs are important for associating microtubules with each other or other filaments of the cytoskeleton. The molecular motors (kinesin and dynein) are involved in the transport of vesicles and organelles, including mitochondria, around the cell (Alberts *et al.*, 2015).

Actin filaments are also known as microfilaments, as at approximately 7nm in diameter they are the thinnest component of the cytoskeleton. Actin monomers (G-actin) polymerise to form long fibres (F-actin) which are often found below the cell cortex, providing mechanical

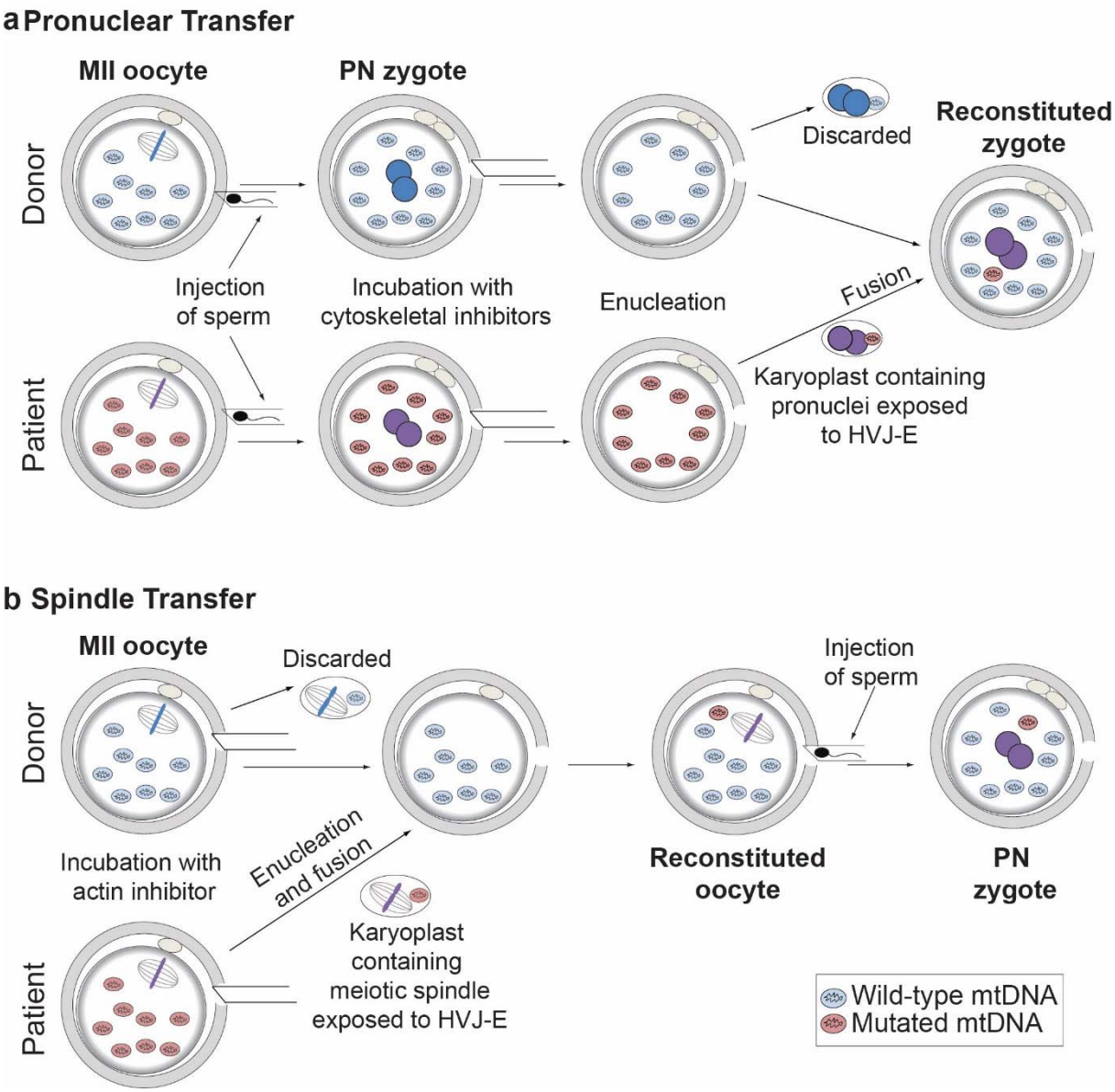


Figure 1.6: Pronuclear transfer (PNT) and metaphase II spindle transfer (MST). a) Schematic showing fertilisation of donor and patient oocytes using sperm from the patient's partner or a donor. The PNT procedure results in the production of a reconstituted zygote containing patient nuclear DNA and healthy mtDNA from a donor. **b)** Schematic showing the MST procedure resulting in the production of a reconstituted oocyte composed of patient nuclear DNA aligned on the MII spindle and healthy mtDNA from a donor. The oocyte can be fertilised using sperm from the patient's partner or a donor.

strength and playing a role in cytokinesis (Alberts *et al.*, 2015). As with microtubules, actin filaments have plus ends and minus ends; with increased growth powered by ATP occurring at the plus end. It is common for cells to have an excess of actin monomers which are not assembled into filaments, as they are bound by actin binding proteins such as profilin. The activity of profilin is controlled by stimuli; certain stimuli can prompt profilin to release G-actin, enabling F-actin formation (Lodish *et al.*, 2000). This allows regulation of the amount of G-actin and F-actin present within the cell.

Finally, there are several types of intermediate filaments which are composed of a number of different proteins. Intermediate filaments differ from actin filaments and microtubules as they are less dynamic and not polarised. These filaments have a role in maintaining the structure of the cell; including the maintenance of cell shape and anchorage of organelles.

The use of cytoskeletal inhibitors during nuclear genome transplantation improves survival, as less stress is put on the oocyte/zygote during enucleation. Cytoskeletal inhibitors also enable the nuclear genetic material to be removed more easily and helps to reduce the size of the karyoplast, thereby reducing carryover of mtDNA. Several inhibitors are available which target either the actin or microtubule cytoskeleton. Inhibitors can either promote or inhibit polymerisation, in turn disrupting cytoskeletal dynamics. For the purposes of PNT and MST, inhibitors which depolymerise the cytoskeleton are appropriate. However, inhibitors of microtubule depolymerisation cannot be used during MST as this will result in disruption of the MII spindle. Research investigating MST commonly uses the actin polymerisation inhibitor cytochalasin B (Tachibana *et al.*, 2009; Paull *et al.*, 2013; Tachibana *et al.*, 2013). Proof of concept studies for the use of PNT in human zygotes used the actin polymerisation inhibitor cytochalasin B alongside nocodazole, an inhibitor of microtubule polymerisation (Craven *et al.*, 2010).

In addition to cytoskeletal inhibitors, during nuclear genome transplantation manipulations inactivated hemagglutinating virus of Japan envelope (HVJ-E), also known as the Sendai virus, is used to facilitate fusion (Rocheleau and Petersen, 2001). Membrane fusion may also be facilitated by applying an electrical pulse, however this has been found to be poorly tolerated by human oocytes and zygotes (Craven *et al.*, 2010; Greggains *et al.*, 2014).

It is essential that the reagents oocyte/zygotes are exposed to during nuclear genome transplantation techniques are reversible and do not have a negative effect on subsequent preimplantation development. For example, due to the important role of actin in the extrusion of the second polar body during meiosis, it is imperative that the effects of actin inhibitors

used during MST are reversed before fertilisation. Slow reversibility of the inhibitor effects or lack of sufficient time for recovery between manipulations and fertilisation may cause abnormal fertilisation, which has been reported in a study of MST using human oocytes (Tachibana *et al.*, 2013). An aim of my research project is to investigate the effect and reversibility of cytoskeletal inhibitors used during nuclear genome transplantation.

1.6 Legal issues related to mitochondrial replacement

The introduction of novel nuclear genome transplantation techniques as clinical treatments to prevent the transmission of mtDNA disease requires changes to existing regulations governing fertility treatments in the UK and in a number of other countries (Richardson *et al.*, 2015). In the UK, amendments to the Human Fertilisation and Embryology Act in 2008 included the provision for Parliament to change the law to enable the Human Fertilisation and Embryology Authority (HFEA) to licence the use of nuclear genome transplantation techniques for prevention of mtDNA disease transmission. Following the publication of a study of PNT using abnormally fertilised human zygotes from our group (Craven *et al.*, 2010), the HFEA convened an expert panel to report on the safety and efficacy of IVF-based techniques to prevent transmission of mtDNA disease. This was followed by a number of public consultations, which were broadly supportive. In February 2015, following a debate the houses of Parliament in the UK voted in favour of approving regulations to permit the use of nuclear genome transplantation techniques, MST and PNT, to prevent the transmission of mtDNA mutations from mother to child. The regulations state that the techniques can only be used in cases to prevent the transmission of mtDNA disease, they cannot be used in general fertility treatment.

The new regulations came into force in October 2015, however the Expert Panel convened by the HFEA required further safety and efficacy tests before the HFEA is able to grant licences to fertility centres to offer these techniques in clinical treatments. The required tests were specified in the third scientific review of methods to avoid mitochondrial disease through assisted conception (HFEA, 2014). As a number of these experiments have been performed for MST (Paull *et al.*, 2013; Tachibana *et al.*, 2013), I will focus on the requirements for PNT studies, which is relevant to this thesis.

The panel recommend the minimal, critical experiment required to assess the safety of PNT is the performance of PNT using normally fertilised human oocytes and subsequent

development compared to normal ICSI fertilised human oocytes (HFEA, 2014). Proof of concept studies were performed using only abnormally fertilised human zygotes (Craven *et al.*, 2010). It was also recommended that karyotype, gene expression analysis and studies on oocyte, zygote and karyoplast vitrification be carried out (HFEA, 2014). With regards to mtDNA carryover during PNT, the panel requested studies on heteroplasmic mosaicism in human morulae and human ESCs derived from blastocysts following PNT using zygotes with different variants of mtDNA (HFEA, 2014). This would enable insights into whether subsequent amplification of carried over mtDNA occurs.

In the report, the HFEA also state that complete reassurance cannot be achieved from testing MST/PNT using animal models and human oocytes *in vitro;* therefore it should be accepted that there will be risk and unknowns associated with the use of these techniques in humans until it is tried in the clinic (HFEA, 2014). This has also been discussed in reviews into the ethical issues surrounding mitochondrial gene replacement (Bredenoord *et al.*, 2008; Bredenoord and Braude, 2010).

Currently, nuclear genome transplantation techniques to prevent the transmission of mtDNA disease are not licenced for use in the USA. However, in February 2016 a committee of experts were convened to report to the US Food and Drug Administration (FDA) regarding the use of these techniques, after the FDA received applications from groups hoping to use the techniques (Claiborne *et al.*, 2016). The report provided by the committee urges a cautious approach, and recommends the techniques should only be used in cases where there is a high risk of transmitting serious mtDNA disease from mother to child (Claiborne *et al.*, 2016). Furthermore, it was recommended that the techniques only be used in male embryos until more is known about possible effects of this technique on future generations (Claiborne *et al.*, 2016). The recommendations are currently under review by the FDA.

1.7 Testing the safety and efficiency of PNT

Proof of concept studies have shown that IVF based techniques have the potential to reduce the risk of mtDNA disease transmission by separating the inheritance of nuclear and mtDNA, allowing females carrying pathogenic mtDNA mutations to have a genetically related child unaffected by mitochondrial disease. However, before any of these techniques can be offered as a clinical treatment, preclinical studies must be performed to test whether they are safe, efficient and compatible with preimplantation development. A number of parameters can be

tested to investigate whether PNT disrupts subsequent development. The expert panel convened by the HFEA also requested the performance of specific critical experiments to investigate the safety of PNT, as discussed above (section 1.6). Of course, it must be demonstrated that the techniques can effectively reduce the risk of disease transmission by minimising the carryover of mtDNA. This is outside the scope of this thesis, but mtDNA carryover experiments following PNT are discussed in detail in our recent publication (Hyslop *et al.*, 2016).

1.7.1 Preimplantation development

As discussed previously (section 1.3), human preimplantation development involves a number of cleavage divisions, compaction and cavitation ultimately resulting in the production of a blastocyst at 5-6 days post-fertilisation. It is important to determine that PNT does not disrupt preimplantation development. At the 1-cell stage the embryo is in a dynamic state in which maternal and paternal genomes are undergoing molecular changes and events associated with the transition from gamete to embryo. It is possible that PNT could disrupt events such as DNA replication and paternal genome demethylation. Disturbance of DNA replication would likely cause developmental arrest, which would reduce PNT efficiency. The blastocyst is an important developmental milestone, as it this stage the embryo is capable of implantation and the formation of a pregnancy. If this stage is not reached due to poor survival following manipulations, developmental arrest or disruption of lineage-specification, the efficiency of PNT would be reduced as the chance of achieving a pregnancy per treatment cycle would be reduced.

It is also possible to assess the quality of human blastocysts according to a grading scheme that is used in the clinical laboratory at Newcastle Fertility Centre, which assesses quality according to blastocyst morphology (Stephenson *et al.*, 2007; Cutting *et al.*, 2008). It is known that blastocyst quality correlates closely with implantation potential (Hardarson *et al.*, 2003; Ahlstrom *et al.*, 2011), therefore this would give a good indication of whether blastocysts produced following PNT are compatible with the establishment of a pregnancy.

Thus, as specified by the expert panel convened by the HFEA (HFEA, 2014), assessing the development of normally fertilised human zygotes following PNT is an essential experiment in the investigation of the safety and efficiency of PNT. Due to the similarities in preimplantation development between mouse and human blastocysts, it may also be possible to performed experiments alongside human PNT using the mouse to further assess the effects of PNT and associated reagents and procedures.

1.7.2 Whole genome analysis

It will also be important to assess the effect of PNT on chromosome segregation, which can be performed using array-based comparative genomic hybridisation (array-CGH) to analyse the incidence of aneuploidy in samples from human blastocysts. Although it is known that chromosomal mosaicism is common in human preimplantation embryos (van Echten-Arends *et al.*, 2011), uniform aneuploidy affecting multiple chromosomes is usually not compatible with development and results in failed implantation or miscarriage. Some aneuploidies can progress to later stages, such as trisomy 21 that results in Down's syndrome, which is usually caused by an error during female meiosis. However, it is important to determine that PNT does not disrupt chromosome segregation during embryonic division.

1.7.3 Gene expression analysis

As discussed previously (section 1.3), lineage specification in human blastocysts has recently been studied in detail using scRNA-seq technologies. Therefore, we are able to use this technology to analyse the expression of lineage-associated genes in PNT blastocysts, which would give further indication as to whether cells are allocated to the ICM and TE correctly during preimplantation development following PNT. It will also be possible to investigate global and mitochondrial gene expression patterns in PNT blastocysts compared to unmanipulated controls. Disruption of events such as paternal genome demethylation occurring in the zygote around the time of PNT may cause gene expression and/or developmental abnormalities that may not be identified until after birth. Comparing gene expression patterns between unmanipulated controls and PNT blastocysts may give an indication on whether PNT has had any effect on gene expression in the embryo.

Chapter 2. Aims

The overarching aim of this project is to perform preclinical studies to investigate the safety and efficiency of PNT using normally fertilised human zygotes.

The specific aims are:

1. Investigate the effect of PNT on human preimplantation development, by assessing survival, blastocyst development, quality, cell number and incidence of aneuploidy. I will also perform additional experiments in parallel to human PNT using mouse zygotes.

2. Investigate the effect and reversibility of cytoskeletal inhibitors used during nuclear genome transplantation techniques, in order to select inhibitors that are safe for use in human PNT.

3. Analyse single-cell RNA-sequencing data from unmanipulated and PNT blastocysts to investigate whether global, lineage-associated and mitochondrial gene expression patterns in the blastocyst are disrupted following PNT.

Chapter 3. Materials and methods

3.1 Human oocytes and embryos

This project was approved by the Newcastle and North Tyneside Research Ethics Committee (REC reference 10/H0906/13) and licensed by the UK Human Fertilisation and Embryology Authority ((HFEA) licence reference R0152-5-B, project reference R0152, centre reference 0017) and the NHS Trust (R&D project 5245). Informed consent was obtained from all donors by research nurses who were not directly involved in the research or patient treatment.

3.1.1 Donated human oocytes

Oocytes were donated by females either undergoing fertility treatment as part of an 'egg sharing' programme, or non-patient donors. Compensation under the 'egg share' programme consisted of a subsidy (£1,500) from research funds towards the cost of treatment for self-funded patients, or an additional funded treatment cycle for those who did not become pregnant after NHS-funded treatment. For non-patient donors, financial compensation of £500 per donation cycle was given. This is in accordance with current HFEA guidelines on payments for donors.

Oocytes were collected by ultrasound-guided follicle aspiration. Hyaluronidase (HYASE™; Vitrolife, Sweden) was used to remove cumulus cells surrounding the oocytes. MII oocytes identified by the presence of the first polar body were fertilised using donated sperm by intracytoplasmic sperm injection (ICSI). This was performed by doctors and embryologists at Newcastle Fertility Centre.

3.1.2 Human abnormally fertilised zygotes

Human abnormally fertilised zygotes are not used in fertility treatment, therefore were available from patients at Newcastle Fertility Centre if consent was given to be used for research. Abnormally fertilised zygotes were identified by the presence of 1 pronucleus or 3 pronuclei on day 1 of the IVF/ICSI cycle.

3.2 Mouse strains

Mouse strains used in this project were CD1 and C57BL/6, housed at the Institute of Genetic Medicine, Newcastle University. Oocytes and embryos were collected from female mice between 2 and 4 months of age. Mouse work was carried out under a license issued by the Home Office (project licence number PPL707960) and according to regulations.

3.2.1 Collection of mouse oocytes

For the collection of metaphase II mouse oocytes, mice were injected with pregnant mare serum gonadotrophin (PMSG) followed 48 hours later by human chorionic gonadotrophin (hCG). Oocytes were harvested approximately 12 hours post-hCG injection. Mice were sacrificed by cervical dislocation. Ovaries and oviducts were dissected and transported to the lab in pre-warmed M2 medium (Sigma Aldrich, UK). In the lab ovaries were transferred to a dish containing pre-warmed M2 medium and insulin needles were used to release oocytes from the oviduct. Oocytes were incubated in 40µl droplets M2 medium overlaid with mineral oil (Sigma-Aldrich, UK) at 37°C until used for experiments.

In vitro matured metaphase II mouse oocytes were also used; in this case mice were not superovulated. Mice were sacrificed by cervical dislocation, ovaries dissected and transported to the lab in pre-warmed M2 medium. In the lab oocytes were released from the ovaries by puncturing follicles using insulin needles. This was performed in a dish containing M2 medium supplemented with 0.022µg/ml 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, UK), to maintain oocytes at the germinal vesicle (GV) stage. GV stage oocytes were cultured overnight in 40µl droplets of G-IVF™ (Vitrolife, Sweden) overlaid with OVOIL™ (Vitrolife, Sweden) and metaphase II stage oocytes, identified by the presence of a polar body, were used for experiments the following morning.

3.2.2 Collection of mouse zygotes

Mice were mated overnight. The following morning the female mice were checked for the presence of a copulatory plug, if present female mice were sacrificed using cervical dislocation. Ovaries and oviducts were dissected and transported to the lab in pre-warmed M2 medium. Pronuclear stage zygotes were harvested from the oviduct by gentle dissection using insulin needles. Incubation with HYASE™ (Vitrolife, Sweden) removed cumulus cells from zygotes. Zygotes were incubated in 40µl droplets of pre-warmed M2 medium overlaid with mineral oil at 37°C until used for experiments.

3.3 Cytoskeletal inhibitor treatments

Oocytes and zygotes were treated with cytoskeletal inhibitors for the purpose of testing the effect and reversibility of inhibitors used for nuclear genome transplantation, in addition to analysing subsequent blastocyst formation and cell number. Inhibitor stock solutions were prepared by dissolving the compound in dimethyl sulfoxide (DMSO; Sigma-Aldrich, UK). The final concentration used is stated below.

3.3.1 Inhibitor treatment of mouse oocytes

Oocytes were treated with either latrunculin A (Merck Millipore, UK) or cytochalasin B (Sigma-Aldrich, UK) which disrupt the actin cytoskeleton. Inhibitors were diluted in G-PGD™ (Vitolife, Sweden) supplemented with 5% HSA-SOLUTION™ (Vitrolife, Sweden), pre-warmed at 37°C. Latrunculin A was used at a final concentration of 1.25µM. Cytochalasin B was used at a final concentration of 5µg/ml. Oocytes were incubated in 5µl droplets of the inhibitor solution overlaid with mineral oil (Sigma-Aldrich, UK) for ten minutes at 37°C. Oocytes were washed in droplets of G-PGD™/HSA-SOLUTION™, then either fixed (section 3.7.1) or cultured in 100µl droplets of equilibrated G-IVF™ (Vitrolife, Sweden) overlaid with equilibrated OVOIL™ (Vitrolife, Sweden), at 37°C and 5% CO_2 until they were fixed at a set time point.

3.3.2 Inhibitor treatment of mouse zygotes and abnormally fertilised human zygotes

Mouse zygotes and abnormally fertilised human zygotes were treated with nocodazole (Merck Millipore, UK), which disrupts microtubules. Actin inhibitors compared were; latrunculin A (Merck Millipore, UK), latrunculin B (Merck Millipore, UK) and cytochalasin C (Abcam, UK). Inhibitors were diluted in G-PGD™ supplemented with 5% HSA-SOLUTION™ and sucrose (0.125µM), pre-warmed at 37°C. The following concentrations were used; nocodazole (10µg/ml), latrunculin A (2.5µM), latrunculin B (2µM) and cytochalasin C (2.5µg/ml). Zygotes were incubated in 5µl droplets of the inhibitor solution overlaid with mineral oil for ten minutes at 37°C. Zygotes were then washed in droplets of G-PGD™/HSA-SOLUTION™, then either fixed (section 3.7.1) or cultured in 100µl droplets of equilibrated G-TL™ (Vitrolife, Sweden) overlaid with equilibrated OVOIL™ (Vitrolife, Sweden), at 37°C and 5% CO_2. Zygotes were fixed at a set time points.

For the experiment in which mouse blastocyst cell number was analysed following exposure to latrunculin A and nocodazole, the previously described conditions applied but a hole was also created in the zona pellucida using a laser (Saturn Active, Research Instruments).

Zygotes were cultured and fixed if they developed to the blastocyst stage on day 4.5 (section 3.7.2).

3.4 MitoTracker® staining

Mouse zygotes from the CD1 strain were stained with MitoTracker® Red CMXRos (Invitrogen, UK) to investigate the effect of cytoskeletal inhibitors on mitochondrial distribution. Zygotes were incubated in 50µl droplets of equilibrated G-TL™ culture medium containing 100nM MitoTracker® Red CMXRos overlaid with OVOIL™, for 45 minutes at 37°C and 5% CO_2. After incubation, zygotes were washed through droplets of G-TL™ culture medium. Before live-cell confocal imaging (section 3.8), zygotes were transferred to 2µl drops of G-TL™ overlaid with mineral oil in a glass-bottom dish. Glass-bottom dishes were also prepared with 2µl drops of G-PGD™/HSA-SOLUTION™/sucrose and G-PGD™/HSA-SOLUTION™/latrunculin A (2.5µM)/nocodazole (10µg/ml). Zygotes were sequentially moved through the dishes and confocal images captured.

3.5 Pronuclear transfer (PNT)

3.5.1 Human PNT

Number of oocytes, zygotes and donors

A total of 523 donated human oocytes from 63 donors were used for human PNT experiments (Table 3.1**Table**). Of these oocytes, 382 were used immediately, 107 were vitrified at the metaphase II (MII) stage and 34 were vitrified following MII completion (2PB stage).

Types of PNT and vitrification

Human PNT was performed by Dr Louise Hyslop, an experienced embryologist, using donated human oocytes (section 3.1.1) ~8-10 hours (ePNT) or 16-20 hours (LtPNT) after fertilisation by ICSI. Three types of PNT were performed; autologous PNT involved removal and replacement of pronuclei in the same zygote, homologous PNT involved transfer of pronuclei between zygotes from the same donor and heterologous PNT involved reciprocal transfer of pronuclei between pairs of zygotes originating from fresh and vitrified oocytes from different donors. The majority of oocytes were vitrified at MII, a small number were vitrified at 2PB stage. The RapidVit™ and RapidWarm™ oocyte kits (Vitrolife, Sweden)

were used for vitrification and warming. Oocytes were stored in liquid nitrogen until used for ePNT.

	Oocytes (n)	**Donors (n)**	**Age range**	
Egg share donors	44	6	25-36 years	
Non-patient donors	479	57	21-36 years	
	Zygotes (n)	**Donors (n)**	**Control (n)**	**PNT (n)**
LtPNT	51	10	12	39
Series I ePNT	58	13	19	39
Series II ePNT	131	30	30	101
Series II ePNT (2PB)	40	13	6	34

Table 3.1: The number of oocytes, zygotes and donors for PNT.

Equipment

PNT was performed in an isolator based workstation (Vitrosafe, UK) with control of temperature, O_2 and CO_2, using an inverted microscope (TE2000-U, Nikon, Japan) fitted with micromanipulators (Integra Ti, Research Instruments, UK) and a laser objective (Saturn Active, Research Instruments, UK). Manipulations took approximately 15 minutes to complete.

Enucleation

Zygotes with two visible pronuclei were placed in droplets of enucleation medium containing the cytoskeletal inhibitors cytochalasin B (2.5µg/ml)/ latrunculin A (2.5µM) (LtPNT) or latrunculin A (2.5µM) (ePNT), and nocodazole (10µg/ml). A set of experiments were performed alongside series II ePNT using latrunculin B (0.1-2.5 µM) instead of latrunculin A in order to compare subsequent survival and embryo development. In LtPNT and the first

series of ePNT experiments enucleation was performed in G-1™ PLUS medium (Vitrolife, Sweden). For series II ePNT G-1™ PLUS was replaced by Sydney IVF Embryo Biopsy Medium (Cook Medical, USA) for enucleation procedures, which does not contain Ca^{2+} or Mg^{2+}. Enucleation was performed in the presence or absence of sucrose (0.125μM). The presence of sucrose increased osmolarity of the enucleation medium from 280 mosm l^{-1} to 449 mosm l^{-1}, inducing shrinkage of the cytoplasm. The laser objective was used to create a hole in the zona pellucida prior to insertion of the enucleation/fusion pipette with an inner diameter of 17μM. Pronuclei with a small amount of cytoplasm were aspirated into the pipette as a single or two individual karyoplasts.

Fusion

The karyoplast(s) were briefly exposed to a suspension of hemagglutinating virus of Japan envelope (HVJ-E); GenomONE™ HVJ-E (Cosmo Bio, USA). In LtPNT and the first series of ePNT experiments HVJ-E was undiluted, series II ePNT used a 1:10 dilution. Karyoplasts were expelled into the perivitelline space and allowed to fuse with the cytoplast.

Culture

Finally, ePNT and unmanipulated control zygotes in LtPNT and series I ePNT were cultured in a sequential medium; G-1™ PLUS (day 1-3) G-2™ PLUS (day 3-6; Vitrolife, Sweden). Series II ePNT zygotes were cultured in G-TL™, a single step culture medium.

Subsequent experiments on PNT blastocysts

In addition to monitoring development following PNT to analyse survival, blastocyst formation and blastocyst quality (section 3.6), a number of experiments were performed on zygotes developing to the blastocyst stage. Cells counts were obtained from LtPNT (n=6), series I ePNT (n=8) and series II ePNT (n=5) blastocysts. Multiple analyses were performed on individual blastocysts developing following series II ePNT, including aneuploidy screening and gene expression analysis (Table 3.2). Stem cell derivation and analysis of mtDNA carryover was performed on a number of blastocysts, but data is not included in this thesis.

Analysis	Group	Blastocysts (n)	Donors (n)
Aneuploidy screening	Control	11	10
	ePNT	30	20
Gene expression	Control	3	3
	ePNT	11	10

Table 3.2: Number of blastocysts used for aneuploidy screening and gene expression analysis.

3.5.2 Mouse PNT

I performed mouse PNT in collaboration with Dr Laura Irving. Mouse PNT was performed using CD1 mouse zygotes (section 3.2.2). The protocol used was the same as human series II autologous ePNT, but using an undiluted HVJ-E suspension to facilitate fusion. After manipulation, zygotes were cultured in 100µl drops of G-TL™ overlaid with OVOIL™ at 37°C and 5% CO_2. Blastocyst formation was assessed. A proportion of blastocysts were fixed and immunofluorescence labelling performed (section 3.6.1) for cell number analysis.

3.6 Human blastocyst grading

Embryo development was monitored daily throughout the duration of culture. On days 5 and 6 of development blastocysts were graded using the UK National External Quality Assessment Service (NEQAS) grading schemes for embryos and blastocysts (Cutting *et al.*, 2008). A three digit grade was assigned representing a score of: 1-6 for blastocoel expansion, 1-5 for inner cell mass morphology and 1-3 for trophectoderm morphology. This 3-digit grade was assigned to the corresponding quality category.

3.7 Immunofluorescence labelling

I performed immunofluorescence labelling of oocytes and zygotes to assess the effect and reversibility of cytoskeletal inhibitors (section 3.3), and blastocysts in order to perform cell count analysis following cytoskeletal inhibitor treatment (section 3.3) or PNT (section 3.5).

3.7.1 Whole oocyte and zygote fixation and staining

Oocytes and zygotes which were treated with cytoskeletal inhibitors (section 3.3) were fixed immediately after treatment or following a recovery period at set time points. Untreated controls and DMSO controls were also fixed. Fixation was performed using a free-oocyte fixation buffer containing 2% formaldehyde at 37°C for 1 hour. Zygotes were briefly exposed to acid Tyrode's solution to dissolve the zona pellucida before fixation. Oocytes and zygotes were stored in PBS at 4°C until immunofluorescence labelling.

Samples were permeabilised using PBTT (0.2% Triton-X and Tween in PBS) for 1 hour at room temperature on a shaking platform at 100rpm. Then a 1% milk solution was used for blocking for 1.5 hours at room temperature on a shaking platform. Samples were incubated with primary antibody solution (Table 3.3) overnight at room temperature. Following incubation with primary antibody, samples were washed (1x10 minutes, 3x15 minutes) in PBTT before incubation with the secondary antibody solution (Table 3.3) for 1.5 hours at room temperature on a shaking platform. For actin staining, Alexa Fluor® 488 phalloidin (ThermoFisher Scientific, UK) was resolved at 5% in the secondary antibody solution. Samples were washed again as before in PBTT. An additional 2 minute wash in PBS was performed before transferring samples to a 2µl droplet of VectaShield DAPI Mounting Medium (H-1200, Vector Laboratories, USA) diluted 1:5 in PBS in a glass-bottom dish.

3.7.2 Blastocyst fixation and staining

Human blastocysts were fixed on day 6 and mouse blastocysts on day 4.5 of development with 4% PFA at pH 7.4, either for one hour on ice or overnight at 4°C. Blastocysts were then stored in PBS at 4°C until immunofluorescence labelling.

The staining procedure for blastocysts was as described in section 3.7.1. However, an additional step was included after the secondary antibody washes for Click-iT® TUNEL Alexa Fluor® 647 Imaging Assay for microscopy (ThermoFisher Scientific, UK), which was performed according to manufacturer's instructions.

Primary antibody	Source	Dilution	Secondary antibody	Source	Dilution
CDX2	BioGenex CDX2-88	1:500	Goat-anti-mouse Alexa Fluor® 488 Or Donkey-anti-mouse Alexa Fluor® 555	Invitrogen A-11001 Or Invitrogen A-31570	1:800
NANOG	R&D Systems AF1997 Or Abcam Ab80892	1:200	Donkey-anti-goat Alexa Fluor® 488 Or Donkey-anti-rabbit Alexa Fluor® 488	Invitrogen A-11001 Or Invitrogen A-21206	1:800
GATA6	R&D Systems AF1700	1:200	Donkey-anti-goat Alexa Fluor® 555	Invitrogen A-21432	1:800
Alpha-tubulin	Abcam Ab52866	1:400	Goat-anti-rabbit Alexa Fluor® 555	Invitrogen A-21429	1:800

Table 3.3: Antibody source and dilutions for immunofluorescence labelling.

3.8 Confocal imaging

Oocytes/ embryos prepared as described in section 3.7 were imaged using an inverted confocal microscope (Nikon A1R, Japan) with NIS-elements image software. Sequential excitation at 405nm, 488nm, 561nm and 642nm was provided by the 405nm Cube Laser (Coherent Inc., USA), 488nm Argon Laser (Melles Griot, USA), Sapphire 561nm Laser (Coherent Inc., USA) and Red Diode 642nm Laser (Melles Griot, USA), respectively. Emission filters were as follows: DAPI (BP 425-475nm), Alexa 488 (BP 525-555nm), Alexa 555 (BP 570-620nm) and Cy5 (BP 662-737nm).

For imaging of blastocysts (human and mouse) and abnormally fertilised human zygotes, the Plan Apo VC 20x DIC N2 objective was used; images were obtained at Z-steps of ~1µm. For mouse oocytes the Plan Apo λ 40x objective was used; images were obtained at Z-steps of 1µm. For mouse zygotes the Plan Apo VC 20x DIC N2 objective was used; images were obtained at Z-steps of 0.75µm. Images with a frame size 1024x1024 pixels were captured using Nikon Elements AR software package.

Live-cell imaging of MitoTracker® stained zygotes (section 3.4) used an S Fluor 40x Oil DIC H N2 objective. Images were obtained at Z-steps of 2µm. Images with a frame size 512x512 pixels were captured using Nikon Elements AR software package. The microscope chamber was maintained at 37°C and 5% CO_2.

3.9 Image processing and analysis

ImageJ software was used for image processing and analysis. All figures and images in this thesis were produced using Adobe Illustrator and Microsoft PowerPoint.

3.10 Aneuploidy screening

Aneuploidy screening of human ePNT blastocysts was performed by Professor Dagan Wells and team at Reprogenetics and the University of Oxford. Cells obtained from the inner cell mass and/or trophectoderm of unmanipulated control and ePNT blastocysts underwent lysis and whole-genome amplification, performed using the SurePlex kit (Illumina) according to manufacturer's instructions and blind to sample origin. Subsequently, microarray-CGH analysis was performed using the previously validated 24Sure Cytochip (Illumina). After

washing and drying, microarrays were analysed using a laser scanner (InnoScan 710, Innopsys). Images were analysed using BlueFuse Multi analysis software (Illumina).

3.11 Gene expression analysis by single cell RNA-Seq

Single cell RNA-Seq (scRNA-Seq) was performed by Dr Kathy Niakan's lab at the Francis Crick Institute, London.

Blastocyst disaggregation

Blastocysts were disaggregated using an Olympus IX73 microscope, Saturn 5 laser (Research Instruments, UK) and Narishige micromanipulators (Narishige, Japan). During manipulation embryos were placed in drops of G-MOPS™ solution (Vitrolife, Sweden) overlaid with mineral oil. The ICM and polar TE were washed in calcium and magnesium free PBS (Invitrogen, UK) before 5-10 minute incubation in 0.05% trypsin/EDTA (Invitrogen, UK). Trypsin was quenched using Global Media supplemented with 5mg/ml LifeGlobal Protein Supplement and single cells isolated using a 30μm inner diameter blastomere biopsy pipette (Research Instruments, UK).

cDNA synthesis and amplification

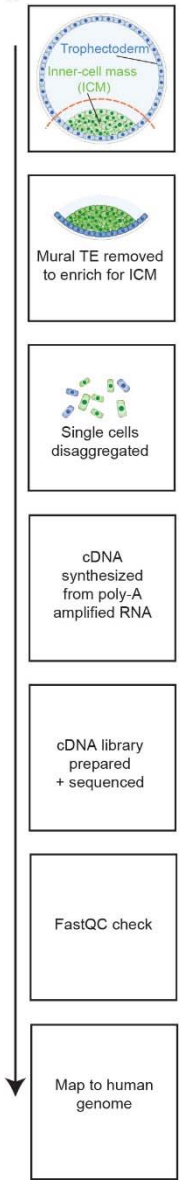
cDNA was synthesised as previously published (Blakeley *et al.*, 2015), using SMARTer Ultra Low Input RNA for Illumina Sequencing-HV kit (Clontech Laboratories, USA), according to manufacturer's guidelines. cDNA was sheared using Covaris S2 with the modified settings 10% duty, intensity 5, burst cycle 200 for 2 minutes. Libraries were prepared using Low Input Library Prep Kit (Clontech laboratories, USA) according to manufacturer's instructions. An Agilent 2100 BioAnalyser was used to assess library quality and concentration measured with a Qubit 2.0 Fluorometer (Invitrogen, UK). Libraries were submitted for 50bp paired-end sequencing using standard Illumina adapters on Illumina HiSeq 2500.

RNA-seq data analysis

Initial RNA-seq data analysis was performed by Dr Paul Blakeley, Francis Crick Institute.

The quality of the RNA-seq data was evaluated using the FastQC tool. Samples with primer contamination and amplification bias, identified by an unequal proportion of ATGC nucleotide percentages, were excluded from subsequent analysis.

a



b

			Blastocyst	Grade	Karyotype	Number passing each stage				
						Samples	cDNA	Library	FastQC	Mapping
Control					***Totals:***	***18***	***18***	***13***	***12***	***8***
			iCtr	B	Normal	6	6	6	6	5
			jCtr	B	Normal	5	5	5	5	2
			kCtr	A	Normal	7	7	2	1	1
ePNT					***Totals:***	***127***	***103***	***91***	***65***	***56***
Autologous ePNT			10PNT	B	Normal	10	10	10	3	2
			11PNT	C	Normal	14	0	0	0	0
Homologous ePNT			20PNT	B	Mosaic euploid/aneuploid	8	8	6	5	5
Heterologous ePNT	Cytoplast origin: fresh MII stage oocyte		3PNT	D	Mosaic euploid/aneuploid	11	9	7	6	5
			4PNT	B	Normal	13	13	12	11	11
			8PNT	F	Uniform aneuploid	16	16	15	8	8
			9PNT	C	Normal	13	13	12	9	9
	Cytoplast origin:	Vitrified MII stage oocyte	1PNT	E	Normal	7	7	5	5	5
			2PNT	E	Mosaic euploid/aneuploid	14	12	9	8	2
			7PNT	F	Mosaic aneuploid	3	3	3	3	2
		Vitrified post-ICSI (2PB stage)	27PNT	B	Mosaic euploid/aneuploid	10	6	6	5	5
			28PNT	B	Mosaic euploid/aneuploid	8	6	6	2	2

Figure 3.1: Experimental approach and samples included in scRNA-seq analysis of control and ePNT blastocysts. a) Diagram showing the steps involved in RNA-seq of single cells microdissected from human blastocysts. **b)** Summary table of unmanipulated control and ePNT blastocysts submitted to scRNA-seq analysis. ePNT blastocysts included those with the same nuclear and mitochondrial genomes (autologous/homologous). Homologous ePNT involved transfer of pronuclei between a zygote pair donated by two sisters. Blastocysts arising from heterologous ePNT represent new combinations of nuclear and mitochondrial genomes and are subgrouped according to cytoplast origin. The reference population of control blastocysts from previously published data (Blakeley *et al.*, 2015) is not included in this table. Blastocyst grade and karyotype is also shown.

Reads were aligned to the human genome sequence hg19 using Tophat2 (Kim *et al.*, 2013) and the number of reads mapping uniquely to each gene was counted using the program htseq-count (Anders *et al.*, 2015). Samples with percentage mapping <50% were excluded from subsequent analysis. Individual count files for each sample were normalised using the RPKM (Mortazavi *et al.*, 2008) function in the edgeR package (Robinson *et al.*, 2010) and a principal component analysis (PCA) of the top 12,000 most variably expressed genes performed blind to sample origin on all control and ePNT samples to investigate differences in global gene expression. The PCA was generated using the R package prcomp, applying both the scaling and centering options. An R script was used to perform unsupervised hierarchical clustering and generate a heatmap using the R package pheatmap. The t-SNE algorithm (van der Maaten and Hinton, 2008) was used for an alternative approach for data dimensionality reduction, and the top 5 principal components of the DESeq2 (Love *et al.*, 2014) normalised count data used for the R implementation of the t-SNE. DESeq2 was applied to read counts for control and ePNT data to identify differentially expression genes between the lineages (epiblast, primitive endoderm and trophectoderm).

I performed the following gene expression analysis using raw read count files from Dr Paul Blakeley.

Global gene expression

PCA was repeated using DESeq2 normalised counts and RPKM normalised counts grouping samples according to experimental origin (control/ePNT) and grade (blastocysts grade A-C/D-F). To create the PCAs, the R packages ggfortify and ggrepel were used and scaling and centering options applied. Outliers were analysed according to experimental origin and blastocyst grade and results plotted on a bar graph. To investigate differences in global gene expression according to morphological characteristics, differential gene expression analysis was performed using DESeq2, comparing samples from blastocysts grades A-C to grades D-F. Genes from the DESeq2 output with an adjusted P value of <0.05 were used for Gene Ontology (GO) analysis using the R package GOstats. Genes with a positive logarithmic fold change were considered upregulated and genes with a negative logarithmic fold change were considered downregulated according to the assigned group. The GOstats output for downregulated and upregulated biological process GOs are presented in tables.

Lineage-associated gene expression

Heatmap was repeated including samples from blastocysts of all grades and samples showing mixed expression of lineage associated genes analysed according to experimental origin and blastocyst grade. Differential gene expression analysis was performed using DEseq2 grouping samples by lineage. Genes from the DESeq2 output with an adjusted P value of <0.05 were used for GO analysis using the R package GOstats and results presented as a table and/or dendrogram using the hclust function in R.

Mitochondrial gene expression

PCA was performed comparing mitochondrial gene expression (nuclear and mtDNA encoded) grouping samples according to experimental origin (control/good quality ePNT) and grade (blastocysts grade A-C/D-F). The list of genes included in this analysis was downloaded from MitoCarta 2.0 (Calvo *et al.*, 2016). To create the PCAs, the R packages ggfortify and ggrepel were used and scaling and centering options applied and a t-distribution added to the plot. Heatmaps were created using the R package pheatmap. Further analysis specifically investigated expression of mtDNA encoded OXPHOS genes, using heatmap, PCA and plotting median RPKMs per sample on a graph. Expression of OXPHOS genes, both mtDNA and nuclear encoded, was analysed using heatmaps including all samples and only samples from good quality blastocysts. To investigate whether a relationship exists between lineage and mitochondrial gene expression, median mtDNA encoded OXPHOS gene expression (RPKM) was plotted by sample according to lineage. The lineage was determined using the lineage heatmap which included samples of all quality and verified using the t-SNE.

3.12 Statistical analysis

Statistical analysis was performed using Minitab, GraphPad Prism and IBM SPSS software. The statistical tests used to analyse data presented in this thesis include: chi-squared test, Fisher's exact test, one-way ANOVA with Tukey's HSD test and unpaired *t*-test. The test used is indicated in the corresponding text and/or figure legend.

Chapter 4. Results I: The effect of pronuclear transfer on preimplantation development

4.1 Introduction

4.1.1 Proof of concept

Proof of concept studies performed using abnormally fertilised human zygotes indicated that pronuclear transfer (PNT) may be a feasible option to prevent transmission of mitochondrial DNA disease (Craven *et al.*, 2010). These experiments were performed ~16-18 hours post-fertilisation, when zygotes are expected to be in the G2 phase of the first mitotic cell cycle (Balakier *et al.*, 1993; Capmany *et al.*, 1996). However, the limited developmental potential of abnormally fertilised zygotes hindered investigation of the therapeutic potential of PNT. Therefore it is essential to use normally fertilised zygotes in order to investigate the effect of PNT on preimplantation development.

4.1.2 Outcome measures

To investigate the effect of PNT on the preimplantation development of normally fertilised human zygotes we used several outcome measures. Firstly, I analysed the number of zygotes surviving the PNT procedure, enabling us to determine if modifications to the procedure are having a positive effect on survival, which would improve PNT efficiency. I also analysed the number of PNT zygotes developing to the blastocyst stage compared to unmanipulated controls. The blastocyst is composed of an inner cell mass which will form the foetus and trophectoderm layer which will become the placenta. This is an important developmental milestone which occurs on day 5-6 of development; at this stage the embryo is capable of implantation.

In clinical IVF blastocysts are selected for use in treatment based on their morphological characteristics, which are assigned a quality score. The grading scheme used at Newcastle Fertility Centre is the UK National External Quality Assessment Service (NEQAS) for embryos and blastocysts (Cutting *et al.*, 2008). Figure 4.1 shows blastocyst quality scoring criteria and grading scheme (Stephenson *et al.*, 2007), which we adapted by assigning alphabetical grades (A-F). Using clinical data I have found a strong correlation between blastocyst quality and implantation, with grade A blastocysts having a significantly higher implantation rate than blastocysts of grade B or below ($P<0.01$; Figure 4.1d). Therefore we decided that blastocyst quality should be assessed using the clinical grading scheme in order

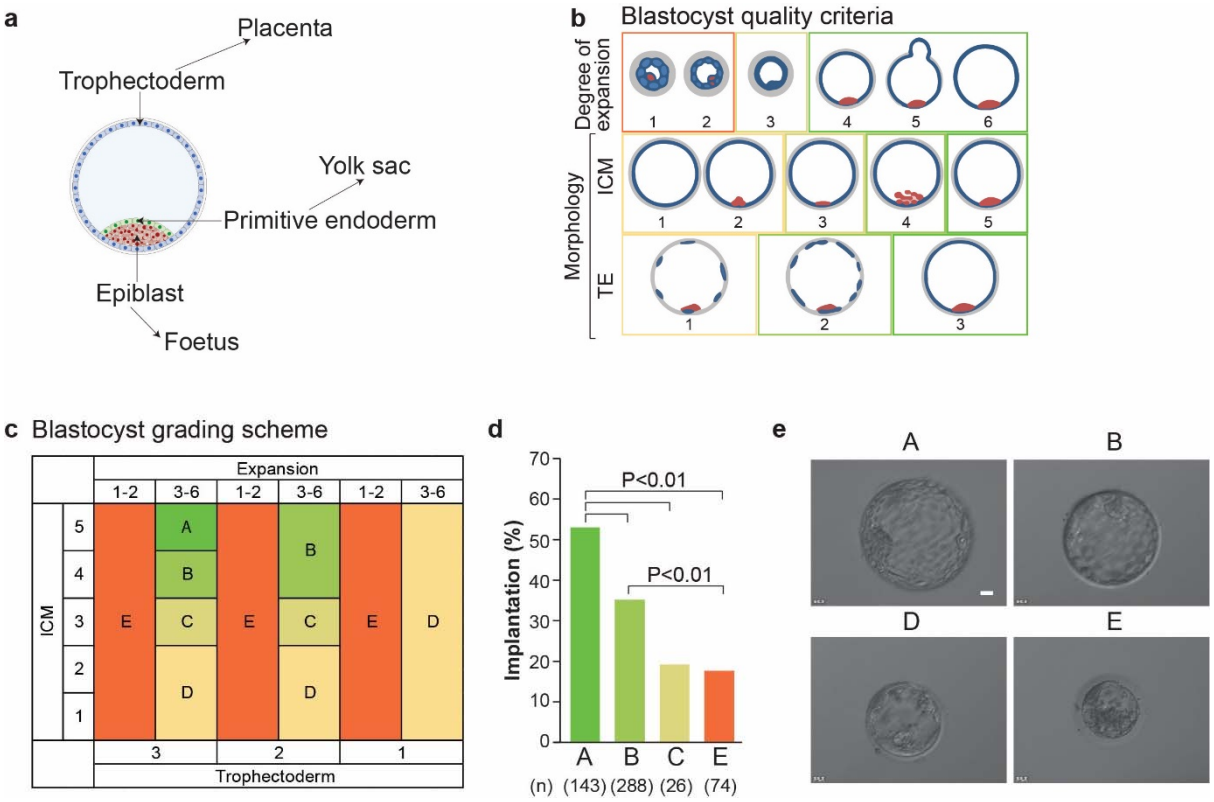


Figure 4.1: Blastocyst morphology and quality scoring criteria. a) Schematic showing cell lineages in a mammalian blastocyst: epiblast, primitive endoderm and trophectoderm. **b)** Morphological criteria and scoring system used for grading human blastocysts. This takes into account the degree of expansion (score 1: early, unexpanded blastocyst; to score 6: fully expanded), inner cell mass (ICM) morphology (score 1: absent ICM; to score 5: large, tightly packed cells) and trophectoderm (TE) morphology (score 1: few discontinuous cells; to score 3; fully formed continuous layer). **c)** Table used to assign blastocyst grades. Grade A-D is top/good quality to poor quality, grade E is early. The table does not include grade F, assigned to embryos which developed to the blastocyst stage but subsequently showed signs of degeneration. **d)** Graph showing the relationship between blastocyst grade and implantation ($P<0.01$, chi-squared test). Data was obtained from clinical IVF blastocysts replaced on day 5 of development. Grades D and F are not included as there were no cases where these were replaced. Implantation is defined as the detection of a foetal heartbeat 6 weeks post-IVF treatment. **e)** Images showing examples of blastocysts of different grades. Scale bar = 20µM.

to compare quality of unmanipulated control and PNT blastocysts. This will also give an indication of whether the blastocysts produced following PNT are compatible with implantation and therefore the establishment of a pregnancy if they were to be used in the clinic.

4.1.3 Types of pronuclear transfer experiments

The three types of PNT experiment performed are shown in Figure 4.2. Autologous PNT is a technical control in which pronuclei are removed and replaced back into the same zygote. Homologous PNT involves transfer of pronuclei between different zygotes obtained from the same oocyte donor. Finally, heterologous transfer involves transfer of pronuclei between zygotes from different oocyte donors. This results in different combinations of nuclear and mitochondrial genomes, mimicking the situation which would arise in any future clinical treatments. Due to the unpredictability in the response to ovarian stimulation, which makes it difficult to synchronise egg donors, heterologous PNT is performed between zygotes originating from fresh and vitrified oocytes.

The number of eggs donated to research is limited. In order to minimise loss of material and any learning curve effect, human PNT was performed by Dr Louise Hyslop; an experienced clinical embryologist. I analysed the human PNT data and performed additional experiments in parallel to human PNT using mouse zygotes. I also performed experiments on human blastocysts.

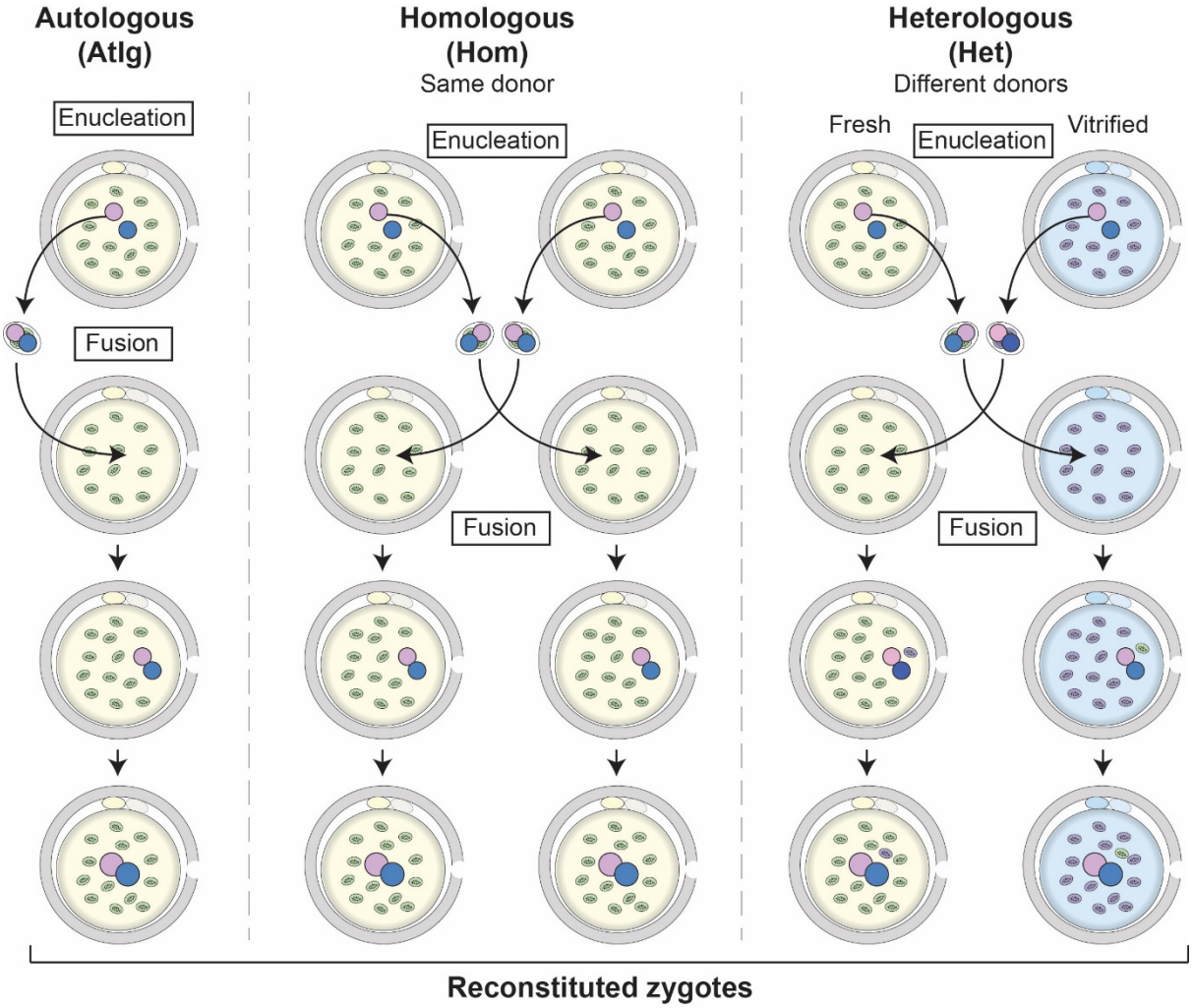


Figure 4.2: Pronuclear transfer (PNT) experiments performed. Schematic showing three types of PNT experiment performed. Autologous (Atlg) PNT involves removal and replacement of pronuclei into the same zygote. Homologous (Hom) PNT involves exchanging pronuclei between zygotes from the same donor. Heterologous (Het) PNT involves exchanging pronuclei between zygotes from different donors, from fresh and vitrified oocytes.

4.2 Initial findings with normally fertilised human zygotes

Surprisingly, techniques developed in proof of concept studies using abnormally fertilised eggs (Craven *et al.*, 2010) were not well tolerated when applied to normally fertilised human zygotes. We found that survival of reconstituted zygotes was low. Only half of the technical controls (autologous) survived PNT (Figure 4.3a). Failure to survive was generally due to lysis of the karyoplast, excessive leakage of cytoplasm, or degeneration after fusion, indicating a problem with the enucleation and fusion procedures.

Consistent with reduced survival, blastocyst formation was significantly reduced following PNT (autologous and homologous) compared to unmanipulated controls ($P<0.05$; Figure 4.3b). Calculating blastocyst formation as a percentage of zygotes that survived PNT resulted in loss of statistical significance, but blastocyst formation remains reduced compared to unmanipulated controls (Figure 4.3c). As the experimental groups involved transferring pronuclei between zygotes from the same donor, this suggests an issue with the PNT procedure is causing reduced blastocyst development. The majority of controls which did not form blastocysts arrested after the 8-cell stage. However, a high proportion of PNT embryos did not develop beyond the 4-cell stage (Figure 4.3d), indicating a detrimental effect on the earliest cell divisions.

Blastocyst formation of unmanipulated controls was 66.67% (8/12); 37.5% of these blastocysts were good quality (grades A/B). However, autologous PNT resulted in the production of only 1 blastocyst of grade D, which is poor quality (Figure 4.3e). Of the 7 blastocysts formed following homologous PNT, 1 of these was grade B (14.29%), the remainder were grade C or below. Therefore in addition to causing poor blastocyst formation, blastocysts produced following PNT are of reduced quality compared to unmanipulated controls.

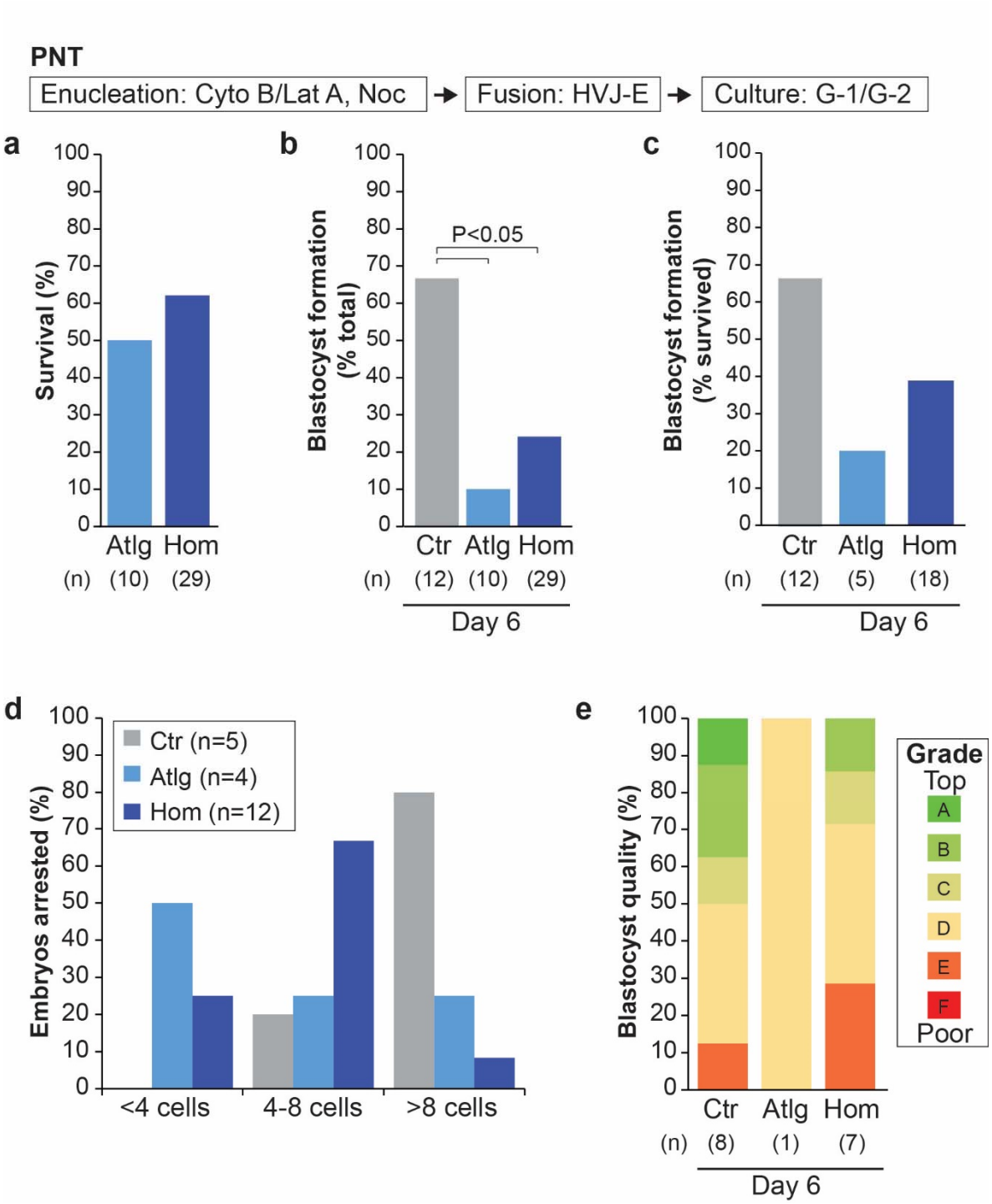


Figure 4.3: Effect of PNT on survival, blastocyst development and quality. PNT experimental conditions are shown. **a)** Survival of reconstituted zygotes following autologous (Altg) and homologous (Hom) PNT (not significant; chi-squared test). **b)** Blastocyst formation of unmanipulated controls (Ctr), autologous (Altg) and homologous (Hom) PNT as a percentage of total number of zygotes (P<0.05, chi-squared test). **c)** Blastocyst formation as a percentage of number of zygotes surviving PNT (not significant; chi-squared test). **d)** Developmental stage reached of zygotes which did not form blastocysts. **e)** Blastocyst quality according to the scoring criteria in Figure 4. (not significant; Fisher's exact test). Quality scores were assigned on day 6 of development. For statistical analysis, grades were grouped (A/B versus C-F).

One possible explanation for the poor survival and early developmental arrest following PNT in normally fertilised zygotes is that there was insufficient recovery time between the manipulations and division to the 2-cell stage. In support of this, our lab (Fenwick *et al.*, 2002) has previously reported that zygotes with the potential to develop to the blastocyst stage have a reduced interval from fertilisation to division to the 2-cell stage compared with those that do not. Moreover, only 17% of abnormally fertilised eggs develop to the blastocyst stage (Craven *et al.*, 2010) compared with over 60% of normally fertilised eggs donated to this study (Fig. 4.3b). To test the possibility that poor survival and development was due to insufficient time to recover from the manipulations before undergoing the 1st mitotic division, we conducted a series of experiments in which the pronuclei were transplanted shortly after they first appear (8 hours post-fertilisation) instead of shortly before they disappear (16-20 hours post-fertilisation). In cell cycle terms this corresponds to performing PNT during early G1 phase of the first mitotic cell cycle rather than in G2 phase (Figure 4.4). The modified technique is referred to as early pronuclear transfer (ePNT), and previously described experiments, performed ~16-18 hours post-fertilisation, are referred to as late PNT (LtPNT).

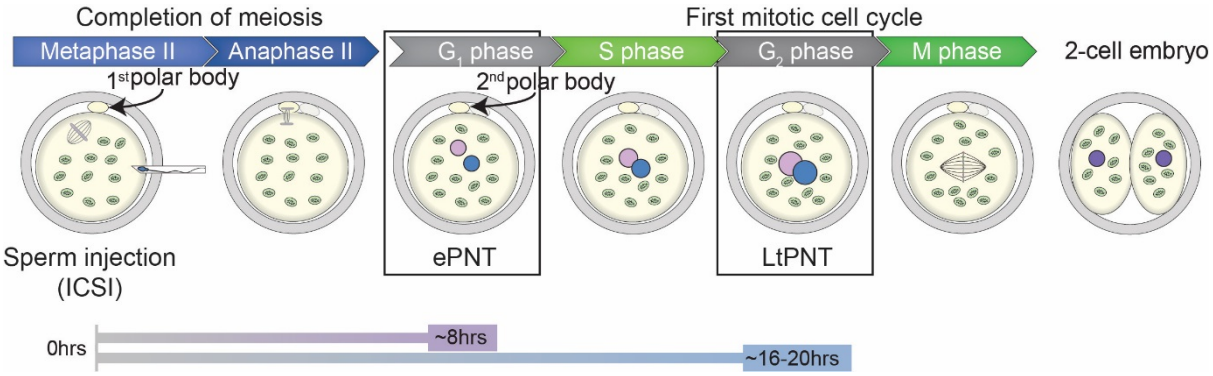


Figure 4.4: Timings of late PNT (LtPNT) and early PNT (ePNT). Schematic showing the fertilisation and progression to completion of the first mitotic cycle. Timings of PNT are shown.

4.3 Preimplantation development following ePNT

4.3.1 Modifications of experimental procedures

We hypothesised that modification to the timing of PNT may improve survival and blastocyst development as zygotes would have more time for recovery between the manipulations and the first mitotic division. A further modification, designed to reduce the risk of membrane damage, in these experiments was the addition of sucrose to the manipulation medium. Due to an osmotic effect, this shrinks the cytoplasm and facilitates enucleation and fusion by increasing the size of the peri-vitelline space.

We also investigated the possibility that the inhibitors used to disable the zygote cytoskeleton might have a detrimental effect on blastocyst formation. This topic is covered in detail in Chapter 5. For the purpose of these experiments, we used latrunculin A, which reversibly inhibits the actin cytoskeleton in human and mouse zygotes, and nocodazole, which reversibly inhibits polymerisation of beta-tubulin and thereby disables the microtubule network. Cytoskeletal inhibitors are used during PNT as they facilitate enucleation by relaxing the cytoskeleton, without inhibitors excess stress placed on the zygotes causes increase lysis and makes it difficult to control the size of the karyoplast.

4.3.2 Survival and blastocyst formation following ePNT

Analysis of the survival data revealed a significant improvement following modification to the timing of PNT; 92% of ePNT zygotes survived the procedures compared to 59% of LtPNT zygotes ($P<0.01$; Figure 4.5a). When the data are broken down to show the different experimental groups, there is a non-significant trend towards increased survival in heterologous ePNT compared with homologous and autologous ePNT (Figure 4.5b). However, this was not reflected in the blastocyst development data.

Generally blastocyst formation on day 6 of development was improved in ePNT (Figure 4.5c, d) compared to LtPNT (section 5.2, Figure 4.3). Although heterologous ePNT zygotes had the highest survival, this group had reduced percentage blastocyst formation compared to the other experimental groups. Therefore it is possible that ePNT between zygotes from different donors has a negative effect on embryo development. There is a non-significant trend towards reduced blastocyst formation of autologous ePNT embryos compared to controls on day 5 of development. However, on day 6 blastocyst formation of autologous and homologous ePNT embryos is comparable to controls (Figure 4.5c, d).

As observed in LtPNT, I found that the majority of ePNT zygotes that did not form blastocysts arrested at an earlier stage than unmanipulated controls (Figure 4.d); 60% of unmanipulated controls reached >8 cells, whereas only a small proportion of ePNT embryos from each experimental group reached this stage before arrest (autologous, 25%; homologous, 16.67%; heterologous, 40%). This suggests that ePNT could have a negative effect on the earliest cell divisions.

Overall, blastocyst formation is improved following ePNT compared to LtPNT, but the proportion of good quality blastocysts remains low (Figure 4.5e). Of the small number of blastocysts produced following heterologous ePNT, all were of poor quality (grade D). By contrast, 55.56% of blastocysts from unmanipulated controls were good quality (grades A/B) on day 6. Furthermore, we observe a delay in embryo development; a higher percentage of ePNT blastocysts are grade E (early) on days 5 and 6 compared with controls. A blastocyst which is grade E on day 5 does have the potential to implant (Figure 4.1; section 5.1.2), but implantation is unlikely for blastocysts that have not yet undergone expansion (grade E) by day 6.

To summarise, adjusting the PNT manipulations to be performed sooner after fertilisation instead of close to the onset of the first mitotic division promoted an increase in survival and blastocyst formation. However, the majority of blastocysts produced following ePNT are of poor quality, this indicated that further optimisation of the technique was required.

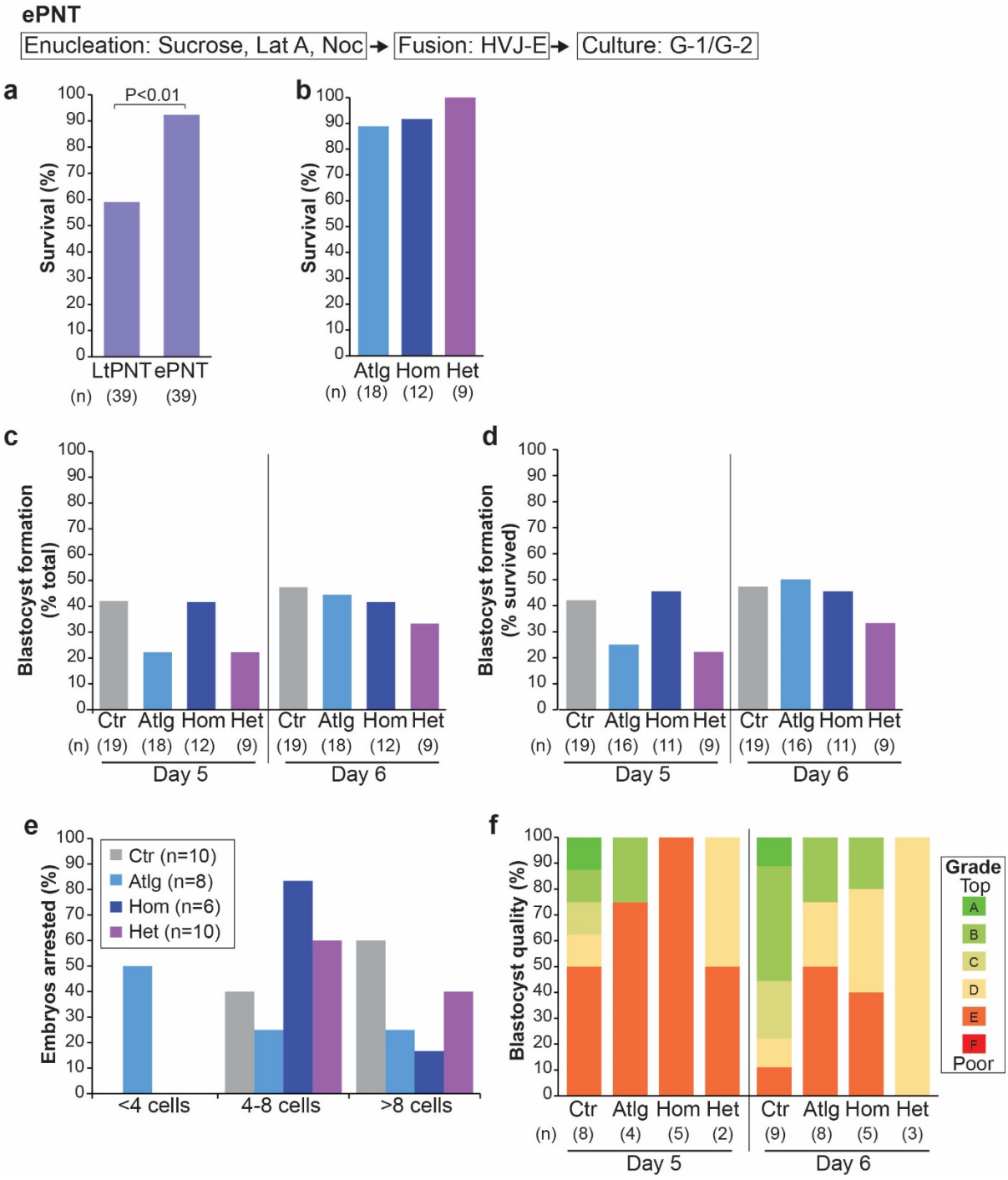


Figure 4.5: Effect of ePNT on survival, blastocyst development and quality. ePNT experimental conditions are shown. **a)** Graph showing an improvement in survival following ePNT compared to LtPNT (P<0.01; chi-squared test). **b)** Graph showing percentage survival for each experimental group; autologous (Atlg), homologous (Hom) and heterologous (Het) (not significant; chi-squared test). **c)** Blastocyst formation of unmanipulated controls (Ctr) and PNT embryos on days 5 and 6 of development as a percentage of total zygotes and **d)** **b**lastocyst formation as a percentage of number of zygotes surviving PNT (not significant; chi-squared test). **e)** Graph showing the developmental stage reached of those embryos that did not form blastocysts. **f)** Blastocyst quality (not significant; Fisher's exact test). Quality scores were assigned on days 5 and 6 of development. For statistical analysis, grades were grouped (A/B versus C-F).

4.4.3 Effect of ePNT on blastocyst cell number

To further investigate the reasons for the generally poor blastocyst quality, I asked whether cell numbers differed between unmanipulated control and PNT blastocysts. To answer this I fixed blastocysts on day 6 of development and stained with DAPI in order to perform nuclear counts using ImageJ software.

Data shown in Figure 4.6 compares cell numbers from unmanipulated controls to LtPNT and ePNT blastocysts. Staining of LtPNT and a proportion of the control blastocysts was performed by Dr Qi Zhang; in order to control for operator variability I repeated nuclear counts on saved 3D images of these blastocysts and found the results to be comparable. I discovered a reduction in the total cell number of both LtPNT ($P<0.01$) and ePNT ($P<0.05$) blastocysts compared to unmanipulated controls (Figure 4.6a).

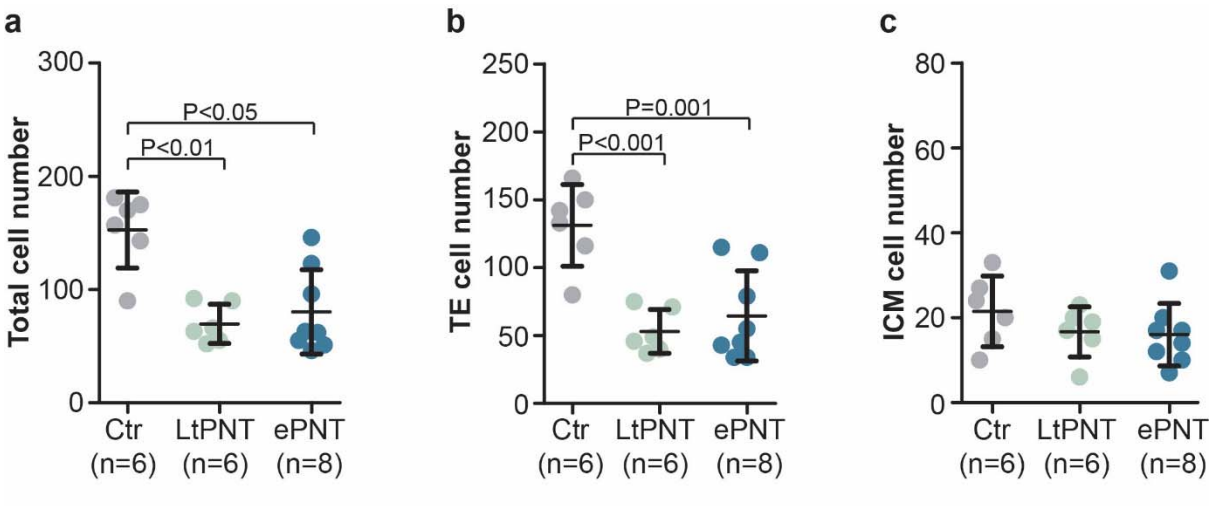


Figure 4.6: Effect of PNT on blastocyst cell number. Blastocyst cell number was assessed by nuclear counts. **a)** Total, **b)** trophectoderm (TE) and **c)** inner cell mass (ICM), cell numbers of unmanipulated controls (Ctr), LtPNT and ePNT blastocysts. Bars show the mean ± standard deviation. Statistical significance is indicated, the test used was one-way analysis of variance (ANOVA) with Tukey's HSD.

I next asked whether the reduced cell number affected the trophectoderm (TE), inner cell mass (ICM) or both. Both LtPNT and ePNT blastocysts display reduced TE cell number, which is statistically significant when compared to controls ($P\leq0.001$; Figure 4.6b). However there is no significant reduction in ICM cell number of ePNT or LtPNT blastocysts compared to controls (Figure 4.6c). These results indicate that PNT effects TE cell number but has no

effect on the ICM. It is important to identify the cause as a reduced TE cell number would decrease blastocyst implantation and therefore reduce PNT efficiency.

To determine whether the decreased cell number observed in PNT blastocysts was a consequence of increased cell death, I used Terminal Uridine Nick-End Labelling (TUNEL); an *in situ* cell death detection kit. I found that the percentage of total TUNEL positive cells was significantly increased in LtPNT (P=0.01) and ePNT (P<0.05) blastocysts compared to controls (Figure 4.7a). I asked if this increase in cell death could be occurring in the TE, which could explain the reduced trophectoderm cell number. However, both the TE and ICM show increased cell death compared to controls, and there was a slight increase in cell death in the ICM compared with the TE (Figure 4.7a).

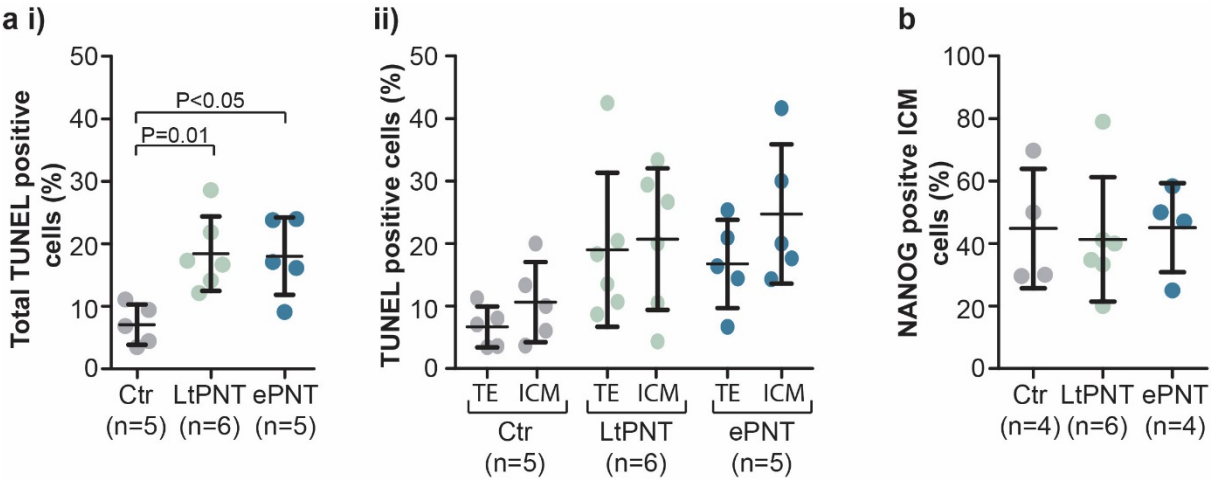


Figure 4.7: Cell death and NANOG expression in control and PNT blastocysts. a) i) The percentage of total TUNEL positive cells in control (Ctr) and PNT blastocysts. **ii)** The percentage of TUNEL positive cells in the trophectoderm (TE) and inner cell mass (ICM). **b)** Percentage of NANOG positive cells in the ICM of control and PNT blastocysts. Bars show the mean ± standard deviation. Statistical significance is indicated, the test used was one-way analysis of variance (ANOVA) with Tukey's HSD.

NANOG is a transcription factor which is upregulated in the ICM and specifies epiblast cells, also known as primitive ectoderm cells, which give rise to all foetal tissues. Analysis of the percentage of NANOG positive cells in the ICM shows no statistically significant difference between controls and LtPNT or ePNT blastocysts (Figure 4.7b). Thus, the modified ePNT procedure does not appear to affect allocation of cells to the ICM, or specification to the epiblast lineage within the ICM.

To summarise, these results indicate that survival and blastocyst formation have been improved by transferring pronuclei shortly after they first appear compared with shortly before they disappear (in the G1 vs G2 stage of the cell cycle). Moreover, ICM cell number and percentage of NANOG positive cells is consistent between control and PNT blastocysts, which suggests that the PNT procedure does not disrupt allocation of cells to the ICM. However, PNT blastocysts show reduced total cell number which is reflected by an increase in total cell death. The increase in total cell death cannot explain the reduced TE cell number, as cell death is increased in both the TE and ICM. Further optimisation of the technique is required in order to improve blastocyst quality and cell number.

4.4 Using the mouse to investigate reduced blastocyst cell number

As the number of eggs donated to research is limited, I decided to use the mouse to investigate possible causes of reduced blastocyst cell number. A mouse model would also be a useful tool for further fine tuning of the PNT technical procedures.

4.4.1 Cytoskeletal inhibitors and laser-induced hole in the zona pellucida

To investigate the possible causes of reduced blastocyst quality and cell number following human PNT, I asked whether it could be due to the removal and transfer of pronuclei or the use of cytoskeletal inhibitors and creation of a hole in the zona. In relation to cytoskeletal inhibitors, it is conceivable that residual effects of latrunculin A and nocodazole could inhibit allocation to the TE layer. Furthermore, premature hatching through the laser-induced hole in the zona pellucida could limit TE proliferation.

To test these possibilities, I harvested C57BL/6 mouse zygotes from the oviduct 12 hours after mating. To determine the effects of exposure to cytoskeletal inhibitors, I incubated zygotes with latrunculin A and nocodazole and created a laser induced hole in the zona pellucida. Control zygotes were incubated in culture medium only. In the mouse, the early blastocyst forms at day 3.5 (E3.5) of development. I fixed late blastocysts at E4.5 and performed immunofluorescence labelling and confocal imaging to obtain cell counts using ImageJ software (Figure 4.8a).

Analysis of total, TE and ICM cell number in control and treated mouse blastocysts indicates that cell number is consistent between experimental groups ($P>0.05$; Figure 4.8b). However, I did observe premature hatching caused by the creation of a hole in the zona-pellucida. This finding raises the possibility that the reduced cell number observed in PNT blastocysts could

be due to effects of the manipulation rather than the cytoskeletal inhibitors and premature hatching through the laser-induced hole in the zona pellucida. Alternatively, it is possible that the mouse is not a useful model for optimising ePNT conditions for human zygotes.

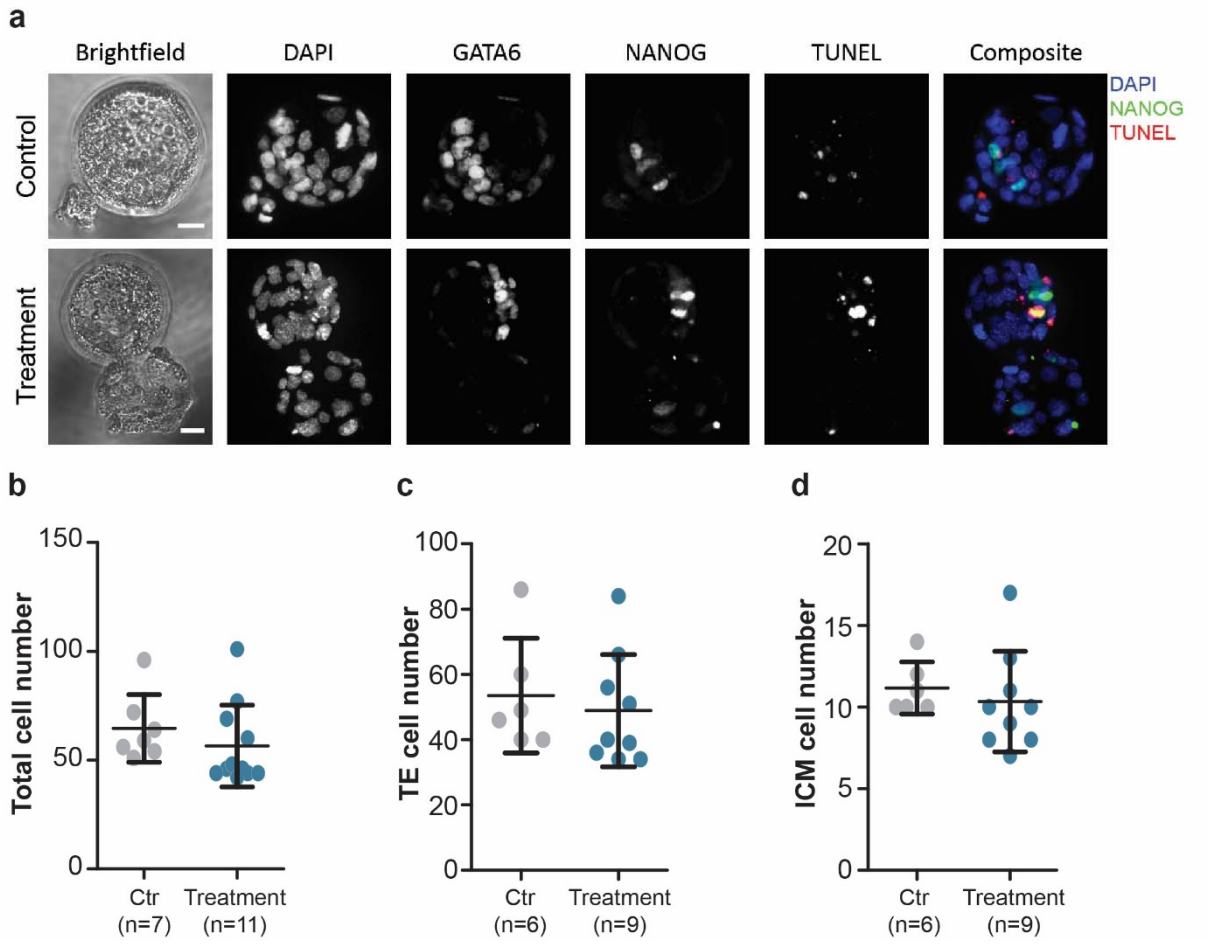


Figure 4.8: Immunofluorescence labelling and cell number analysis of mouse blastocysts exposed to cytoskeletal inhibitors and a laser-induced hole created in the zona pellucida. **a)** Examples of confocal images of control and treated zygotes which were fixed at the blastocyst stage for immunofluorescence labelling and cell number analysis. Premature hatching occurred in blastocysts from zygotes exposed to cytoskeletal inhibitors and a hole created in the zona (bottom left). Scale bar = 20µM. **b)** Total, **c)** trophectoderm (TE) and **d)** inner cell mass (ICM) cell number of control and treated blastocysts. Bars show the mean ± standard deviation. No statistical significance was found using unpaired t-test.

4.5 Series II ePNT

4.5.1 Modifications to ePNT

In order to address the problem of poor blastocyst quality following ePNT, a second series of ePNT experiments were performed using human zygotes. As in the first series, PNT was performed at ~8 hours post-fertilisation. However the following modifications to the enucleation/fusion procedures and culture conditions were introduced.

Firstly, we switched to a calcium free medium for the manipulations. When the sperm and oocyte membranes fuse, phospholipase C zeta (PLCζ) is released from the sperm into the oocyte cytoplasm, causing calcium release (Saunders *et al.*, 2002). Downstream events cause release of calcium from endoplasmic reticulum (ER) stores and generation of calcium oscillations, which continue for a number of hours leading to egg activation (Miyazaki *et al.*, 1986). Therefore, in the case of spindle transfer, calcium-free medium is used to reduce the risk of premature activation of the unfertilised eggs during the manipulations (Paull *et al.*, 2013). There is also evidence that calcium signals play a role in early embryo development, such as embryo compaction (Ducibella and Anderson, 1975; Gumbiner, 2005). Furthermore, the amplitude and frequency of the sperm-induced oscillations influences development to the blastocyst stage (Ozil *et al.*, 2006). Importantly, sperm-induced calcium oscillations continue until formation of pronuclei (Marangos *et al.*, 2003). It is therefore possible that disruption of calcium homeostasis due to influx from the external medium shortly after pronuclei formation might disrupt the downstream events triggered by sperm-induced calcium oscillations and thereby contribute to reduced blastocyst development and quality. We therefore decided to use a calcium-free manipulation medium. Based on evidence from a study on spindle transfer (Paull *et al.*, 2013), we also reduced the concentration of HVJ-E (1:10). This parameter was not tested in the mouse because, for reasons that are unclear, the reduced concentration of HVJ-E was not effective in inducing karyoplast/cytoplast fusion in mouse zygotes.

On the basis of observations that development of ePNT embryos appeared to suffer when they were transferred to the second stage of the sequential media (G-1/G-2) used in LtPNT and series I ePNT experiments, we switched to a single-step medium (G-TL), in which embryos remained undisturbed for the duration of culture. This culture medium is relatively new and was initially tested in our laboratory using mouse embryos, giving encouraging results showing increased blastocyst formation compared to when sequential medium was used (unpublished data).

4.5.2 Blastocyst development following ePNT in human zygotes

I analysed survival and blastocyst development following series II ePNT and found that under these conditions the majority of autologous (90.48%) and heterologous (82.5%) ePNT zygotes survived the procedure (Figure 4.9a).

Blastocyst formation on days 5 and 6 was comparable between unmanipulated controls and autologous ePNT embryos. However, there was a significant reduction in blastocyst formation following heterologous ePNT compared to controls on day 6 ($P<0.05$; Figure 4.9b). When blastocyst formation is calculated as a percentage of reconstituted zygotes surviving the manipulations, the statistical significance is lost but a trend towards reduced blastocyst formation following heterologous ePNT remains (Figure 4.9c). Heterologous ePNT involved transfer of pronuclei between zygotes originating from fresh oocytes and oocytes vitrified at the MII stage. Therefore, reduced blastocyst formation could be due to an effect of vitrification. Another possibility is asynchrony between zygotes, which could disrupt processes occurring in the zygote such as DNA replication and paternal genome demethylation, subsequently negatively affecting blastocyst formation.

Following heterologous ePNT, 60% of zygotes failed to go on to form a blastocyst. Analysis of the stage these zygotes reached shows that 14.29% did not develop beyond 4 cells, 53.57% developed to the 4-8 cell stage and 32.14% developed past the 8 cell stage (Figure 4.9d). Aside from the small proportion arresting before the 4-cell stage, these results are consistent with unmanipulated controls, and indicates that modifications to the procedure introduced during series II ePNT overcame the negative impact of ePNT on the very early embryonic divisions.

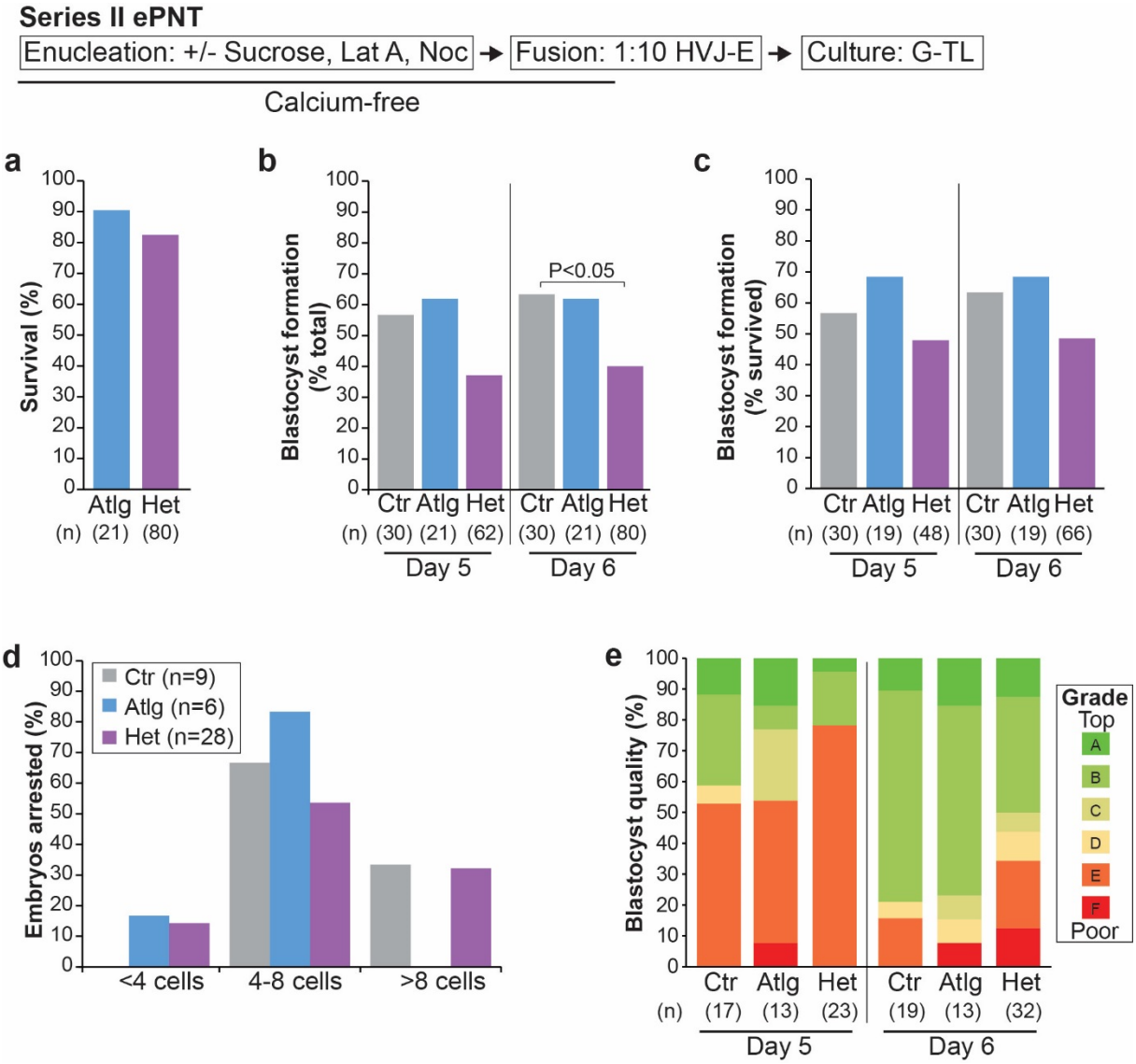


Figure 4.9: Effect of series II ePNT on survival, blastocyst development and quality. Series II ePNT experimental conditions are shown. **a)** Graph showing percentage survival following autologous (Atlg) and heterologous (Het) ePNT (not significant; chi-squared test). **b)** Blastocyst formation of control (Ctr) and ePNT (Atlg/Het) embryos on days 5 and 6 of development as a percentage of total zygotes submitted to ePNT (significance is shown; chi-squared test) and **c)** blastocyst formation as a percentage of zygotes surviving ePNT (not significant; chi-squared test). **d)** Graph showing the developmental stage reached of the embryos which did not form blastocysts. **e)** Blastocyst quality (not significant; Fisher's exact test). Grades were assigned on days 5 and 6 of development. For statistical analysis, grades were grouped (A/B versus C-F).

Overall blastocyst quality was improved compared to LtPNT (Figure 4.3) and the first series of ePNT (Figure 4.5) experiments. The majority of autologous ePNT blastocysts were grades A or B, consistent with controls (Figure 4.9e). Also, despite the reduction in blastocyst formation following heterologous ePNT, 50% of these blastocysts were of good quality (grades A or B) on day 6 (Figure 4.9e). This is a vast improvement compared to the first series of ePNT experiments, in which all blastocysts produced following heterologous ePNT only achieved grade D on day 6. However, again we observed developmental delay in ePNT blastocysts, with a high proportion (not significant) of heterologous ePNT blastocysts grade E on day 5 of development. Again, this may be due to an effect of vitrification, or asynchrony between zygotes.

4.5.3 Effect of vitrification on survival and blastocyst development following ePNT in human zygotes

Heterologous ePNT experiments are performed between zygotes originating from fresh oocytes and oocytes that were vitrified at the metaphase II (MII) stage. This results in reconstituted zygotes containing a vitrified component; zygotes are composed of either a fresh cytoplast (FreshCy) and vitrified karyoplast or vitrified cytoplast (VitCy) and fresh karyoplast. It is possible that this contributes to the reduced blastocyst development following heterologous ePNT. It is important to determine which zygotes result in the best blastocyst development of those composed of FreshCy or VitCy. This will inform whether best clinical practice would be to vitrify patient or donor oocytes.

Data indicated that the osmotic effect of sucrose was causing increased carryover of mitochondrial DNA within the karyoplast to the recipient cytoplast (Hyslop *et al.*, 2016), therefore during series II ePNT sucrose was omitted from the manipulation medium. I have analysed survival between FreshCy and VitCy embryos in the presence and absence of sucrose and found that removal of sucrose from the manipulation medium did not affect survival. I did find that survival was reduced in VitCy zygotes in both conditions, but this was not statistically significant (Figure 4.10a).

I have found that blastocyst formation is significantly reduced following heterologous ePNT compared to controls (Figure 4.9). I analysed blastocyst development to determine whether there was a difference in blastocyst formation between FreshCy and VitCy heterologous ePNT embryos. Analysis revealed reduced blastocyst formation in both FreshCy and VitCy heterologous ePNT embryos compared to technical controls (autologous ePNT) on days 5 and 6 of development (Figure 4.10b). When accounting for survival of reconstituted zygotes in the

calculation of blastocyst formation, I found blastocyst formation to be increased in VitCy embryos compared to FreshCy (Figure 4.10c; not statistically significant). However, a high proportion of VitCy blastocysts were grade E on day 5. By day 6, blastocyst quality is comparable between FreshCy and VitCy blastocysts (Figure 4.10d). Although fewer FreshCy and VitCy heterologous ePNT blastocysts were of top and good quality (grades A/B) compared to technical controls (autologous), the difference is not statistically significant.

These experiments involved the vitrification of oocytes at the MII stage, before fertilisation (Figure 4.11). Therefore, when these oocytes are used for ePNT there are several sequential manipulations; MII vitrified oocytes must be warmed and fertilised by ICSI before ePNT. It is possible that this could place too much stress on the zygote and have a negative effect on subsequent embryo development. It may be possible to improve blastocyst formation by altering the timing of vitrification. Another series of experiments were performed to test the effect of using oocytes that were vitrified after fertilisation, at the 2PB stage (Figure 4.11). This would enable zygotes to be manipulated after warming, as ICSI has already been performed. This method may also improve synchrony between zygotes used for heterologous ePNT transfer.

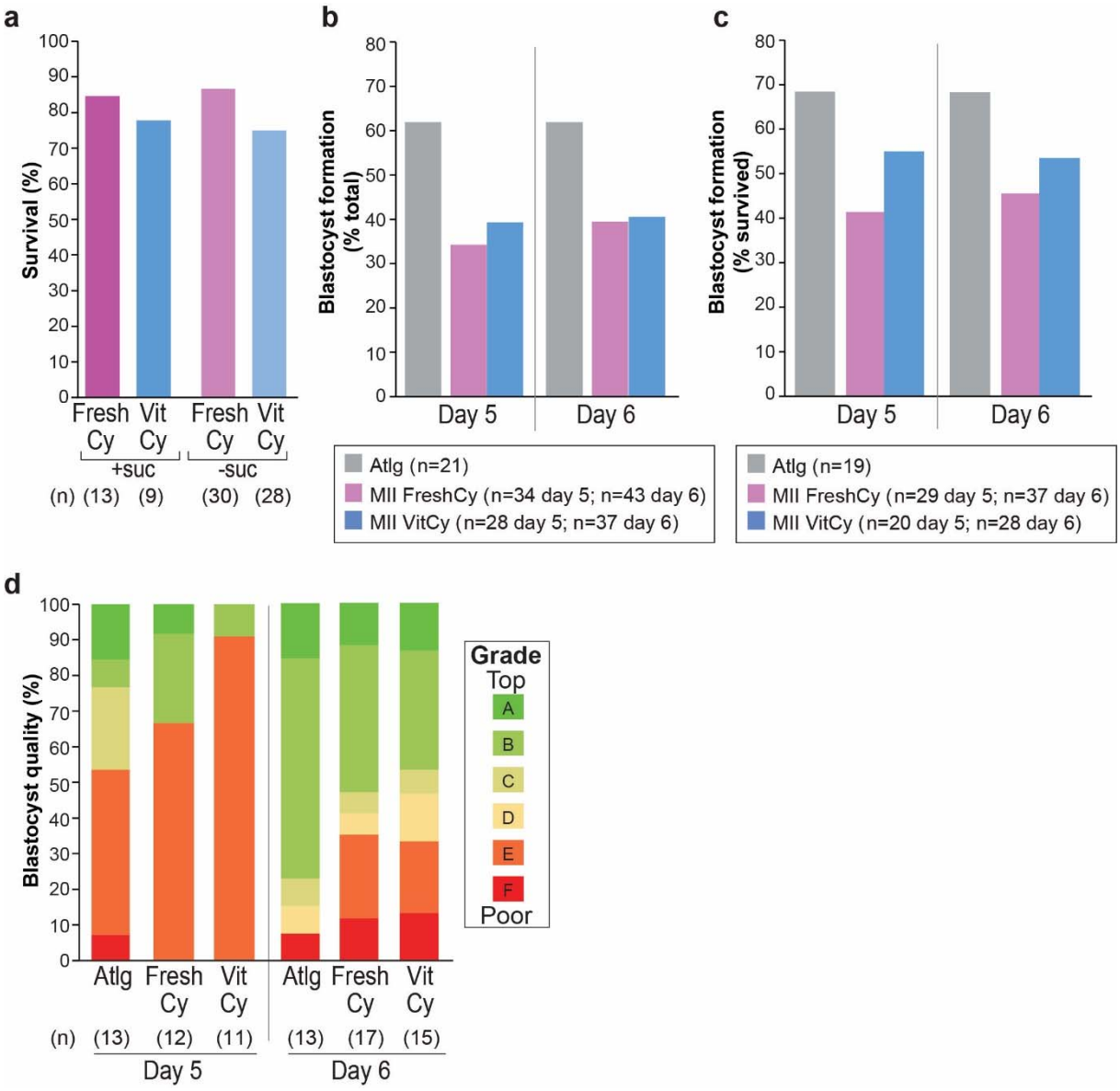


Figure 4.10: Survival and blastocyst development after heterologous ePNT between zygotes from freshly harvested and MII vitrified oocytes. a) Survival of zygotes according to whether the cytoplast was derived from a fresh (FreshCy) or vitrified (VitCy) oocyte, in the presence and absence of sucrose (not significant; chi-squared test). **b)** Blastocyst formation as a percentage of total for autologous (Atlg) and heterologous (Het) ePNT FreshCy/ VitCy zygotes and **c)** blastocyst formation as a percentage of zygotes surviving ePNT (not significant; chi-squared test). **d)** Quality of autologous (Atlg) and heterologous (Het) ePNT blastocysts according to stage of vitrification and cytoplast origin (not significant; Fisher's exact test). Grades were assigned on days 5 and 6 of development. For statistical analysis, grades were grouped (A/B versus C-F).

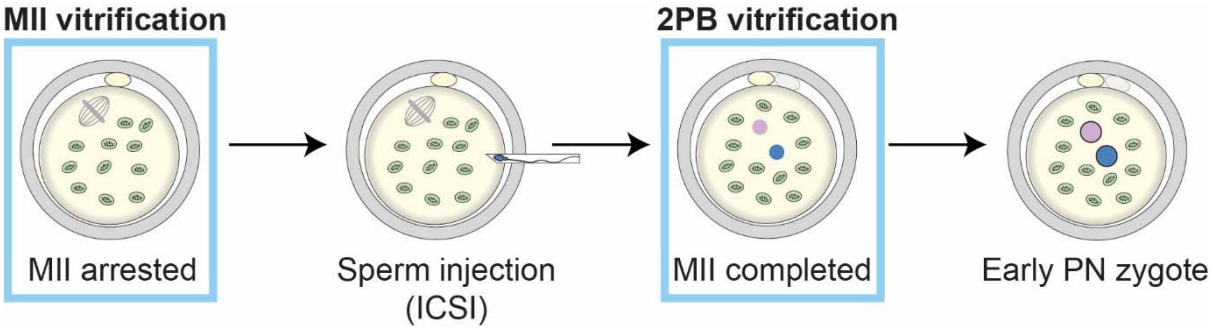


Figure 4.11: Timing of vitrification for oocytes used in heterologous ePNT experiments. Schematic showing development from MII arrest and fertilisation (sperm injection by ICSI) to completion and formation of the PN stage zygote. Oocytes were vitrified at the MII stage (MII arrest) or 2PB stage (MII completion). 2PB refers to the formation of a second polar body.

Analysis of blastocyst formation following heterologous ePNT using 2PB vitrified oocytes revealed a significant reduction in FreshCy embryos compared to technical controls ($P<0.05$) on days 5 and 6 (Figure 4.12b, c). Overall, vitrification at the 2PB stage has failed to improve blastocyst formation compared to when MII vitrified oocytes are used (Figure 4.10b, c). When taking into account survival, blastocyst formation of MII FreshCy (45.9%) and MII VitCy (53.6%) is increased compared to 2PB Fresh Cy (21.4%) and VitCy (42.9%) on day 6. Blastocyst quality was improved following 2PB vitrification, and the developmental delay was reduced; no blastocysts were grade E on day 6 of development. However, the numbers are very small. Vitrification at the 2PB stage was not investigated further as the main aim was to increase blastocyst formation, but we found it to have the opposite effect, especially when the karyoplast component was from a vitrified egg (FreshCy). This raises the possibility that a nuclear component may be sensitive to the effects of vitrification at this stage. For example, reprogramming and/or duplication of the sperm centriole could be disrupted. In support of this, a higher percentage of 2PB FreshCy embryos arrested at an early stage (<4 cells) compared to technical controls and 2PB VitCy embryos (Figure 4.12d).

These results suggest that oocytes should be vitrified at the MII stage, and blastocyst formation and quality is comparable between FreshCy and VitCy embryos. However, we found a reduction in the carryover of mtDNA in blastocysts originating from FreshCy reconstituted zygotes (Hyslop *et al.*, 2016). Therefore, in clinical treatment patient oocytes should be vitrified.

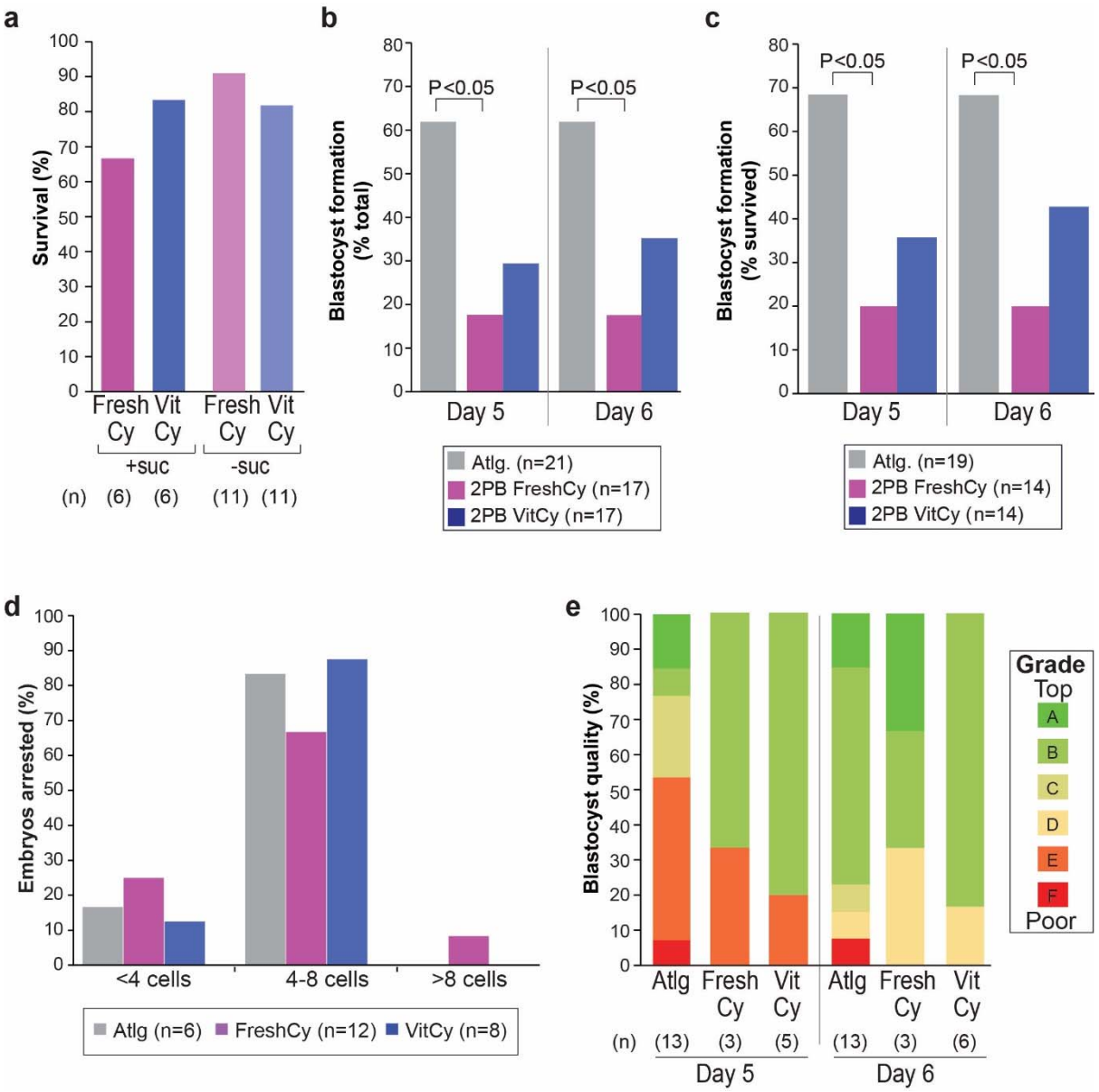


Figure 4.12: Survival and blastocyst development after heterologous ePNT between zygotes from freshly harvested and 2PB vitrified oocytes. a) Survival of zygotes according to whether the cytoplast was derived from a fresh (FreshCy) or vitrified (VitCy) oocyte, in the presence and absence of sucrose (not significant; chi-squared test). **b)** blastocyst formation as a percentage of total for autologous (Atlg) and heterologous (Het) ePNT FreshCy/ VitCy zygotes and **c)** blastocyst formation as a percentage of zygotes surviving ePNT (significance is shown; chi-squared test). **d)** Graph showing the developmental stages reached of embryos that did not form blastocysts. **e)** Quality of autologous (Atlg) and heterologous (Het) ePNT blastocysts according to stage of vitrification and cytoplast origin. Grades were assigned on days 5 and 6 of development. Statistical testing was not performed on this data due to low numbers and limited clinical significance.

4.5.4 Effect of modified ePNT procedures on blastocyst cell number

A proportion of blastocysts resulting from series II ePNT were fixed in order to obtain cell counts and determine whether the modifications to the procedure solved the issue of reduced trophectoderm cell number following PNT. The number of blastocysts used for these experiments were small as fixation precluded the use of blastocysts for other purposes, such as measurement of mtDNA carryover, aneuploidy or gene expression analysis.

The total cell number of series II ePNT blastocysts was equivalent to unmanipulated controls, and significantly increased compared to LtPNT and ePNT blastocysts (P=0.001; Figure 4.13b). This increase in cell number is also observed in the TE compared to previous experimental conditions (P<0.001), with no significant difference between control and series II ePNT blastocyst cell number (Figure 4.13c). Furthermore, cell number of the ICM in all PNT experiments remain equivalent to unmanipulated controls (Figure 4.13d). These results suggest that the modifications to the ePNT procedure have resolved the problem of reduced cell number in blastocysts following PNT.

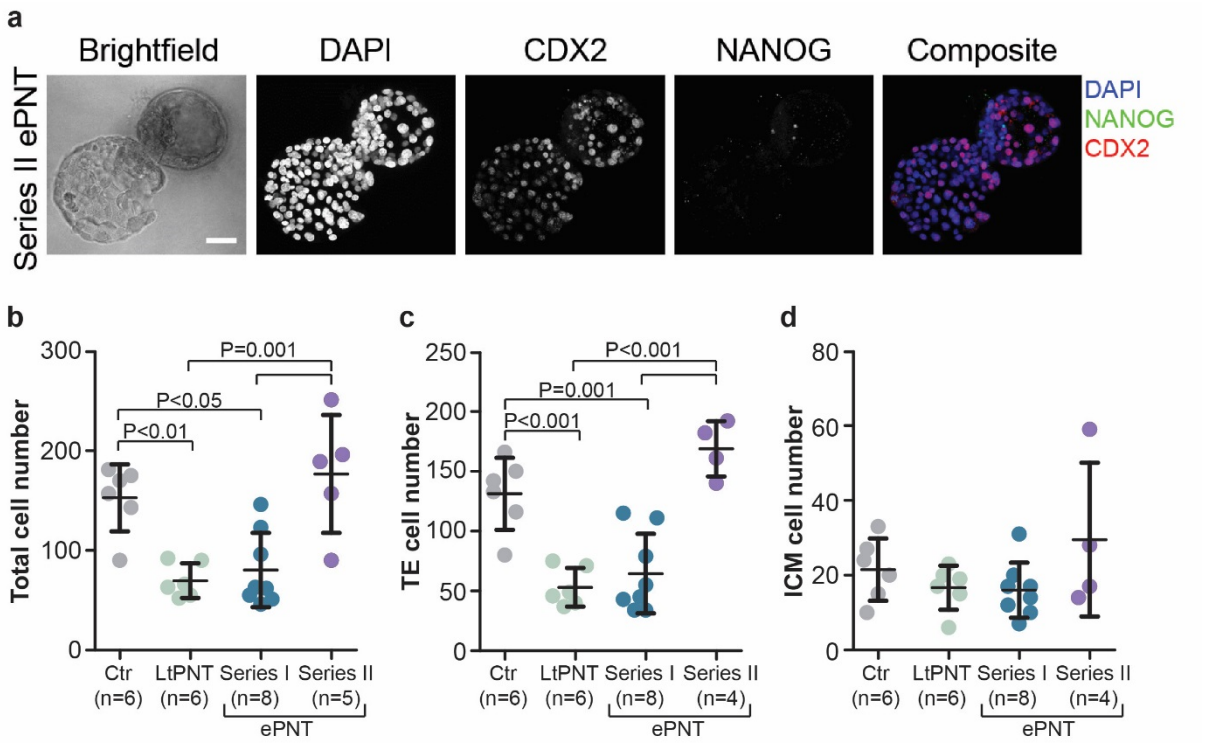


Figure 4.13: Immunofluorescence labelling and cell number analysis of series II ePNT blastocysts. a) Confocal image of a series II ePNT blastocyst fixed on day 6. DAPI staining was used to obtain nuclear counts for cell count analysis. Scale bar = 50µM. **b)** Total, **c)** trophectoderm (TE) and **d)** inner cell mass (ICM) cell number of control (Ctr), LtPNT, series I and series II ePNT. Bars show the mean ± standard deviation. Statistical significance is indicated, the test used was one-way analysis of variance (ANOVA) with Tukey's HSD.

4.5.5 Mouse PNT

Due to the limited number of human ePNT blastocysts allocated for cell number analysis, in parallel with the series II ePNT experiments I performed mouse PNT in collaboration with Dr Laura Irving. The experimental conditions of mouse PNT were consistent with series II ePNT; using calcium-free manipulation medium and single-step culture medium. However, we did not reduce the concentration of HVJ-E as this resulted in unsuccessful fusion of the karyoplast and cytoplast. Instead of the C57BL/6 mouse strain we switched to the CD1 strain for mouse PNT experiments; pronuclei are easier to see as the cytoplasm is less granular.

The PNT procedure was well tolerated by mouse zygotes, with a high percentage (86.18%) surviving the procedure (Figure 4.14a). However there is a reduction in blastocyst formation following PNT compared to unmanipulated controls (P<0.005; Figure 4.14b). Quality of mouse blastocysts was not recorded as we found limited variability between blastocysts, which made it difficult to assign scores consistent with the grading scheme used for human blastocysts.

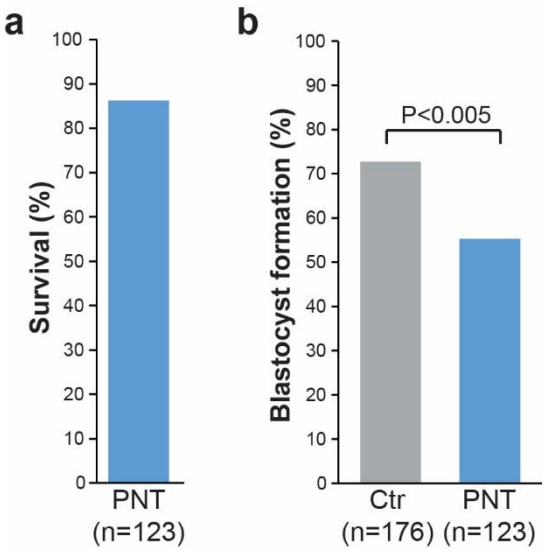


Figure 4.14: Survival and blastocyst formation of mouse PNT zygotes. a) Survival of mouse zygotes following autologous PNT. **b)** Formation of blastocysts following autologous PNT using mouse zygotes compared to unmanipulated controls (P<0.005; chi-squared test).

A proportion of zygotes which developed to the blastocyst stage were fixed and stained with DAPI to obtain nuclear counts by confocal imaging. A small number of these counts were performed by MRes student Jessica Neilson (control, n=5 and PNT, n=3). A cell count validation exercise conducted by Jessica Neilson showed that cell counts were comparable between operators. I found a statistically significant reduction in total cell number of PNT blastocysts compared to unmanipulated controls ($P<0.05$). This was apparent also in the TE ($P<0.01$) but ICM cell number was consistent with controls; reflecting observations in LtPNT and the first series of ePNT experiments using human zygotes (Figure 4.6). However, the cell number of blastocysts which were fixed for nuclear counts during series II human ePNT is significantly improved and comparable to unmanipulated controls (Figure 4.13). Taken together, this indicates that the higher concentration of HVJ-E could contribute to reduced cell number, as conditions used in mouse PNT mimicked that of series II ePNT except the reduced HVJ-E concentration. It is conceivable that a high concentration of HVJ-E could have residual effects causing reduced cell number, due to its membrane fusion properties.

4.5.6 Effect of modified ePNT procedures on chromosome segregation

To determine whether ePNT effects chromosome segregation, in collaboration with Professor Dagan Wells (Oxford), the incidence of aneuploidy was analysed by array-based comparative genomic hybridisation (array-CGH). Cells for analysis were obtained predominately from the TE but in some cases cells from the ICM were also sampled. We analysed samples from both unmanipulated controls and series II ePNT blastocysts and were able to compare data to a large reference population of blastocysts resulting from normal IVF treatment and tested under the same conditions. Data from the reference population was age-matched to the donors from the ePNT experiments.

Following analysis of the array-CGH data, I found that incidence of aneuploidy was highest in ePNT blastocysts, with aneuploidy detected in 50% of ePNT blastocysts (Figure 4.16a). However this was not statistically significant when compared to unmanipulated controls and a reference population of 17,443 samples from IVF blastocysts.

On further investigation of the origin of the aneuploid blastocysts, I found aneuploidy was detected in the majority (83.3%) of poor quality (grade E/F) ePNT blastocysts (Figure 4.16b). However, aneuploidy in good quality (grade A/B) ePNT blastocysts was exactly equivalent to unmanipulated controls, which were all of good quality. The increased incidence of aneuploidy in poor quality blastocysts is consistent with recent findings (Minasi *et al.*, 2016).

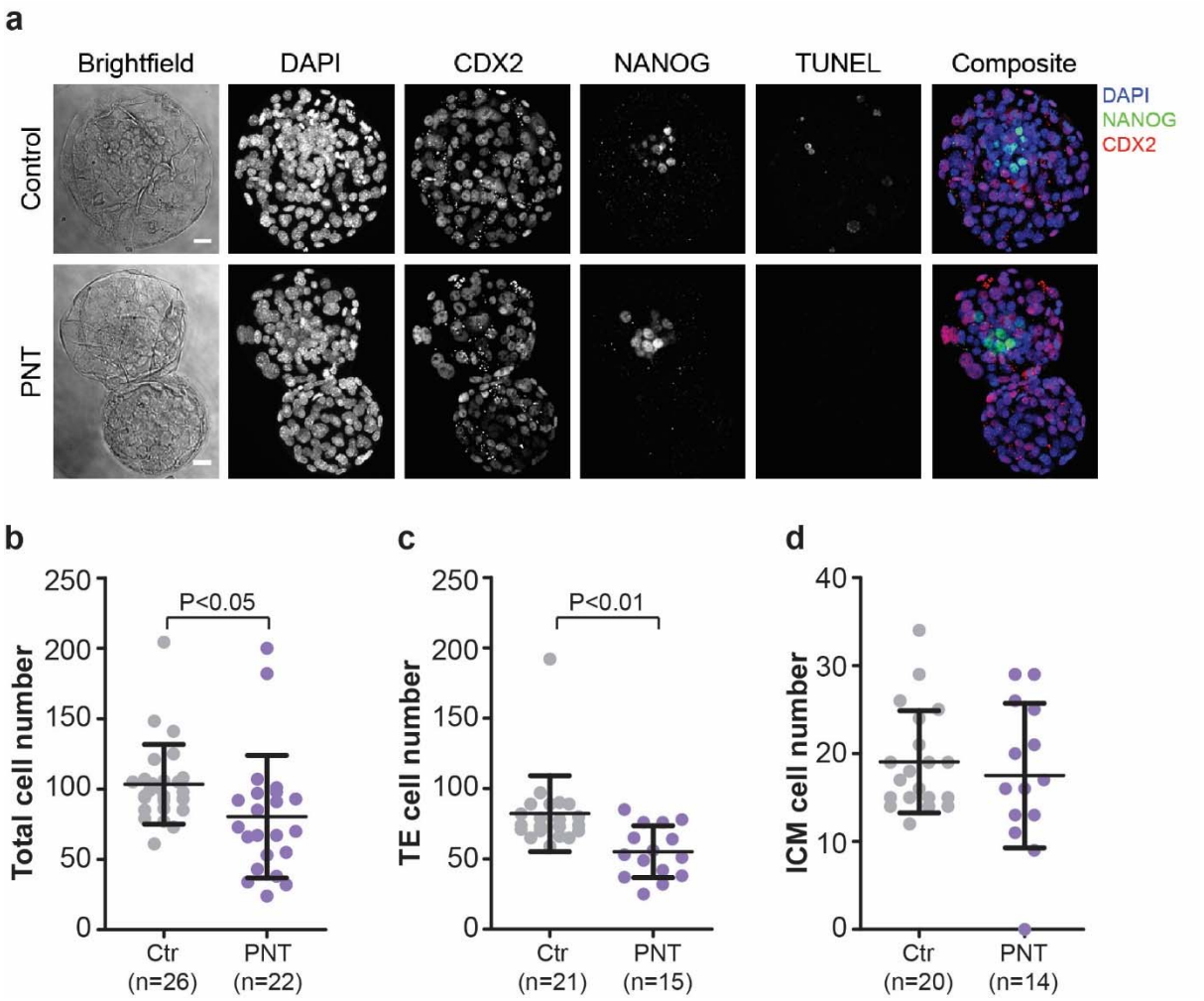


Figure 4.15: Mouse PNT blastocyst cell number analysis. a) Confocal images showing examples of control and mouse PNT blastocysts which were fixed for cell number analysis using DAPI staining to obtain nuclear counts. Scale bar = 20µM. **b)** Total, **c)** trophectoderm (TE) and **d)** inner cell mass (ICM) cell number of unmanipulated controls and mouse PNT blastocysts submitted for analysis. Bars show the mean ± standard deviation. Significance is shown and was calculated using unpaired t-test.

It should be noted that poor quality blastocysts are not normally analysed for aneuploidy in clinical programmes.

In blastocysts where more than one sample was analysed by array-CGH, aneuploidy was categorised to reflect the degree of concordance between different samples. Blastocysts were defined as mosaic euploid/aneuploid if they contained a mixture of aneuploid and euploid samples. If multiple samples were aneuploid but different chromosomes affected, blastocysts were defined as mosaic aneuploid. Finally, if multiple samples were analysed and aneuploidy detected affecting the same chromosomes, they are described as uniform aneuploid, which is indicative of a meiotic error. The proportion of euploid and aneuploid samples according to these definitions is shown in Figure 4.16c. As expected, the highest proportion of uniform aneuploidy is observed in poor quality ePNT blastocysts (20% of blastocysts uniform aneuploid). In total, 60% of poor quality ePNT blastocysts are uniform or mosaic aneuploid. However, the majority of controls and ePNT blastocysts grades A-D are euploid in all or at least one sample analysed (Figure 4.16c). The array-CGH results are also shown in Table 4.1.

In summary, given that eggs were donated by young women with no known fertility problems, the incidence of aneuploidy was higher than expected compared with the reference population of IVF blastocysts. This may have been due to the fact that we tested multiple samples in most cases, whereas in clinical IVF only one sample is tested. Given the apparently high incidence of mosaicism in the TE, the findings raise a question mark over the reliability of TE analysis for pre-implantation genetic screening. However, the main finding of this part of the work is that the incidence of aneuploidy was not increased in those ePNT blastocysts whose morphological features are compatible with implantation.

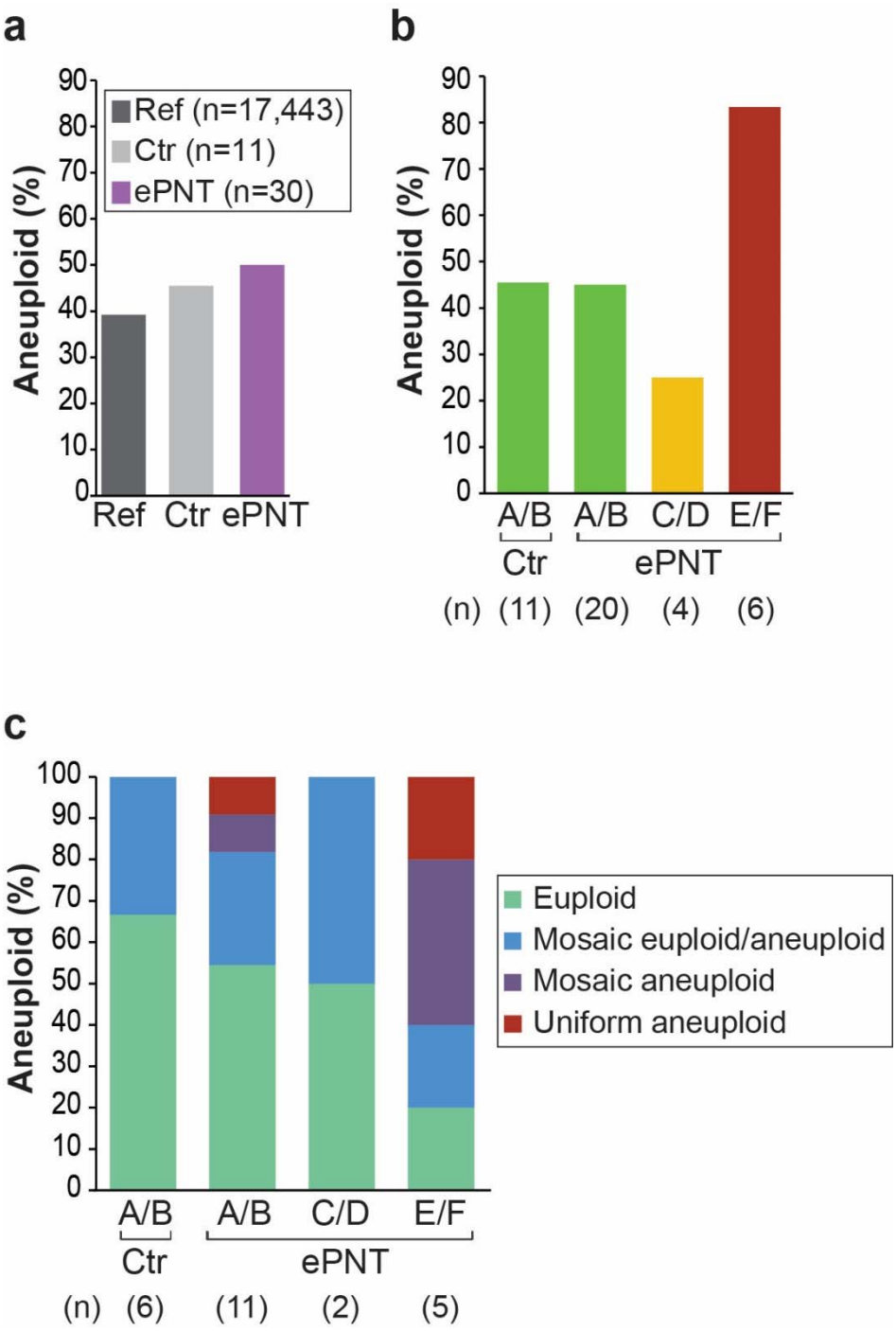


Figure 4.16: Incidence of aneuploidy in ePNT blastocysts. a) Graph showing percentage of aneuploid samples from a reference population of IVF blastocysts, unmanipulated controls and series II ePNT. **b)** Percentage of aneuploid samples in unmanipulated controls and series II ePNT blastocysts according to blastocyst grade. **c)** Graph showing the type of aneuploidy observed in blastocysts for which 2 or more samples were analysed. Results were not statistically significant (chi-squared test).

		Chromosome																						
Embryo	Grade	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	XY	Interpretation
Control																									
21Ctr	A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
50Ctr	A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
6Ctr	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
12Ctr	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
25Ctr	B	gain	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	part	✓	✓	loss	✓	✓	✓	✓	part	✓	✓	✓	Mosaic
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	euploid/aneuplo
34Ctr	B	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	gain	Triploid
39Ctr	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	gain	✓	Aneuploid
40Ctr	B	✓	✓	✓	✓	part	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Aneuploid
43Ctr	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
44Ctr	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
49Ctr	B	✓	part	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Mosaic
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	euploid/aneuplo
ePNT																									
36PNT	A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
51PNT	A	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
4PNT	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
10PNT	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
13PNT	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
14PNT	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
15PNT	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
20PNT	B	✓	✓	✓	✓	✓	gain	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Mosaic
		✓	✓	✓	✓	✓	gain	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	euploid/aneuplo
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	id
23PNT	B	✓	✓	✓	✓	✓	✓	loss	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Aneuploid
27PNT	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	gain	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Mosaic
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	euploid/aneuplo
28PNT	B	✓	✓	✓	✓	✓	✓	✓	part	✓	✓	✓	✓	✓	✓	✓	✓	gain	✓	✓	✓	loss	✓	✓	Mosaic
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	euploid/aneuplo
31PNT	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
38PNT	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
41PNT	B	✓	✓	✓	✓	gain	✓	✓	gain	✓	✓	✓	✓	✓	gain	✓	✓	✓	✓	✓	✓	loss	✓	✓	Aneuploid
45PNT	B	✓	✓	✓	✓	✓	✓	✓	loss	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Aneuploid
46PNT	B	✓	✓	part	✓	✓	✓	✓	✓	✓	✓	✓	✓	gain	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Aneuploid
47PNT	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	part	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Mosaic
		✓	✓	✓	✓	✓	✓	✓	part	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	aneuploid
48PNT	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
54PNT	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
55PNT	B	✓	✓	✓	✓	✓	gain	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Uniform
		✓	✓	✓	✓	✓	gain	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	aneuploid
9PNT	C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
11PNT	C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
3PNT	D	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Mosaic
		part	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	euploid/aneuplo
37PNT	D	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
1PNT	E	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Euploid
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
2PNT	E	✓	✓	✓	✓	✓	✓	✓	✓	✓	loss	part	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	Mosaic
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	euploid/aneuplo
52PNT	E	✓	✓	✓	gain	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	gain	gain	gain	✓	✓	✓	Aneuploid
56PNT	E	✓	✓	✓	✓	gain	loss	loss	✓	✓	loss	✓	loss	✓	✓	gain	✓	✓	✓	✓	gain	✓	gain	✓	Mosaic
		✓	✓	part	loss	✓	loss	✓	gain	✓	✓	loss	✓	loss	✓	gain	✓	part	✓	✓	gain	gain	✓	✓	aneuploid
7PNT	F	part	part	part	loss	✓	part	✓	✓	part	part	✓	✓	✓	gain	✓	gain	✓	✓	✓	loss	✓	part	✓	Mosaic
		✓	✓	✓	loss	✓	✓	✓	gain	✓	✓	✓	✓	✓	✓	✓	✓	loss	✓	gain	✓	gain	✓	✓	aneuploid
8PNT	F	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	loss	✓	loss	✓	✓	✓	✓	✓	✓	✓	✓	loss	✓	Uniform
		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	loss	✓	loss	✓	✓	✓	✓	✓	✓	✓	✓	loss	✓	aneuploid
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	XY	
		Chromosome																							

Table 4.1: Array-CGH results for ePNT blastocysts. Summary of results obtained from samples from unmanipulated control (n=11) and ePNT (n=30) blastocysts. Blastocysts are ordered by quality (grade).

4.6 Predicting the chance of achieving a pregnancy following ePNT

Our experiments and numerous analyses performed on ePNT blastocysts indicate that a high proportion of blastocysts formed following ePNT are of good quality, and do not exhibit an increased incidence of chromosome segregation errors compared with controls. However, these are preclinical studies and PNT is not yet available as a treatment. Thus, we have no data on the success of PNT based on clinical outcome. It would be helpful to give patients an idea of the likelihood of successfully achieving a pregnancy following the PNT procedure prior to commencing treatment.

Data from IVF/ICSI treatment at Newcastle Fertility Centre shows that blastocyst grade correlates with implantation (Figure 4.1). Percentage implantation ranges from 17.6% for grade E blastocysts, 35.1% for grade B blastocysts to 53.1% for grade A blastocysts, when replaced on day 5.

In the context of clinical treatment, each individual series II ePNT heterologous experiment would correspond to one treatment cycle. Our mtDNA carryover data (Hyslop *et al.*, 2016) indicates that the best option is to vitrify patient oocytes. Therefore, reconstituted zygotes would be composed of fresh cytoplast (FreshCy) and vitrified karyoplast. In Figure 4.17 I present the data for each heterologous ePNT experiment, including only data for FreshCy embryos and blastocyst development on day 5. I found that 28.6% of experiments resulted in the production of at least one blastocyst with an implantation potential of 35.1% to 53.1%. Furthermore, 42.9% of experiments produced at least one blastocyst with an implantation potential of 17.6%. However, a number of cases (28.6%) resulted in the production of no blastocysts. Therefore, it is likely that patients would require more than one treatment cycle to produce blastocysts compatible with implantation.

a

Heterologous ePNT: FreshCy only					Day 5 Grade (%)						Implantation
Experiment	Zygotes	Survived	Blastocyst	Blastocyst (%)	A	B	C	D	E	F	potential (%)
1	3	3	2	66.67	0	1	0	0	1	0	35.1
2	3	2	1	33.3	0	0	0	0	1	0	17.6
3	1	1	1	100	0	1	0	0	0	0	35.1
4	1	1	0	0	0	0	0	0	0	0	0
5	2	2	0	0	0	0	0	0	0	0	0
6	3	2	1	33.3	0	0	0	0	1	0	17.6
7	4	4	2	50	0	1	0	0	1	0	35.1
8	1	1	1	100	0	0	0	0	1	0	17.6
9	3	3	1	33.3	0	0	0	0	1	0	17.6
10	2	2	0	0	0	0	0	0	0	0	0
11	1	1	1	100	0	0	0	0	1	0	17.6
12	3	2	1	33.3	1	0	0	0	0	0	53.1
13	3	3	0	0	0	0	0	0	0	0	0
14	3	3	1	33.33	0	0	0	0	1	0	17.6

b

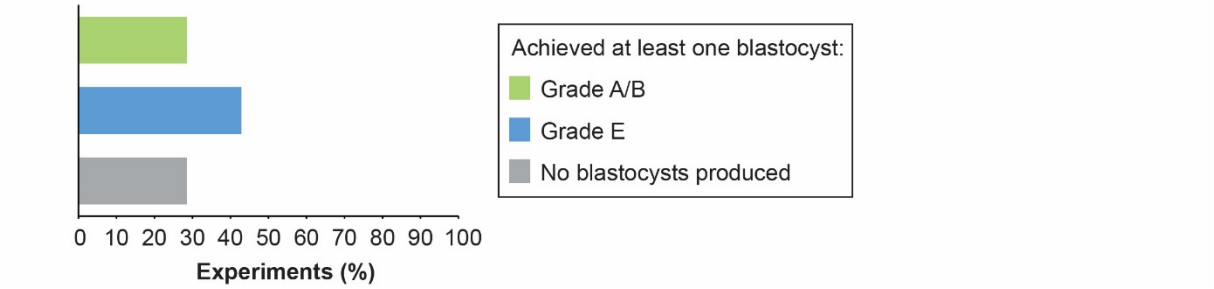


Figure 4.17: Predicting the chance of achieving a pregnancy following ePNT. a) Table showing the number of blastocysts and grade produced per experiment. Only heterologous ePNT (FreshCy) are included, as this mimics what would be performed clinically. Implantation potential, calculated from clinical IVF/ICSI data is shown, according to the highest quality blastocyst produced. **b)** Graph showing the percentage of heterologous ePNT (FreshCy) experiments which achieved at least one blastocyst of grade A/B, E or no blastocysts.

4.7 Discussion

Our initial findings using normally fertilised human zygotes for PNT showed poor survival of reconstituted zygotes. Blastocyst formation was also significantly lower than unmanipulated controls, and the majority of blastocysts formed were poor quality. The finding that only 50% of technical controls (autologous PNT) survived the manipulations indicated a possible issue with the enucleation and fusion procedures. We found that switching the timing of PNT from the G2 to the G1 phase of the 1st mitotic cell cycle improved survival of zygotes (ePNT). However, despite the increase in survival, we found that the quality of ePNT blastocysts was not improved, even in the case of autologous transfers. We therefore performed a second series of ePNT experiments with further modifications. Modifications to the enucleation/fusion procedures in series II ePNT were to reduce the concentration of HVJ-E and use of a calcium-free manipulation medium. We also altered the culture conditions, using a single-step (G-TL) instead of sequential (G-1/G-2) medium, due to observations indicating that sequential medium has a negative effect on development and positive results following testing G-TL using mouse embryos. Interestingly, based on results from mouse embryos, G-TL was introduced into the clinical laboratory at Newcastle Fertility Centre. Analysis of clinical data indicates an increase in blastocyst formation and improved blastocyst quality following culture in G-TL compared to sequential culture medium (unpublished data). Therefore, it is likely that this modification has contributed to the improvement in blastocyst formation for unmanipulated controls and technical controls (autologous ePNT). It is possible that it also promoted the development of good quality blastocysts.

Mouse PNT was performed to investigate the causes of reduced blastocyst cell number, which was observed in LtPNT and the first series of human ePNT experiments, and specifically affected the TE. The conditions of these experiments mimicked those of series II ePNT, with the exception of a reduced HVJ-E concentration, as this was not compatible with fusion of mouse karyoplast and cytoplast. Human blastocysts which were used for cell number analysis in series II ePNT showed a statistically significant increase in cell number compared to ePNT (series I) blastocysts and were equivalent to unmanipulated controls. Surprisingly, this was not reproduced following analysis of mouse PNT blastocysts. This suggests that the high concentration of HVJ-E used in previous PNT procedures could have had a negative effect on blastocyst cell number. This is not implausible considering the membrane fusion properties of HVJ-E, it is possible that there were residual effects which were alleviated by reducing the concentration used. To confirm this, mouse PNT should be repeated using a reduced concentration of HVJ-E and blastocyst cell number compared to previous experiments. If it is

not possible to reduce the HVJ-E concentration 1:10, as in human ePNT, it could at least be partially reduced.

The modifications introduced in series II promoted the development of good quality blastocysts. Notably, blastocyst formation and quality following autologous ePNT was exactly equivalent to unmanipulated controls. This gives reassurance that the technical procedures and reagents used do not have a negative impact on early human development. While blastocyst formation was reduced following heterologous ePNT, this was at least in part due to reduced survival following vitrification at the MII stage. However, given that 48.5% of zygotes that survived the procedure developed to the blastocyst stage and that half of these were grade A/B on day 6, with no increase in the incidence of aneuploidy, we are optimistic that the procedure would be compatible with a the establishment of pregnancies in clinical treatment.

Identifying which modification (if not all) is promoting improved formation of good quality blastocysts would require changing each element individually. However this is not possible due to the nature of the experiments and the limited number of donated human oocytes. Therefore, taking these results together these are the current optimal conditions for ePNT.

Further research will focus on bridging the gap in blastocyst formation and quality between heterologous and autologous ePNT. In this regard, it would be interesting to test the effect of vitrification by performing heterologous ePNT between two lots of freshly harvested oocytes. However, it is difficult to synchronise egg donors due to unpredictability in the response to ovarian stimulation. This strategy may therefore not provide a long term solution.

Another possibility is that asynchrony between zygotes used for ePNT contributed to reduced blastocyst formation. Asynchrony could disrupt the critical processes of paternal genome demethylation, DNA replication and sperm centriole duplication, which occur in the zygote. In relation to the effect of DNA replication, it has been reported from studies on mouse embryos (Yamauchi *et al.*, 2009) that fusion of pronuclei from parthenogenetically activated mouse oocytes at the G1 stage with a cytoplast from an S-phase zygotes did not induce premature replication of DNA. However, there may be effects mediated by the sperm centriole. Nothing is known of the mechanisms or timing of centriole duplication in human. Similarly, the timing of paternal genome demethylation, which in mouse is a highly regulated process (Gu *et al.*, 2011; Wossidlo *et al.*, 2011; Nakamura *et al.*, 2012), has not been characterised in human. Characterisation of these events in human zygotes will provide insight into the possible effects on blastocyst formation. It may be informative in defining

morphological correlates and, importantly, the tolerable limits of asynchrony in the likely event that there will be a limited number from which to choose in future clinical treatment.

To conclude, modifications to the PNT procedure have improved survival and blastocyst quality which will aid the efficiency of PNT in the clinic. Furthermore, the analysis we have performed suggests that blastocysts formed following ePNT are compatible with the establishment of a normal pregnancy. However, further investigation is required in order to identify the causes of reduced blastocyst formation following heterologous ePNT.

Chapter 5. Results II: The effect and reversibility of cytoskeletal inhibitors used during nuclear genome transplantation

5.1 Introduction

Nuclear genome transplantation techniques, including pronuclear transfer (PNT) and metaphase II spindle transfer (MST), require the use of cytoskeletal inhibitors during manipulations. Cytoskeletal inhibitors facilitate enucleation and fusion as they relax the cytoskeleton, making the cytoplasm more fluid, by preventing polymerisation of actin and microtubules.

5.1.1 The cytoskeleton

The cytoskeleton is composed of three types of protein filament responsible for maintaining the shape and internal organisation of the cell; microtubules, actin and intermediate filaments. The cytoskeleton also provides mechanical support for essential functions including cell division and trafficking of organelles such as the mitochondria (Alberts *et al.*, 2015).

Microtubules are the largest filament at approximately 25nm in diameter and arranged in arrays extended from microtubule organising centres (MTOCs). Actin filaments are also known as microfilaments, as at approximately 7nm in diameter they are the thinnest component of the cytoskeleton. Actin monomers (G-actin) polymerise to form long fibres (F-actin) which are often found below the cell cortex. Visualisation of actin filaments in fixed samples can be achieved by staining with phalloidin, a member of a family of toxins from the *Amanita phalloides* 'death cap' mushroom (Wieland and Faulstich, 1978), which selectively labels F-actin (Estes *et al.*, 1981). Actin filaments and microtubules have plus and minus ends; with faster growth occurring at the plus ends. Microtubules are constantly growing and shortening; this is known as dynamic instability and contributes to the important roles of microtubules in cell division and organelle transport (Kirschner and Mitchison, 1986; Desai and Mitchison, 1997; Brouhard, 2015).

The structure of the cytoskeleton differs in the oocyte before fertilisation and after fertilisation. This reflects changes occurring in the oocyte following fertilisation, when meiosis is completed and the first mitotic cycle begins. Before fertilisation the microtubules are assembled to form the metaphase II spindle, on which chromosomes align and segregate either to the oocyte or to the second polar body following fertilisation. Actin forms a thick cortical layer adjacent to the spindle, known as the actin cap. The actin cap is responsible for maintaining the position of the spindle and plays an essential role in polar body extrusion

(Longo and Chen, 1985). After fertilisation, the spindle is disassembled as maternal and paternal nuclear genetic material is packaged into pronuclei. Microtubules are involved in positioning pronuclei towards the centre of the zygote (Reinsch and Karsenti, 1997; Deng *et al.*, 2007; Wuhr *et al.*, 2009). Actin filaments in the zygote are enriched at the cortex, providing mechanical strength and in preparation for the first mitotic division.

5.1.2 Cytoskeletal inhibitors

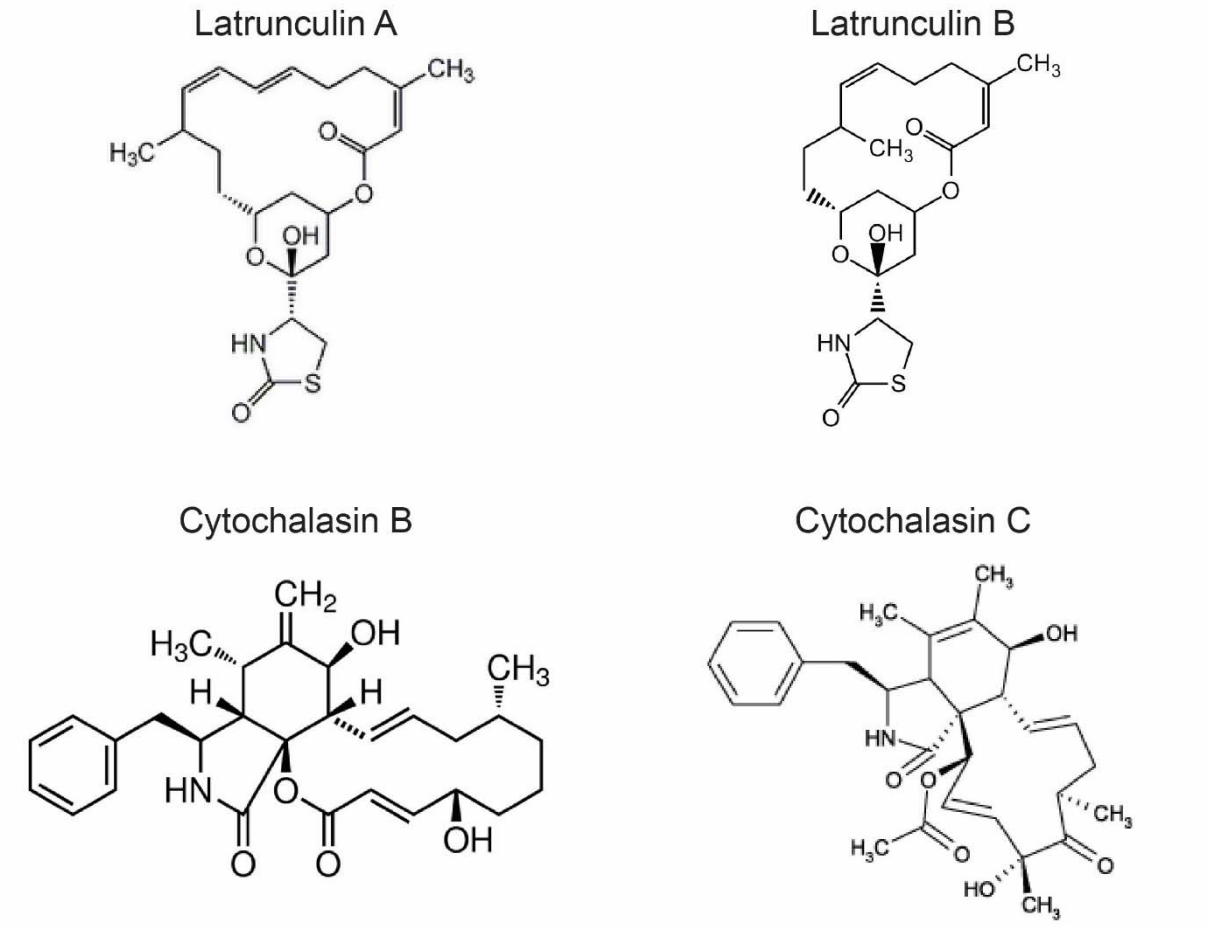
Cytoskeletal inhibitors facilitate enucleation during PNT by inhibiting polymerisation of actin filaments and microtubules which provide strength and structural support to the zygote. The absence of an intact cytoskeletal network facilitates removal of the pronuclei by increasing the fluidity of the cytoplasm. We found that enucleation in the absence of cytoskeletal inhibitors frequently results in lysis of the zygote and makes it difficult to minimise the amount of cytoplasm surrounding the pronuclei, which has implications for the level of mitochondrial DNA carryover. However, it is important that inhibitors are rapidly reversible and do not negatively affect subsequent embryo development.

Cytoskeletal inhibitors which target microtubules cannot be used during MST as they will disrupt the spindle. It is possible to use actin inhibitors but it is critically important that the inhibitor is rapidly reversible due to the essential role of the actin cap in polar body extrusion. The absence of an intact actin cap may result in failed extrusion of the second polar body and abnormal fertilisation, which has been reported to be occur at high frequency following MST in human oocytes (Tachibana *et al.*, 2013).

Several inhibitors are available which target either the actin or microtubule cytoskeleton; either promoting or inhibiting polymerisation. The structures of a number of inhibitors of actin and microtubule polymerisation are shown in Figure 5.1. The inhibitor commonly used in nuclear genome transfer to prevent the polymerisation of actin filaments is cytochalasin B. However, in initial LtPNT experiments (Chapter 4.2) we found that the potency of cytochalasin B varied between batches. Therefore, we decided to test the efficacy and reversibility of alternative actin inhibitors.

Cytoskeletal inhibitors

Inhibitors of actin polymerisation



Inhibitors of microtubule polymerisation

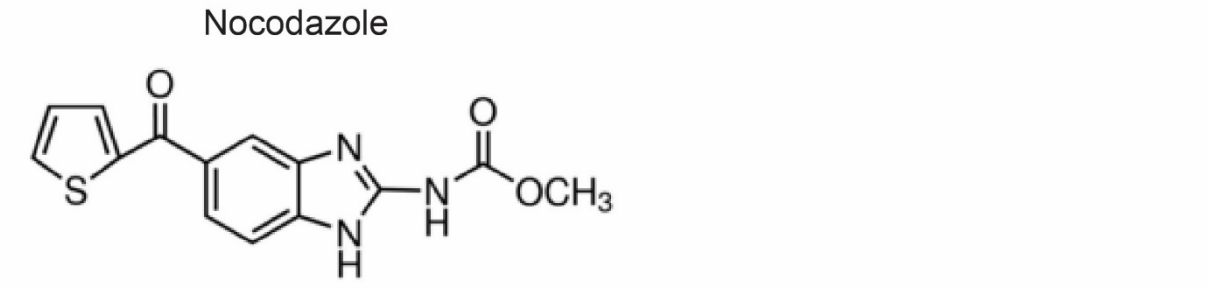


Figure 5.1: Structure of cytoskeletal inhibitors targeting actin and microtubule polymerisation.

The cytochalasins are cell permeable fungal metabolites. Cytochalasin B prevents actin elongation and shortening by binding to the plus ends of actin filaments (Lin *et al.*, 1980). Cytochalasin C is closely related to cytochalasin D, which also prevents actin polymerisation by binding to actin filaments (Lin *et al.*, 1980). These cytochalasins differ from latrunculins as they cannot disrupt polymerisation by binding to actin monomers. Cytochalasin C has been found to be ten times less toxic than cytochalasin D when used in mice, but displays a similar level of biological effectiveness (Walling *et al.*, 1988). We therefore decided to test the effect and reversibility of cytochalasin C in zygotes.

The latrunculins are purified natural marine toxins from the red sea sponge *Latrunculia magnifica* (*Negombata magnifica*). Both latrunculin A and latrunculin B disrupt polymerisation of actin filaments by 1:1 molecular binding of monomeric actin (G-actin) present in the cytoplasm (Spector *et al.*, 1983; Spector *et al.*, 1989; Yarmola *et al.*, 2000). Latrunculin A and latrunculin B have similar short term effects; however latrunculin B is less potent and gradually inactivated by serum, therefore the effects of the inhibitor are transient (Spector *et al.*, 1989). For this reason, latrunculin B may be preferable to latrunculin A for use in PNT and spindle transfer, which require inhibitors to be rapidly reversible.

The lack of specificity of compounds which target actin hinders their use in therapeutic applications, such as chemotherapy. However, a phase I clinical trial has been conducted to test the use of latrunculin B in the treatment of patients with ocular hypertension or early primary open-angle glaucoma (Rasmussen *et al.*, 2014). This trial involved testing different concentrations of Lat-B Opthalmic Solution in 4 cohorts of 14 patients over 3 days, results indicated that latrunculin B may be a potential therapeutic agent for the treatment of glaucoma. The adverse events recorded in this clinical trial were few and those that did occur were mild (Rasmussen *et al.*, 2014).

The microtubule inhibitor selected for use in PNT was nocodazole as we found this inhibitor to be reliable in previous experiments (Craven *et al.*, 2010). Nocodazole is a synthetic compound which disrupts the polymerisation of microtubules by binding beta-tubulin and inhibiting the formation of interchain disulphide linkages. Nocodazole is frequently used in cell biology research laboratories to synchronise the cell cycle, as it arrests cells in the G2/M phase. There are currently no therapeutic uses of nocodazole, however several drugs which act by disrupting the microtubule cytoskeleton are used in chemotherapy. For example, Vinblastine prevents microtubule polymerisation by binding tubulin and inducing the

formation of spiral tubulin aggregates (Gigant *et al.*, 2005). This compound is used in the treatment of several cancers, including: lymphomas, bladder, breast and testicular cancer.

The aim of experiments in this chapter is to investigate the effect and reversibility of cytoskeletal inhibitors, in order to contribute to the selection of optimal inhibitors for use in nuclear genome transplantation.

5.2 The effect and reversibility of inhibitors of actin polymerisation in the oocyte

5.2.1 How effective are inhibitors at rapidly depolymerising the actin cytoskeleton in oocytes?

Inhibitors which target the polymerisation of actin filaments are used during MST to aid removal of the spindle. However, it is important that the inhibitor used is fast-acting to minimise exposure time of oocytes to the inhibitor. I compared the action of the actin inhibitor latrunculin A that we have used for PNT to cytochalasin B, which is commonly used in MST.

Oocytes were incubated with inhibitor for 10 minutes before fixation using 2% PFA. These experiments used mouse oocytes at the metaphase II stage, which were either freshly harvested following superovulation or *in vitro* matured. Following fixation, immunofluorescence labelling and confocal imaging was performed to view the effect of inhibitors on the actin network in mouse oocytes.

Inhibitor stock solutions were prepared using dimethyl sulfoxide (DMSO). To control for any effects of DMSO, a number of oocytes (n=6) were treated with DMSO in the absence of actin inhibitors. A Z-projection of a DMSO treated oocyte is included in Figure 5.2b. I found the cytoskeleton of DMSO treated oocytes to be comparable to untreated controls. However, in the images due to different orientations of the oocytes, the actin cap is more visible in DMSO treated oocytes (Figure 5.2b).

Latrunculin A appears to have a disruptive effect on cortical actin, including the actin cap. This is seen in a Z-section showing a slice and a Z-projection of a whole confocal image of the same oocyte treated with latrunculin A (Figure 5.2a, b). However, cytochalasin B had little effect on the actin cytoskeleton of oocytes (Figure 5.2a, b). These experiments included the use of two separate cytochalasin B stock solutions which were used within two months of preparation.

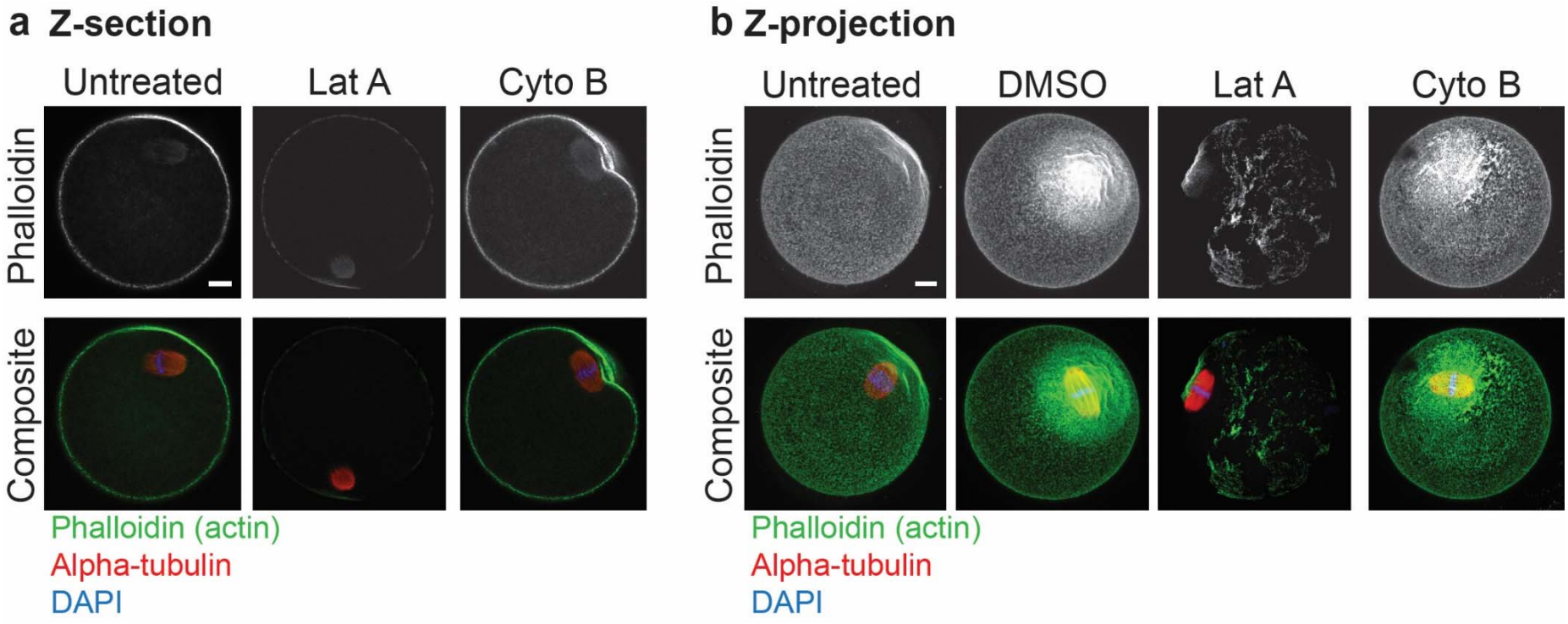


Figure 5.2: The effect of actin inhibitors latrunculin A and cytochalasin B on the cytoskeleton of mouse oocytes. a) Representative confocal images showing single z-sections of metaphase II mouse oocytes which were untreated (n=12), treated with latrunculin A (Lat A; n=7) or cytochalasin B (Cyto B; n=8) for 10 minutes before fixation. **b)** Maximum intensity Z-projections showing untreated (n=12), DMSO (n=6), Lat A (n=7) and Cyto B (n=8) treated mouse oocytes. Note the enriched area of actin is the actin cap, which is not as visible in the untreated oocyte compared to DMSO and Cyto B due to differing orientations. Oocytes were labelled with phalloidin (actin; green), an antibody against alpha-tubulin (microtubules; red) and DAPI (DNA; blue). Scale bar = 10µm.

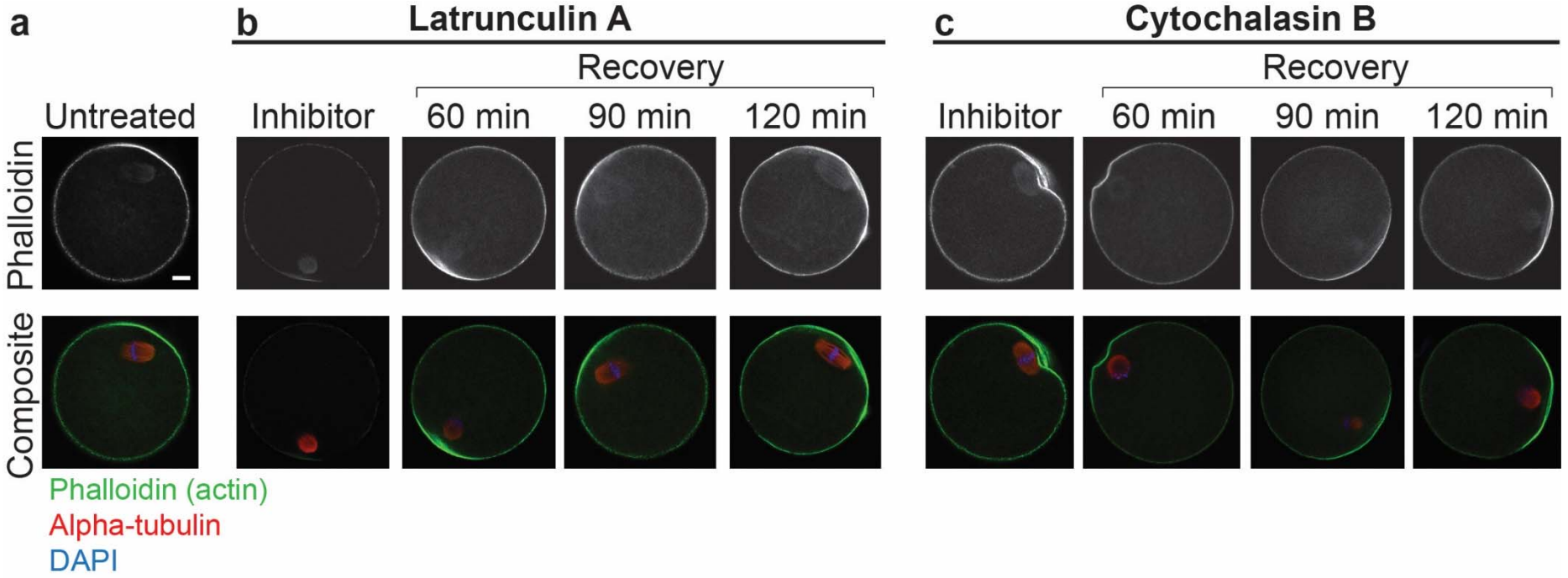


Figure 5.3: Recovery of the mouse oocyte cytoskeleton following exposure to latrunculin A or cytochalasin B. a) Confocal image showing Z-section of an untreated control (n=12) mouse oocyte. **b)** Confocal images showing the cytoskeleton of mouse oocytes treated with latrunculin A (Lat A; n=7) for 10 minutes and allowed to recover for 60 minutes (n=10), 90 minutes (n=9) and 120 minutes (n=5) post-inhibitor wash-out. **c)** Confocal images showing the cytoskeleton of mouse oocytes treated with cytochalasin B (Cyto B; n=8) for 10 minutes and allowed to recover for 60 minutes (n=5), 90 minutes (n=7) and 120 minutes (n=2) post-inhibitor wash-out. Oocytes were labelled with phalloidin (actin; green), an antibody against alpha-tubulin (microtubules; red) and DAPI (DNA; blue). Scale bar = 10µm.

The concentration of cytochalasin B used is the same as reported in the literature (Paull *et al.*, 2013; Tachibana *et al.*, 2013). One possibility is that the ten minute exposure time is not long enough for cytochalasin B to exert its effects. However, when this inhibitor was used in initial PNT experiments, it was found to be unreliable with high batch-to-batch variability.

5.2.2 How quickly are the effects of actin inhibitors reversed in the oocyte?

The actin inhibitor must have low toxicity and be rapidly reversible following inhibitor wash-out. After MST, oocytes must be fertilised by intracytoplasmic sperm injection (ICSI). If the effects of the inhibitor are not reversed prior to fertilisation, this could disrupt polar body extrusion. I compared the reversibility of latrunculin A and cytochalasin B by fixing mouse oocytes at set time points following exposure to the selected actin inhibitor (section 5.2.1; Figure 5.2) and inhibitor wash-out. I performed immunofluorescence labelling and confocal imaging to determine whether the effect inhibitors on the cytoskeleton are reversible.

Although latrunculin A had a disruptive effect on cortical actin in the oocyte, including the actin cap, oocytes treated with latrunculin A show complete recovery of the actin cytoskeleton within 60 minutes (Figure 5.3b). Oocytes incubated with cytochalasin B showed little disruption of the actin cytoskeleton. However, I did observe disruption of the metaphase II spindle in one oocyte 60 minutes post-inhibitor wash-out. This occurred in only 1 of a total of 33 oocytes treated with cytochalasin B, so it could be a random event. However, it was not observed in any of the 12 untreated oocytes or 31 oocytes treated with latrunculin A.

5.2.3 Summary

I have compared the effect and reversibility of cytochalasin B, the actin polymerisation inhibitor commonly used for spindle transfer, with latrunculin A. I observed extensive disruption of cortical actin following a ten minute incubation of mouse oocytes with latrunculin A, which was recovered within 60 minutes post-inhibitor wash out. However, incubation of oocytes with cytochalasin B had little effect; it is possible that a ten minute incubation period is insufficient. A previous study of MST using human oocytes found high levels of abnormal fertilisation (Tachibana *et al.*, 2013). It will be interesting to perform MST using either latrunculin A or cytochalasin B to test whether the incidence of abnormal fertilisation could be reduced by using latrunculin A.

5.3 The effect and reversibility of cytoskeletal inhibitors in the zygote

5.3.1 How effective and reversible are latrunculin A and nocodazole?

Proof of concept studies for PNT (Craven *et al.*, 2010) and LtPNT experiments (Chapter 4.2) were performed using the actin inhibitors latrunculin A and/or cytochalasin B. We found cytochalasin B to be unreliable due to variability between batches, which hindered experiments. Therefore, we chose to use latrunculin A alongside nocodazole which targets the microtubules for ePNT manipulations (chapter 4.3).

I tested the effect of latrunculin A and nocodazole using mouse zygotes and abnormally fertilised human zygotes. These experiments were performed at a time when sucrose was routinely added to the enucleation medium to reduce the risk of damage during the manipulations. Zygotes were incubated with sucrose and cytoskeletal inhibitors for 10 minutes before fixation using 2% PFA. Zygotes that had not been exposed to inhibitor were also fixed for comparison. These experiments included controls for exposure to sucrose which was present in the inhibitor solution. Immunofluorescence labelling and confocal imaging was performed to investigate the effect of inhibitors on the cytoskeleton.

Untreated control zygotes stained with the F-actin probe phalloidin show consistent distribution of actin filaments throughout the cytoplasm, with actin enriched at the cortex (Figure 5.4). I found that latrunculin A rapidly depolymerised cytoplasmic actin in both human and mouse zygotes; a reduction in fluorescence was visible following only a ten minute incubation with inhibitor before fixation (Figure 5.4). However, latrunculin A did not seem to have an effect on cortical actin.

Staining of zygotes with an antibody against alpha-tubulin enables visualisation of microtubules, which radiate from MTOCs close to the pronuclei (Figure 5.4). As would be expected, exposure to sucrose caused 'compaction' of the microtubule network (Figure 5.4). A ten minute incubation with nocodazole appears to cause the microtubule network of mouse zygotes to disassemble (Figure 5.4b). The effect of nocodazole on microtubules in abnormally fertilised human zygotes is not as severe; I observed partial disassembly of microtubules (Figure 5.4a). This may reflect differences in microtubule organisation between mouse and human zygotes. A number of zygotes were incubated and fixed at set time points following inhibitor wash-out, in order to determine the length of time taken for the cytoskeleton to return to control state. Immunofluorescence labelling and confocal imaging allowed me to observe recovery of the cytoskeleton (Figure 5.5).

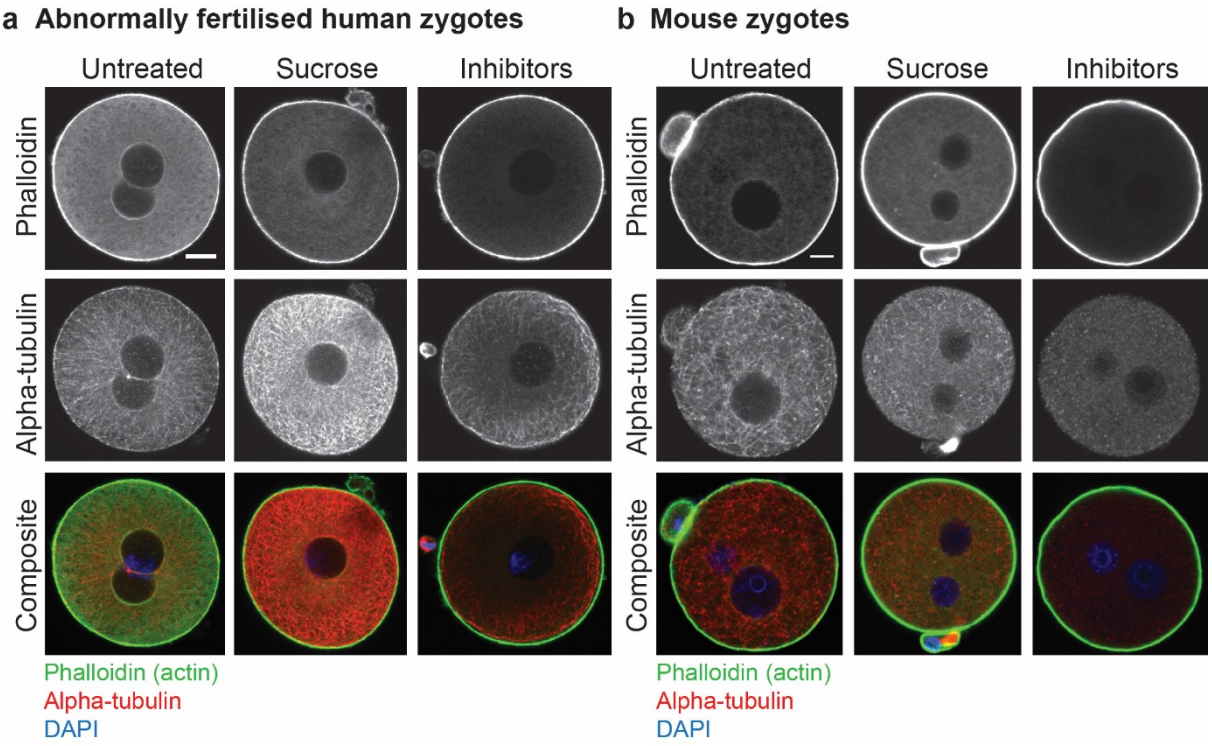


Figure 5.4: The effect of cytoskeletal inhibitors latrunculin A and nocodazole on the cytoskeleton of mouse and abnormally fertilised human zygotes. a) Representative confocal images showing the actin (phalloidin; green) and microtubule (alpha-tubulin; red) cytoskeleton of abnormally fertilised human zygotes which were untreated (n=9), sucrose treated (n=7) or treated with inhibitors (n=12) for 10 minutes before fixation. Scale bar = 20µm. **b)** Representative confocal images showing the actin (phalloidin; green) and microtubule (alpha-tubulin; red) cytoskeleton of mouse zygotes which were untreated (n=11), sucrose treated (n=10) or treated with inhibitors (n=10) for 10 minutes before fixation. Zygotes were also stained with DAPI (DNA; blue). Inhibitor treated zygotes were exposed to latrunculin A and nocodazole that target actin polymerisation and microtubule polymerisation, respectively. Scale bar = 10µm. All images show only one Z-section, therefore not all pronuclei may not be visible.

Firstly, I determined whether the cytoskeleton in abnormally fertilised human zygotes had recovered at 30 minutes and 60 minutes post-inhibitor wash-out. Results indicate that nocodazole is quickly reversible, as the microtubule network appears comparable to controls following a 60 minute recovery period (Figure 5.5). By contrast, recovery of the actin cytoskeleton does not occur within the 60 minute period; the presence of cytoplasmic actin appears to be reduced compared to untreated controls (Figure 5.5). I therefore decided to extend the recovery period to 120 minutes to determine whether the actin cytoskeleton recovered within this time period; I found that at 120 minutes post-inhibitor wash-out the actin network of zygotes appears comparable to untreated controls (Figure 5.6). The number of human zygotes used for extended recovery to 120 minutes is low, this is because abnormally fertilised human zygotes are allocated to research from the clinical laboratory

quite late (~20-22 hours) after fertilisation and the majority of zygotes underwent pronuclear breakdown or spontaneous division during the extended recovery phase.

I also analysed recovery of mouse zygotes following incubation with inhibitors (Figure 5.7). I found that nocodazole did not appear to be as reversible in mouse zygotes as human zygotes, as at 60 minutes recovery images were not comparable to controls (Figure 5.7). This is likely because the effects of nocodazole were greater on mouse zygotes, for reasons discussed previously, thus the recovery period is extended. It is important to note that although microtubules appear completely disassembled in mouse zygotes immediately after a 10 minute incubation, this is effect is fully reversible after 90-120 minutes.

Based on immunofluorescence images, I estimate that recovery of actin filaments in the cytoplasm occurs between 90 minutes and 120 minutes post-inhibitor wash-out. It is interesting that mouse oocytes recovered more quickly than zygotes following exposure to latrunculin A, although extensive disruption to the cytoskeleton was observed in oocytes (Figure 5.2). This may reflect differences in cytoskeleton dynamics in the oocyte before and after fertilisation. For example, the oocyte is in M-phase of the cell cycle whereas the zygote is in interphase; it is known that the actin cytoskeleton is linked to cell cycle progression (reviewed in Heng and Koh (2010)).

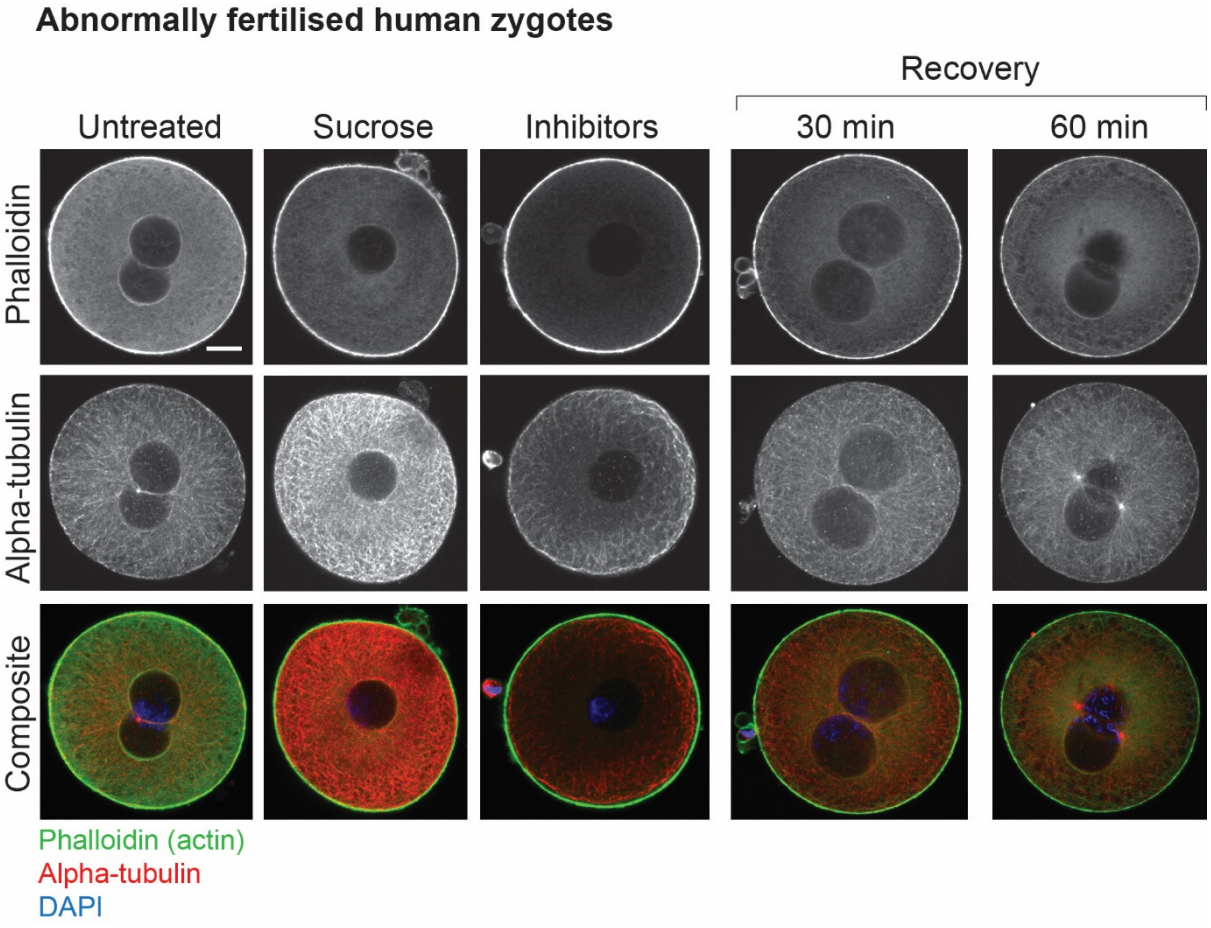


Figure 5.5: Reversibility of cytoskeletal inhibitors latrunculin A and nocodazole in abnormally fertilised human zygotes. Representative confocal images of abnormally fertilised human zygotes treated with cytoskeletal inhibitors latrunculin A which inhibits actin polymerisation and nocodazole which inhibits microtubule polymerisation, for 10 minutes. Zygotes were allowed to recover for 30 minutes (n=7) and 60 minutes (n=6) post-inhibitor wash-out. These images are compared to images of untreated, sucrose and inhibitor treated zygotes shown in figure 5.4. Zygotes were labelled with phalloidin (actin; green), an antibody against alpha-tubulin (microtubules; red) and DAPI (DNA; blue). Scale bar = 20µm. All images show only one Z-section, therefore not all pronuclei may not be visible.

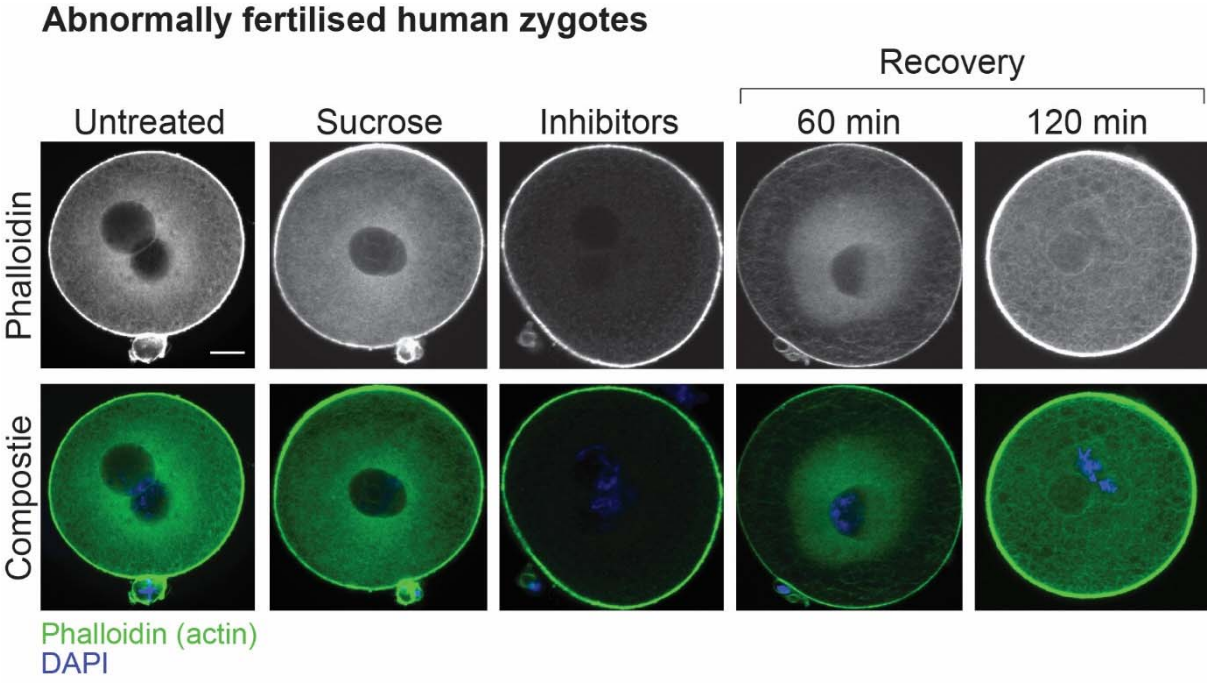


Figure 5.6: Recovery of the actin cytoskeleton of abnormally fertilised human zygotes following treatment with cytoskeletal inhibitors. Representative confocal images showing untreated (n=9), sucrose (n=7), inhibitors (n=12) treated zygotes with 60 minute (n=6) and 120 minute (n=3) recovery periods. Zygotes were incubated with latrunculin A and nocodazole for ten minutes. Zygotes were labelled with phalloidin (actin; green), an antibody against alpha-tubulin (microtubules; red) and DAPI (DNA; blue). Scale bar = 20µm. All images show only one Z-section, therefore not all pronuclei may not be visible.

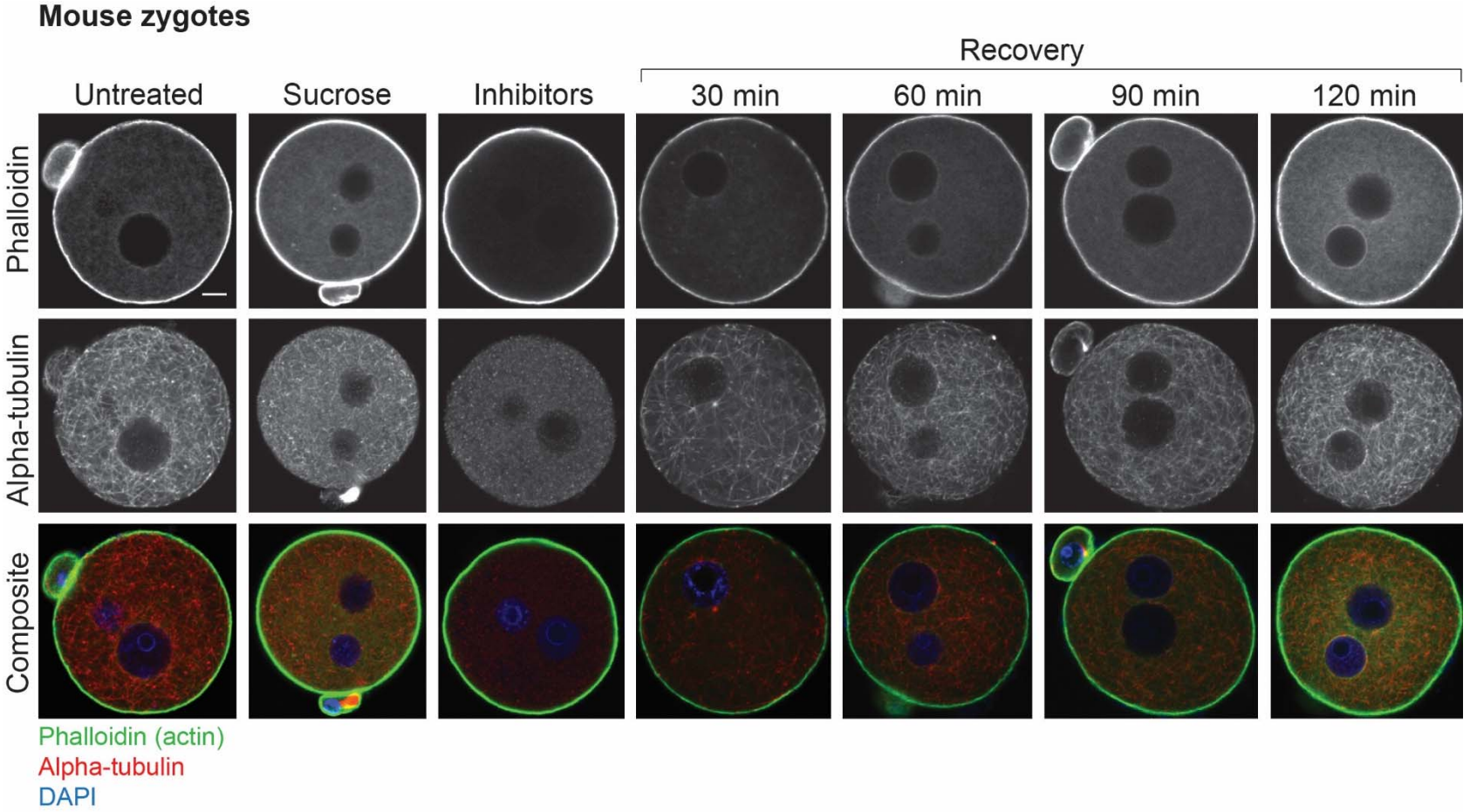


Figure 5.7: Reversibility of cytoskeletal inhibitors latrunculin A and nocodazole in mouse zygotes. Representative confocal images of mouse zygotes treated with cytoskeletal inhibitors latrunculin A and nocodazole for 10 minutes. Zygotes were allowed to recover for 30 minutes (n=6), 60 minutes (n=6), 90 minutes (n=8) and 120 minutes (n=8) post-inhibitor wash-out. These images are compared to images of untreated, sucrose and inhibitor treated zygotes shown in figure 5.4. Zygotes were labelled with phalloidin (actin; green), an antibody against alpha-tubulin (microtubules; red) and DAPI (DNA; blue). Scale bar = 10µm. All images show only one Z-section, therefore not all pronuclei may not be visible.

5.3.2 Can alternative actin inhibitors speed up recovery in zygotes?

Latrunculin B

Experiments described above indicate that nocodazole is fast-acting and rapidly reversible but latrunculin A requires a longer recovery period post-inhibitor wash-out. This could negatively affect events occurring in the zygote, for example cytoplasmic actin filaments may play a role in pronuclear migration and maintenance of a centralised mitotic spindle (Chaigne *et al.*, 2016). Therefore, we decided to test alternative inhibitors which target actin polymerisation. Latrunculin B is less potent than latrunculin A (Spector *et al.*, 1989) and recommended for short term studies as its effects are transient.

I compared the effects of latrunculin A (2.5μM) and latrunculin B (2μM) in abnormally fertilised human zygotes. Again, untreated controls and zygotes exposed to sucrose are included in Figure 5.8a for comparison. I found that latrunculin B had a disruptive effect on cortical actin which was not observed following treatment with latrunculin A (Figure 5.8b, c). Latrunculin B also appeared to depolymerise cytoplasmic actin filaments, as phalloidin fluorescence in the cytoplasm was reduced compared to untreated controls (Figure 5.8a, c).

Zygotes were fixed 15 minutes and 30 minutes after wash-out of latrunculin A or latrunculin B (Figure 5.8b, c). As discussed previously (section 5.3.1), the actin cytoskeleton of abnormally fertilised human zygotes is not fully recovered until approximately 120 minutes after treatment with latrunculin A (Figure 5.6). As expected, in this series of experiments the fluorescence of cytoplasmic phalloidin was not comparable to untreated controls within the 30 minute recovery period (Figure 5.8b). On the other hand, despite latrunculin B appearing to have a more disruptive effect on cortical actin, the actin cytoskeleton of zygotes treated with latrunculin B is quickly reassembled (Figure 5.8c). Zygotes treated with latrunculin B are comparable to controls at 30 minutes post-inhibitor wash-out. The faster recovery time following latrunculin B treatment may make latrunculin B a better option than latrunculin A for PNT.

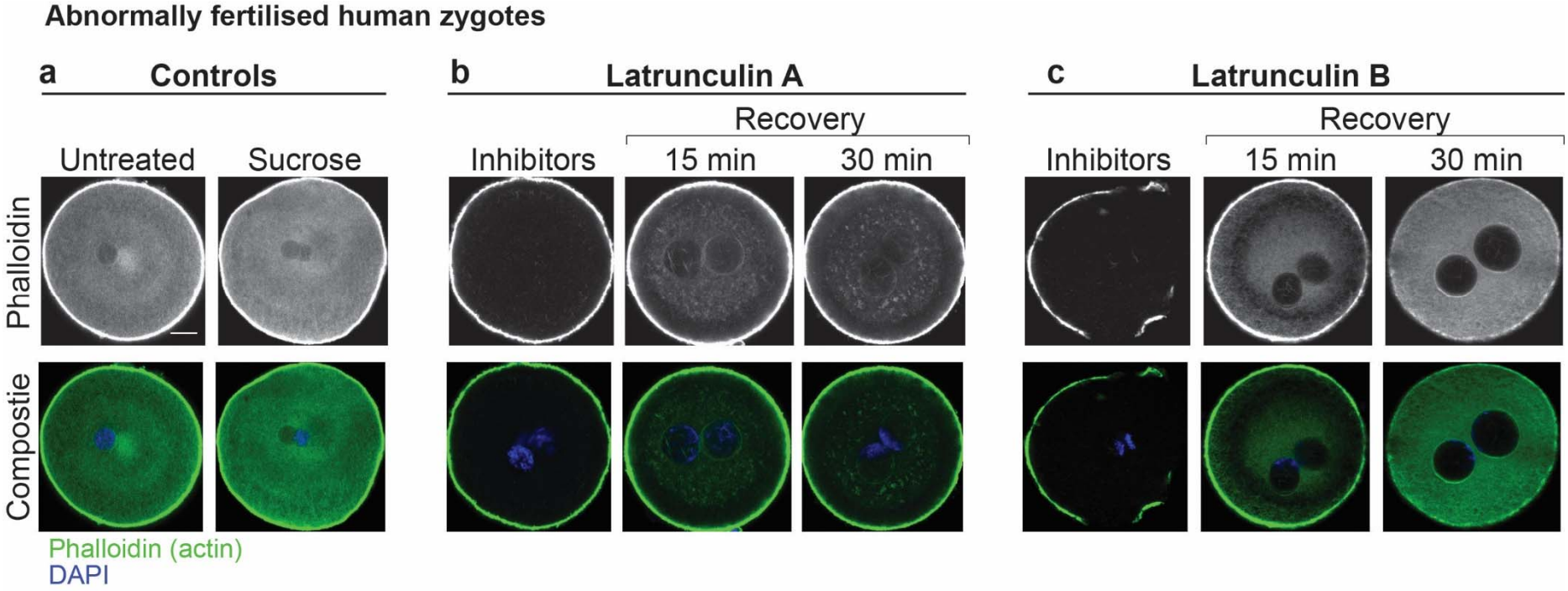


Figure 5.8: The effect and reversibility of actin inhibitors latrunculin A and latrunculin B on abnormally fertilised human zygotes. Representative confocal images of **a)** Untreated (n=13) and sucrose (n=7) controls. **b)** Latrunculin A (n=11) treated zygotes with 15 minute (n=6) and 30 minute (n=7) recovery. **c)** Latrunculin B (n=6) treated zygotes with 15 minute (n=5) and 30 minute (n=6) recovery. Nocodazole was also present in the inhibitor solution, in which zygotes were incubated for 10 minutes. Zygotes were labelled with phalloidin (actin; green), an antibody against alpha-tubulin (microtubules; red) and DAPI (DNA; blue). Scale bar = 20μm. All images show only one Z-section, therefore not all pronuclei may not be visible.

Cytochalasin C

Although we excluded cytochalasin B due to inconsistency between batches, cytochalasin C may be a promising options as it displays reduced toxicity in mice compared to other cytochalasins (Walling *et al.*, 1988). I first tested the effect and reversibility of cytochalasin C using mouse zygotes (Figure 5.9). I found the effect of this inhibitor to be similar to that observed following latrunculin B treatment of abnormally fertilised human zygotes, with disruption of cortical actin in addition to cytoplasmic actin. However, zygotes were recovered approximately 60 minutes following inhibitor wash-out, which is not as prompt as latrunculin B but faster than latrunculin A recovery.

Next, I tested cytochalasin C using abnormally fertilised human zygotes (Figure 5.10). Surprisingly, I found that many zygotes were degenerating during the recovery period following inhibitor treatment. For this reason, I was not able to fix many zygotes. Of those that were fixed after a 60 minute recovery period, zygotes showed signs of degeneration and the actin cytoskeleton was highly abnormal. It is unusual that this inhibitor used at the same concentration and same exposure time would have such different effects on mouse and human zygotes, and may point to differences in the actin cytoskeleton between mouse and human zygotes, or abnormally fertilised zygotes.

Summary

Due to the slow recovery of zygotes treated with the actin polymerisation inhibitor latrunculin A, I decided to test alternative inhibitors which may speed up recovery. The inhibitors selected were latrunculin B and cytochalasin C, as previous studies have shown they are less toxic than other inhibitors of the same family (Walling *et al.*, 1988; Spector *et al.*, 1989). Although mouse zygotes showed relatively quick recovery following incubation with cytochalasin C, this inhibitor seemed to cause degeneration of human zygotes. Due to the negative effect of this inhibitor on human zygotes, it would not be suitable for use in PNT. However, immunofluorescence labelling indicates that actin filaments of human zygotes treated with latrunculin B recover within 30 minutes post-inhibitor wash-out. This is an improvement on the 120 minute recovery period for latrunculin A treated zygotes. The quicker recovery period of zygotes following latrunculin B treatment is likely due to the reduced potency of latrunculin B, and its transient effects due to gradual inactivation by serum (Spector *et al.*, 1989).

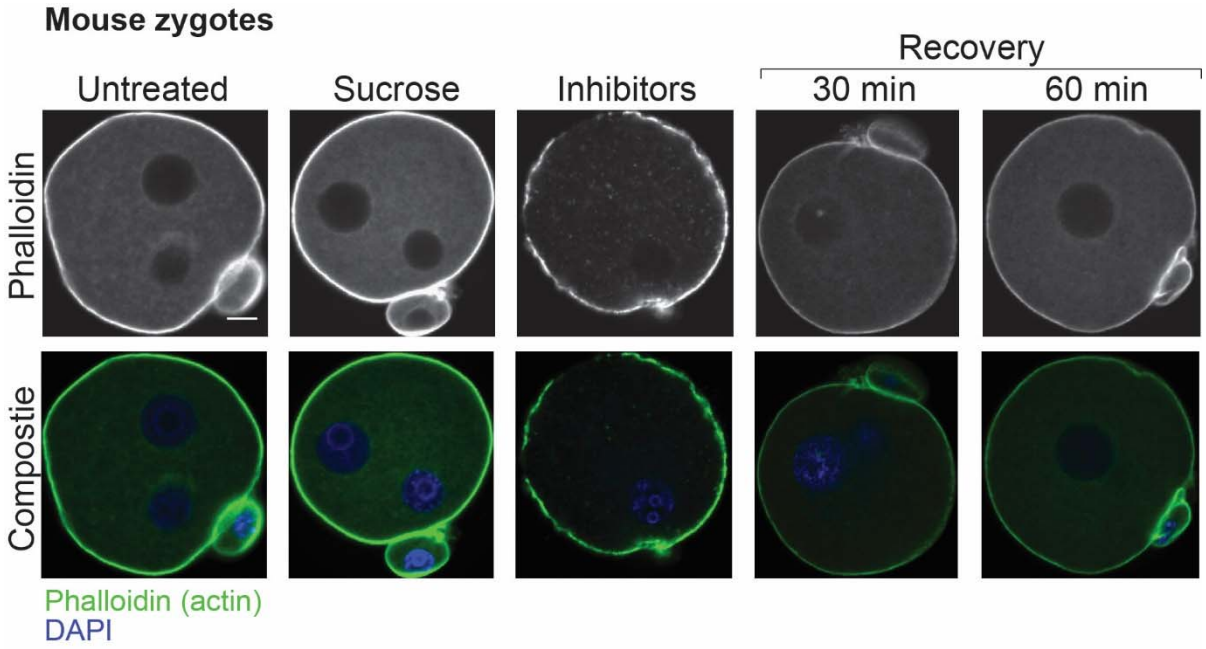


Figure 5.9: The effect and reversibility of actin inhibitor cytochalasin C on mouse zygotes. Representative confocal images of mouse zygotes which were; untreated (n=4), sucrose (n=4), inhibitor (n=3) treated, with 30 minute (n=5) and 60 minute (n=5) recovery periods. Nocodazole was also present in the inhibitor solution, in which zygotes were incubated for 10 minutes. Zygotes were stained with phalloidin (actin; green) and DAPI (DNA; blue). Scale bar = 10µm. All images show only one Z-section, therefore not all pronuclei may not be visible.

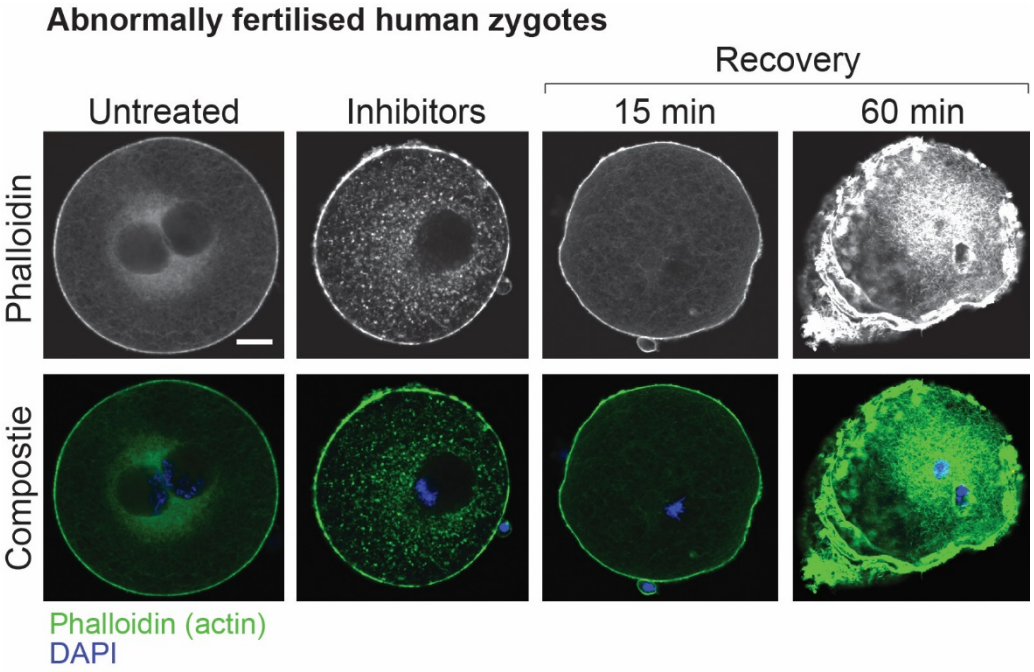


Figure 5.10: The effect and reversibility of actin inhibitor cytochalasin C on abnormally fertilised human zygotes. Representative confocal images of zygotes which were; untreated (n=6), inhibitor (n=9) treated, with 15 minute (n=2) and 60 minute (n=3) recovery periods. Nocodazole was also present in the inhibitor solution, in which zygotes were incubated for 10 minutes. Zygotes were stained with phalloidin (actin; green) and DAPI (DNA; blue). Scale bar = 20µm. All images show only one Z-section, therefore not all pronuclei may not be visible.

5.4 Embryo development following PNT: comparison of actin inhibitors

The experiments described above have investigated the effect and short term reversibility of cytoskeletal inhibitors. I have found that nocodazole is fast-acting and quickly reversible. However, latrunculin B displays faster reversibility than latrunculin A, which is currently used in PNT. The next stage is to test whether latrunculin B may improve blastocyst formation and quality if used as an alternative to latrunculin A in PNT.

5.4.1 Human ePNT: latrunculin A versus latrunculin B

A series of human autologous ePNT experiments using nocodazole and latrunculin B were performed alongside autologous ePNT experiments using nocodazole and latrunculin A. All ePNT experiments using human zygotes were performed by Dr Louise Hyslop, an experienced clinical embryologist. I analysed survival, blastocyst formation and blastocyst quality following ePNT using either latrunculin A or latrunculin B (Figure 5.11).

Comparison of the survival of latrunculin A and latrunculin B ePNT zygotes shows a trend towards reduced survival of latrunculin B treated ePNT zygotes (Figure 5.11a). This reduction in survival is reflected in blastocyst formation data (Figure 5.11b, c). On day 5 and 6 of development, there is a trend towards reduced blastocyst formation of latrunculin B treated ePNT embryos (36.4% day 5; 45.5% day 6) compared to unmanipulated controls (52.4% day 5; 61.9% day 6). This contrasts to latrunculin A ePNT zygotes, which show increased blastocyst formation compared to controls on day 5 (61.9% versus 52.4%) and are comparable to controls on day 6 (61.9%). When I take into account the reduced survival and calculate blastocyst formation as a percentage of zygotes which survived manipulation rather than a percentage of all zygotes submitted to ePNT, the percentage blastocyst formation of latrunculin B is improved but the trend of the data remains the same (Figure 5.11c).

Blastocysts developing following ePNT procedures were given grades reflecting their quality scores. The grading scheme for ePNT blastocysts is consistent with that used in the clinical laboratory at Newcastle Fertility Centre, which was discussed in more detail in Chapter 4.1. The quality scores assigned to ePNT blastocysts treated with latrunculin A are similar to controls on days 5 and 6 of development (Figure 5.11d). Statistical analysis revealed the quality of latrunculin B treated ePNT blastocysts was significantly reduced compared to controls and latrunculin A treated ePNT blastocysts ($P<0.05$). Of the ePNT zygotes developing to the blastocyst stage following treatment with latrunculin B, all were assigned a grade E (early) on day 5. By contrast, 36.4% of controls and 23.1% latrunculin A ePNT blastocysts were good quality (grade A/B) on day 5 (Figure 5.11d). The percentage of good quality blastocysts increased to 76.9% for controls and latrunculin A-treated ePNT blastocysts, whereas only 20% of latrunculin B ePNT blastocysts were good quality on day 6, with a high proportion remaining grade E (40%). This suggests that in addition to reducing blastocyst formation, latrunculin B also has a negative impact on blastocyst quality and rate of development.

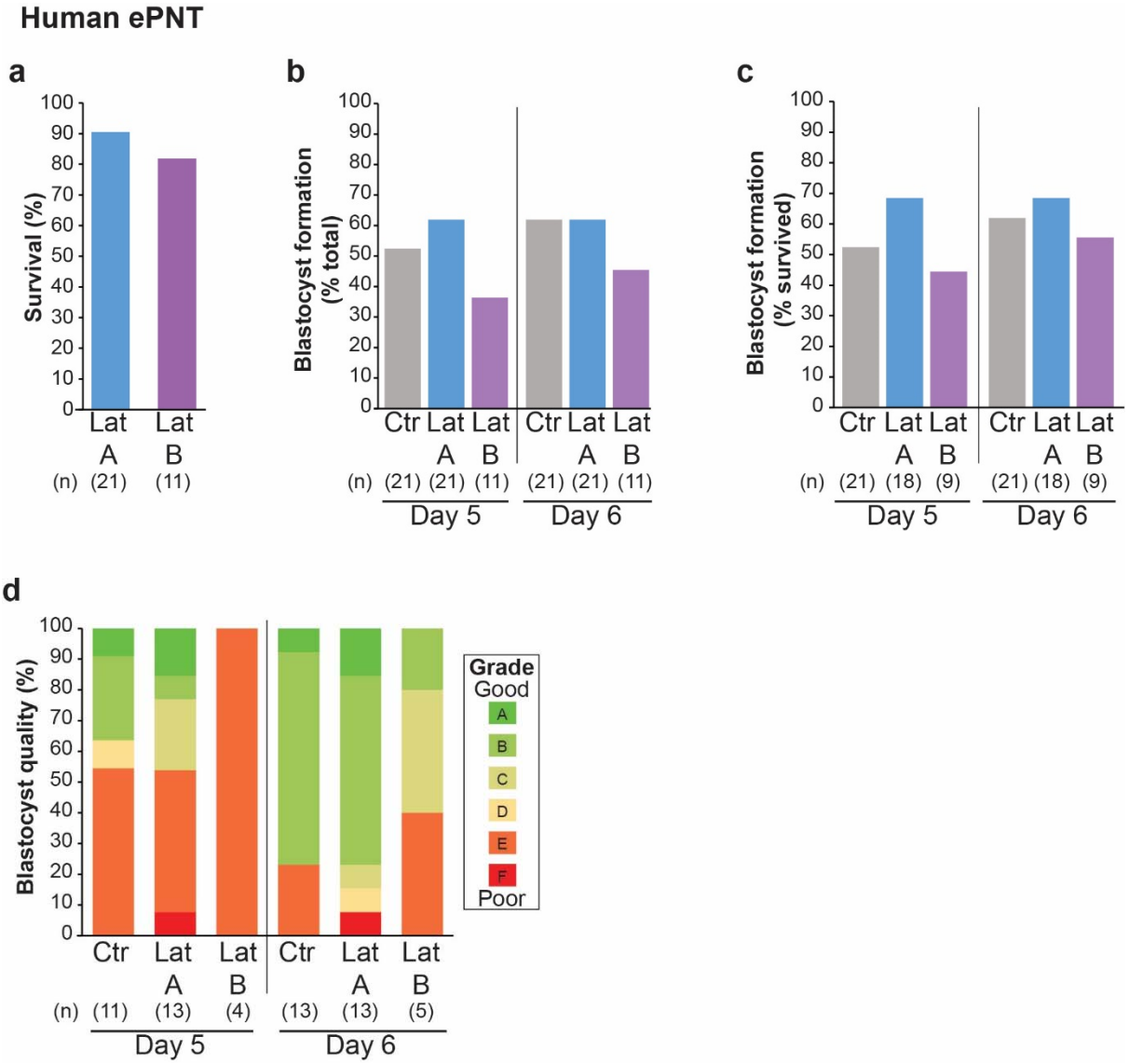


Figure 5.11: Survival and blastocyst development following human ePNT using either latrunculin A or latrunculin B to inhibit actin polymerisation. Human autologous ePNT experiments were performed using cytoskeletal inhibitors latrunculin B and nocodazole. This data was compared to data from unmanipulated controls and autologous ePNT (series II) experiments in which latrunculin A and nocodazole were used. **a)** Survival of zygotes following latrunculin A (Lat A) ePNT and latrunculin B (Lat B) ePNT. Difference is not significant (chi-squared test). **b)** Blastocyst formation as a percentage of total zygotes submitted to ePNT in unmanipulated controls (Ctr), Lat A and Lat B ePNT embryos. Not significant (Fisher's exact test). **c)** Blastocyst formation as a percentage of zygotes surviving manipulations. Not significant (chi-squared test). **d)** Quality of unmanipulated controls (Ctr) and latrunculin A/ latrunculin B (Lat A/ Lat B) ePNT blastocysts. Blastocysts were assigned a grade from A-D (good to poor quality), E (early) and F (blastocyst showing signs of degeneration). Latrunculin B treated ePNT zygotes are of significantly poorer quality than control and latrunculin A treated ePNT zygotes on day 5 and day 6 ($P<0.05$; Fisher's exact test). For this analysis, grades were grouped (grades A/B versus grades C-F).

5.4.2 Mouse PNT: latrunculin A versus latrunculin B

The human ePNT experiments described in section 5.5.1 comparing latrunculin A and latrunculin B were repeated using mouse zygotes, as only a small number (n=11) of human ePNT zygotes were treated with latrunculin B. Mouse PNT experiments discussed in Chapter 4.5 were performed using latrunculin A; I therefore compared these data to latrunculin B mouse PNT development data.

I found survival to be comparable between latrunculin A (88.5%) and latrunculin B (87%) PNT zygotes (Figure 5.12a). Blastocyst formation was reduced following PNT in the presence of latrunculin A or latrunculin B compared to controls ($P<0.05$; Figure 5.12b). However, when accounting for the reduction in survival, blastocyst formation following PNT using latrunculin A is comparable to controls, whereas there remains a significant reduction in blastocyst formation following PNT in the presence of latrunculin B ($P<0.05$; Figure 5.12c).

Taken together with the data from human ePNT, these results raise concerns about the safety of latrunculin B for use in PNT. This is surprising, as zygotes treated with latrunculin B displayed a faster short term recovery than those treated with latrunculin A (section 5.3.2; Figure 5.8). It is possible that the disruptive effect of latrunculin B on cortical actin causes excessive leakage of cytoplasm during enucleation, which could affect embryo development. We conclude that latrunculin A is the preferable inhibitor of actin polymerisation for use in conjunction with the microtubule polymerisation inhibitor nocodazole during ePNT.

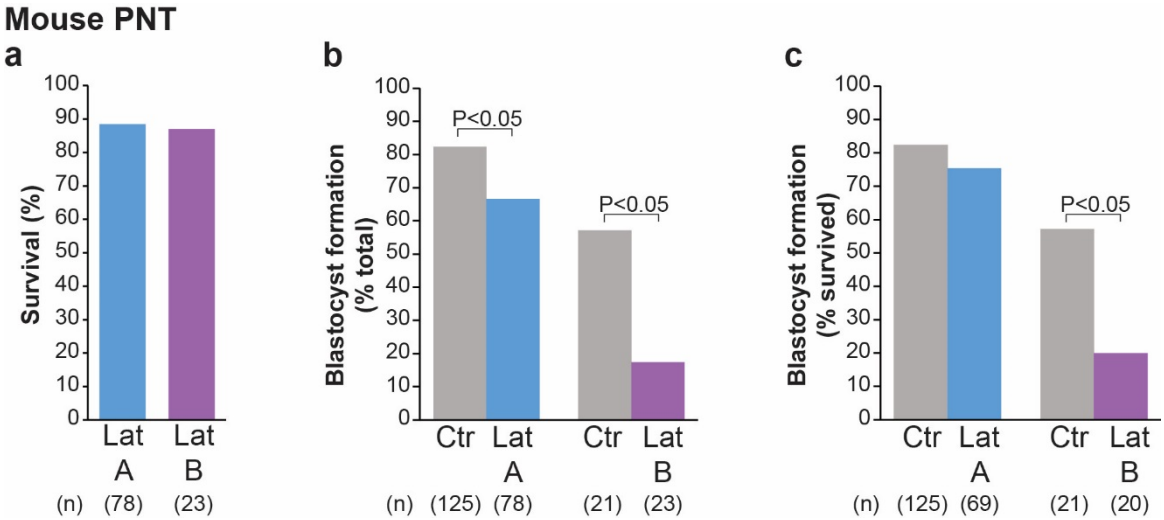


Figure 5.12: Survival and blastocyst formation following mouse PNT using either latrunculin A or latrunculin B to inhibit actin polymerisation. A series of mouse PNT experiments were performed using cytoskeletal inhibitors latrunculin B and nocodazole. This data was compared to data from unmanipulated controls and mouse PNT experiments that used latrunculin A and nocodazole. **a)** Survival of zygotes following latrunculin A (Lat A) PNT and latrunculin B (Lat B) PNT. Difference is not significant (chi-squared test). **b)** Blastocyst formation as a percentage of total zygotes submitted to PNT in unmanipulated controls (Ctr), Lat A and Lat B PNT embryos. Significance is indicated on the graph (chi-squared test). **c)** Blastocyst formation as a percentage of zygotes surviving manipulations. Significance is indicated on the graph (chi-squared test).

5.5 The effect of sucrose and cytoskeletal inhibitors on mitochondrial distribution in zygotes

In addition to investigating the effect of actin and microtubule inhibitors on the cytoskeleton, I performed MitoTracker® staining and live-cell confocal imaging of zygotes to determine the effect of sucrose and cytoskeletal inhibitors on the distribution of mitochondria in the cytoplasm. This is important because mitochondria are trafficked by microtubules (Van Blerkom, 1991). Any aggregation of mitochondria around the pronuclei as a consequence of the reagents would likely result in increased mitochondrial DNA carryover during PNT.

Sucrose was added to the manipulation medium in the first series of ePNT experiments to induce shrinkage of the cytoplasm, increasing the size of the peri-vitelline space thus facilitating enucleation and minimising risk of damage to the membrane. However, during a second series of ePNT experiments (series II) we found that the osmotic effect of sucrose may be contributing to a high level of carryover of karyoplast mtDNA (Hyslop *et al.*, 2016). MitoTracker® staining and live-cell imaging may give some insights regarding whether the use of sucrose causes aggregation of mitochondria in the vicinity of the pronuclei.

Analysis of mitochondrial distribution by live cell imaging of zygotes stained with MitoTracker® Red CMXRos revealed that mitochondria in mouse zygotes appear to be consistently localised to the central area of the zygote, with less mitochondria at the periphery of the zygote (Figure 5.13). Interestingly, I found that mitochondria appeared to be consistently enriched around the smaller pronucleus, which in mouse zygotes is the female pronucleus.

During incubation with sucrose solution, more mitochondria appear to be distributed centrally in the zygote, close to the pronuclei. This would likely contribute to increased carryover of mtDNA during PNT. In zygotes exposed to cytoskeletal inhibitors, MitoTracker® staining appears to show increased aggregation of mitochondria. It is expected that inhibitors would alter mitochondrial distribution, as nocodazole targets microtubules, known to be involved in mitochondrial transport (Van Blerkom, 1991). After a 30 minute recovery period, the mitochondrial distribution of zygotes is comparable to MitoTracker® staining observed before treatment. However, in order to minimise carryover of mtDNA during PNT sucrose should be eliminated from the enucleation medium, as aggregation of mitochondria around the pronuclei at the time of PNT is likely to increase mtDNA carryover.

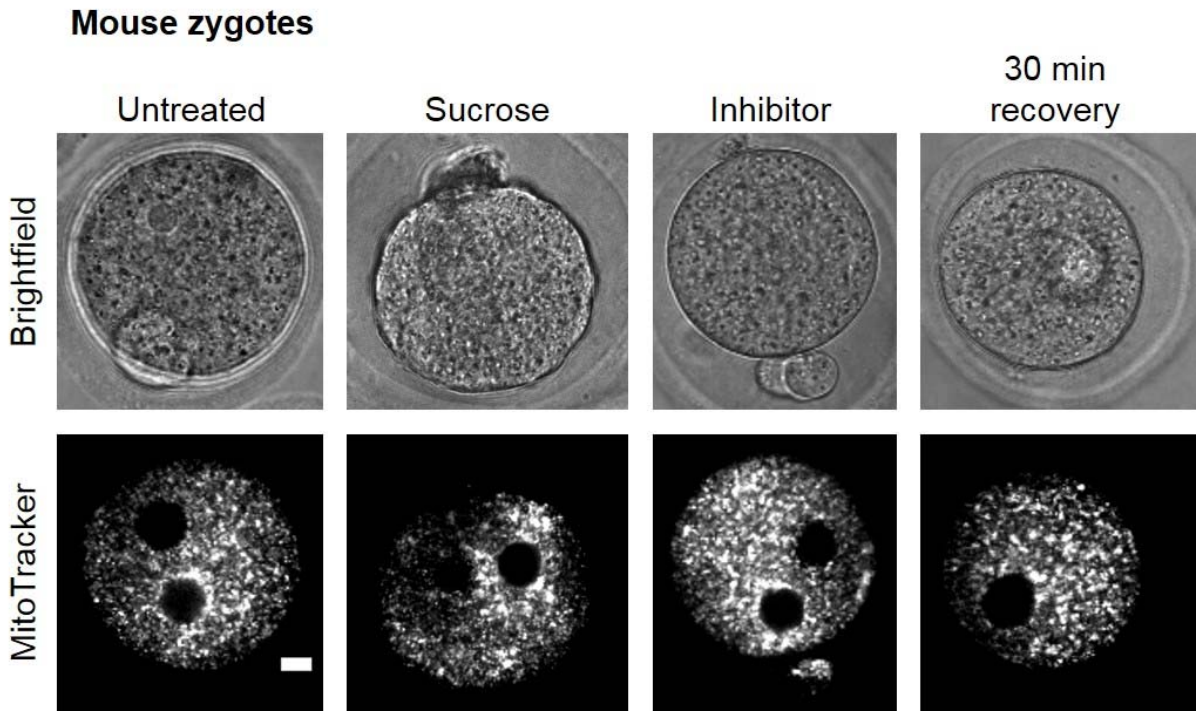


Figure 5.13: The effect of sucrose and cytoskeletal inhibitors on mitochondrial distribution in mouse zygotes. A total of 18 zygotes from 2 mice underwent MitoTracker® Red CMXRos staining and live-cell confocal imaging. Images show MitoTracker® staining before, during and 30 minutes after treatment with sucrose and the cytoskeletal inhibitors nocodazole and latrunculin A. Scale bar = 10µm.

5.6 Discussion

In this chapter I have investigated the effect and reversibility of cytoskeletal inhibitors used for MST and PNT. Firstly, I tested the effect of inhibitors of actin polymerisation in mouse oocytes. Cytochalasin B is commonly used for MST, however, because we found cytochalasin B to be unreliable, we opted to use latrunculin A for the ePNT experiments. Comparison of cytochalasin B and latrunculin A using immunofluorescence labelling and confocal imaging of mouse oocytes indicates that latrunculin A efficiently disrupts the actin cytoskeleton, including the actin cap. Despite this, recovery of oocytes was quick as an intact actin cap was visible in oocytes following a 30 minute recovery period. I found that cytochalasin B seemed to have little effect on the actin cytoskeleton although two batches were tested.

Previous reports of MST in human oocytes involved the use of cytochalasin B at a concentration of 5µg/ml (Paull *et al.*, 2013; Tachibana *et al.*, 2013). At this concentration I

found the effect of cytochalasin B to be minimal. Initially, I suspected this could be due to the short exposure time of 10 minutes. Tachibana *et al.* incubated oocytes for 10-15 minutes with cytochalasin B before performing manipulations. This supports the idea that cytochalasin B may not be effective within 10 minute to facilitate spindle removal. On the other hand, Paull *et al.* state that oocytes were incubated with inhibitor for only 3-5 minutes prior to manipulation. However, neither of the above studies directly investigated the effect of the cytochalasin B on the actin network.

Tachibana *et al.* (2013) reported a high incidence of abnormal fertilisation when human oocytes were fertilised by ICSI following MST. Abnormal fertilisation, identified by the presence of 1 pronucleus or more than 2 pronuclei, was observed in 52% of spindle transfer zygotes in this study (Tachibana *et al.*, 2013). The authors found that this was likely due to the retention of genetic material from the second polar body. In an attempt to reduce any residual effects of the inhibitor, the authors extended the time between spindle transfer and ICSI from 30 minutes to 2 hours. Surprisingly, incidence of abnormal fertilisation was consistent between both groups (44% versus 43%). This may suggest that oocytes had not fully recovered from inhibitor exposure and/or manipulations after two hours.

The possibility of cytochalasin B contributing to abnormal fertilisation was also considered in this study (Tachibana *et al.*, 2013), as actin inhibitors are commonly used to prevent cytokinesis and polar body extrusion in artificial activation procedures. The concentration of cytochalasin B was reduced from 5µg/ml to 2.5µg/ml, or completely excluded. However, abnormal fertilisation persisted in all conditions and the authors conclude that 'abnormal meiotic segregation is not likely to be caused by cytochalasin B exposure' (Tachibana *et al.*, 2013). However, the 100% abnormal fertilisation reported in spindle transfer oocytes not exposed to cytochalasin B refers to only 1/1 oocyte. I expect that removal of the spindle was difficult in the absence of an actin polymerisation inhibitor, which could have put excessive stress on the oocyte. Due to only one oocyte being included in this experimental group, I do not think it can be concluded that exposure to cytochalasin B did not contribute to abnormal fertilisation. It will be interesting to further investigate this, and compare the outcomes of spindle transfer using latrunculin A or cytochalasin B.

I have tested the effect and reversibility cytoskeletal inhibitors in order to select optimal inhibitors for PNT. Early PNT experiments were performed using latrunculin A to inhibit actin polymerisation and nocodazole which targets polymerisation of microtubules. These inhibitors were selected based on observations in initial PNT experiments. Treatment of

mouse zygotes and abnormally fertilised human zygotes with nocodazole and latrunculin A and subsequent fixation and immunofluorescence labelling showed that both inhibitors are fast acting. Nocodazole is rapidly reversible, however the effects of latrunculin A take longer to reverse. Therefore, I tested latrunculin B, which has reduced potency (Spector *et al.*, 1989), and found the recovery period could be reduced to 30 minutes.

However, immunofluorescence labelling showed latrunculin B also affected cortical actin whereas latrunculin A did not. This may have contributed to the adverse effect of latrunculin B on onward development; latrunculin B treated ePNT embryos showed reduced blastocyst formation in human ePNT and mouse PNT. Surprisingly, during human ePNT the effects of latrunculin B were found to be more potent than latrunculin A when used at the same concentration (2.5μM). Therefore, a range of concentrations were tested and a slightly reduced concentration was used (2μM). In mouse PNT we observed excessive cytoplasmic leakage during manipulations using latrunculin B, which may have contributed to the detrimental effect on subsequent embryo development.

An interesting finding was the different effects of cytochalasin C on mouse zygotes and abnormally fertilised human zygotes. Cytochalasin C showed effective depolymerisation and full recovery of the actin cytoskeleton of mouse zygotes within 60 minutes. Furthermore, on one occasion cytochalasin C was used for mouse PNT and resulted in the successful formation of two blastocysts (data not presented due to small numbers). However, abnormally fertilised human zygotes show signs of degeneration following exposure to cytochalasin C. Cytochalasins bind actin filaments to prevent elongation and shortening; they do not bind to actin monomers (Lin *et al.*, 1980). A publication by Walling *et al*. (1988) claims that cytochalasin C is less toxic in mice than its relative cytochalasin D but displays the same biological effectiveness. Cytochalasin C is a relatively new inhibitor, it does not appear to have been commonly used and there is little information in the literature regarding its mechanism of action. The difference in the effect of this inhibitor between mouse and human zygotes may indicate differences in the actin cytoskeleton between mouse and human zygotes. It may be interesting to investigate this further, however these results suggest that cytochalasin C is not a safe inhibitor for the purposes of PNT. Furthermore, the finding underscores the importance of using human oocytes and zygotes to test the safety and efficacy of reagents used for PNT and spindle transfer.

Differences between the microtubule network in mouse and abnormally fertilised human zygotes were also observed when testing nocodazole. There are many factors that may

contribute to the different effect of nocodazole on mouse and human zygotes. Firstly, it could be attributed to the different size of the zygotes and exposure to the same concentrations of inhibitor. Secondly, the zygotes are at different stages; mouse zygotes are at an early stage and pronuclei have only just appeared, whereas abnormally fertilised human zygotes are at a late stage. Furthermore, polyspermy in abnormally fertilised human zygotes may affect the microtubule network; it is possible that the presence of additional microtubule asters from sperm in zygotes with >2 pronuclei would increase microtubule polymerisation. Finally, and importantly, the sperm aster is absent in mouse zygotes as centrosomes are maternally inherited. Therefore, microtubules have different organisations within mouse and human zygotes.

MitoTracker® staining of mouse zygotes revealed the enrichment of mitochondria around the female pronucleus. In mouse zygotes, pronuclear apposition is achieved by both microtubules and microfilaments, as centrosomes are maternally inherited in the mouse. It is possible that mitochondria are also involved in pronuclear apposition, most likely by providing energy for transport.

To conclude, for the purposes of facilitating enucleation and fusion during PNT latrunculin A and nocodazole are the optimal cytoskeletal inhibitors. Sucrose should not be used during manipulations as the increased osmolarity causes aggregation of mitochondria around the pronuclei, which is likely to result in increased mitochondrial DNA carryover.

Chapter 6: Results III: Gene expression patterns in blastocysts following pronuclear transfer

6.1 Introduction

6.1.1 Human blastocyst development and gene expression

The human blastocyst is formed at 5 to 6 days post-fertilisation, following a series of cleavage divisions, compaction and cavitation. This is an important developmental milestone; the embryo cannot implant without developing to the blastocyst stage and hatching from its zona pellucida. The blastocyst is composed of an inner cell mass (ICM) and outer layer known as the trophectoderm (TE). The TE goes on to form the placenta, whereas the ICM is composed of the primitive endoderm (PE) and the epiblast (EPI) which form the yolk sac and foetus, respectively.

Developmental stages from the oocyte to morula are characterised by distinct gene expression profiles (Xue *et al.*, 2013), suggesting stepwise transcriptional changes in cell cycle, metabolism, gene regulation and translation pathways, that are largely conserved between mouse and human. Much of what is known about gene expression and lineage specification in blastocysts is based on findings from the mouse. For example, during blastocyst development in the mouse differential Hippo signalling at compaction triggers the first cell fate decision which separates the ICM and TE (Nishioka *et al.*, 2009). Subsequent segregation of the ICM into the EPI and PE lineages is driven by differential FGF signalling (Guo *et al.*, 2010).

A recent publication (Blakeley *et al.*, 2015) using single-cell RNA-sequencing (scRNA-seq) highlights the similarities and differences between human and mouse lineage-specification during preimplantation development. For example, FOXA2 is restricted to the PE, NANOG to the EPI and CDX2 to the TE lineages of both mouse and human blastocysts. This study also revealed a number of genes and signalling pathways enriched in the human EPI, such as components of the TGF-β signalling pathway. Interestingly, FGF signalling has been shown to not be required for ICM segregation in human (Roode *et al.*, 2012). Another publication using scRNA-seq analysis studied lineage specification using over 1,500 cells from 88 human blastocysts (Petropoulos *et al.*, 2016), and identified a period of co-expression of lineage-associated genes prior to establishment of the TE, PE and EPI lineages. This is consistent with previous reports from immunofluorescence studies indicating mixed expression of lineage-associated transcription factors before ICM cells finally committed to either the EPI or PE

lineage (Chazaud *et al.*, 2006; Plusa *et al.*, 2008; Roode *et al.*, 2012; Niakan and Eggan, 2013).

During preimplantation development it is thought that there is no replication of mtDNA, with the exception of a suggested brief period of mtDNA synthesis in the oocyte post-fertilisation (McConnell and Petrie, 2004). Thus, according to our current understanding, mtDNA is inherited maternally from the oocyte and is segregated between daughter cells during each embryonic division in a regulated manner. In the blastocyst, the trophectoderm has an increased number of mitochondria compared to the inner cell mass, observed following MitoTracker staining, and is responsible for higher oxygen consumption and the production of the majority of ATP (Houghton, 2006). Mitochondria have an essential function in bioenergetics during preimplantation development, with mitochondrial dysfunction compromising developmental success (reviewed in Dumollard *et al.* (2007; Van Blerkom, 2009; Steffann *et al.*, 2015)).

6.1.2 Single-cell RNA-sequencing

As mentioned above, several recent publications investigating gene expression patterns in human preimplantation development have used scRNA-seq (Xue *et al.*, 2013; Yan *et al.*, 2013; Piras *et al.*, 2014; Petropoulos *et al.*, 2016). The ability to obtain gene expression data from single cells allows the investigation of specific biological questions which was not possible using traditional methods. However, there are also challenges associated with this technology. For example, measurements from RNA-seq may be affected by technical variability (Brennecke *et al.*, 2013). Furthermore, as scRNA-seq is a relatively new technology, we are faced with computational challenges and there is currently no standard for scRNA-seq data analysis (Stegle *et al.*, 2015) and numerous computational methods are available for the normalisation and downstream data analysis. Despite these challenges, scRNA-seq provides a powerful tool for defining gene expression during human preimplantation development.

In collaboration with Dr Kathy Niakan's lab at the Francis Crick Institute, London, we have performed scRNA-seq using cells from unmanipulated control and ePNT blastocysts. Importantly, our samples were processed and analysed in the same way as the published dataset consisting of unmanipulated human blastocysts (Blakeley *et al.*, 2015) from Dr Kathy Niakan's lab. This enabled us to use them as a reference population in addressing the question of whether ePNT disrupts gene expression in the blastocyst, with a focus on global, lineage-associated and mitochondrial gene expression.

6.2 Global gene expression in unmanipulated controls and ePNT blastocysts

6.2.1 Global gene expression patterns in ePNT blastocysts compared to unmanipulated controls

To determine whether ePNT has an effect on gene expression in the blastocyst, we first performed principal component analysis (PCA) on scRNA-seq data. In addition to our unmanipulated experimental controls, we included a previously published series of unmanipulated blastocysts as a reference population (Blakeley *et al.*, 2015). Blastocysts produced following ePNT and submitted to RNA-seq included those generated by fusion of cytoplasts and karyoplasts with the same (autologous/homologous) or different (heterologous) mitochondrial genomes.

Our PCA analysis included controls and all ePNT samples, irrespective of blastocyst quality, (Figure 6.1a). By plotting PC1 against PC2, which together account for the highest variation in global gene expression, we found that while the majority of samples clustered together, there were some outliers. Analysis of the origin of outliers revealed a higher percentage of outliers were samples from ePNT blastocysts than unmanipulated controls (Figure 6.1b). However, further investigation discovered an increased percentage of outliers were from poor quality heterologous ePNT blastocysts compared to controls (grades D-F; $P<0.005$) and good quality heterologous ePNT blastocysts (grades A-C; $P<0.01$) (Figure 6.1c). This is supported by analysis of outliers according to blastocyst grade, which shows that a high proportion (47.1%) of samples from poor quality blastocysts (grades E/F) were outliers (Figure 6.1d). This was significantly increased compared to samples from good quality blastocysts (grades A/B; $P<0.0001$) and blastocysts grades C/D ($P<0.05$). These findings indicated that PCA of scRNA-seq data is sufficiently sensitive to detect differences in global gene expression between good and poor quality blastocysts.

It is unsurprising that not all ePNT samples clustered together with unmanipulated controls, as all the control samples came from blastocysts which were top or good quality (grades A/B), whereas ePNT samples analysed included poor quality blastocysts. Furthermore, a high percentage of poor quality blastocysts were aneuploid for multiple chromosomes (Chapter 4.5.6), which would likely affect their gene expression profile compared to normal blastocysts.

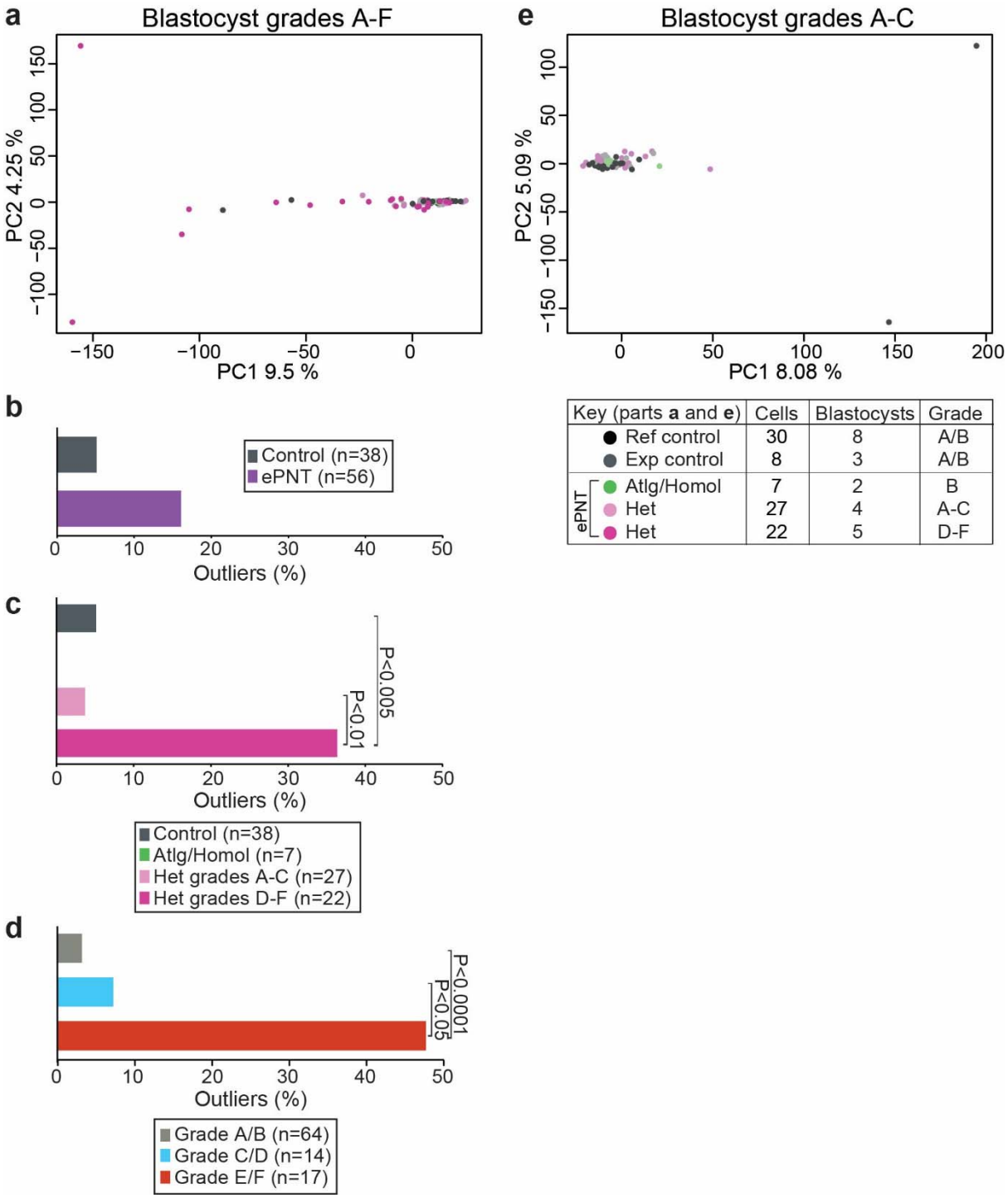


Figure 6.1: Global gene expression patterns in unmanipulated controls and ePNT blastocysts according to RPKM normalised counts. a) PCA of scRNA-seq data comparing global gene expression profiles in unmanipulated controls and ePNT blastocysts (grades A-F). **b)** Graph showing percentage of outliers from control and ePNT samples (not significant; Fisher's exact test). **c)** Graph showing percentage outliers according to origin (P-values are shown; Fisher's exact test). **d)** Graph shows an increased percentage of outliers in samples from poor quality blastocysts (P values are shown; Fisher's exact test). **e)** PCA of scRNA-seq data comparing unmanipulated control samples with samples from good quality ePNT blastocysts. The key shows the numbers of samples and blastocysts included in this analysis.

While the inclusion of samples from poor quality blastocysts provided a useful means of validating our approach, it has little biological relevance. As shown in Chapter 4 (Figure 4.1), the implantation potential is strongly correlated with blastocyst grade and poorer quality blastocysts, particularly (Grades E/F) are highly unlikely to be capable of implanting to form a viable foetus. For this reason, samples from poor quality blastocysts were excluded and PCA was repeated to compare ePNT (grades A-C) to unmanipulated controls. This analysis showed that ePNT samples clustered closely with controls (Figure 6.1e) and the variation accounted for by PC1 and PC2 was reduced, this is shown by the PC1 and PC2 percentages. While there was still a small number of outliers (3/94), these samples were derived from controls (n=2) as well as ePNT (n=1) blastocysts.

The data from the PCAs described above were obtained using counts which were normalised using the reads per kilobase of exon model per million mapped reads (RPKM) method. This approach normalises for transcript length and sequencing depth (Mortazavi *et al.*, 2008). To test whether these results are reproducible, I repeated the analyses using DESeq2 normalised counts (Love *et al.*, 2014), which uses a negative binomial generalised linear model and provides an alternative approach for analysis of scRNA-seq data. PCA performed using DESeq2 normalised counts comparing controls and ePNT samples gave similar results to PCA using RPKM normalised counts (Figure 6.2a). I found that a higher percentage of outliers were samples from ePNT blastocysts compared to unmanipulated controls ($P<0.05$; Figure 6.2b). This was also observed with RPKM normalised counts, however was not statistically significant in this case (Figure 6.1b). Further analysis of outlier origin revealed a high percentage of outliers were from poor quality heterologous ePNT blastocysts (Figure 6.2c). This was significantly increased compared to controls ($P<0.0001$), autologous ePNT ($P<0.05$) and good quality heterologous ePNT blastocysts ($P<0.0005$). As before, a high proportion of samples from poor quality blastocysts (grades E/F) were outliers in the PCA; this was significantly higher in poor quality blastocysts compared to blastocysts of grades A/B ($P<0.0001$) and C/D ($P<0.05$; Figure 6.2d). Removal of poor quality samples from the PCA results in the majority of control and ePNT samples clustering together (Figure 6.2c). As observed in the PCA using RPKM normalised counts, there are 3 outliers from control (n=2) and ePNT (n=1) blastocysts. These samples (hCtr.1, hCtr.2 and 9PNT.12) are identified as outliers in both the PCA using RPKM normalised counts and DESeq2 normalised counts.

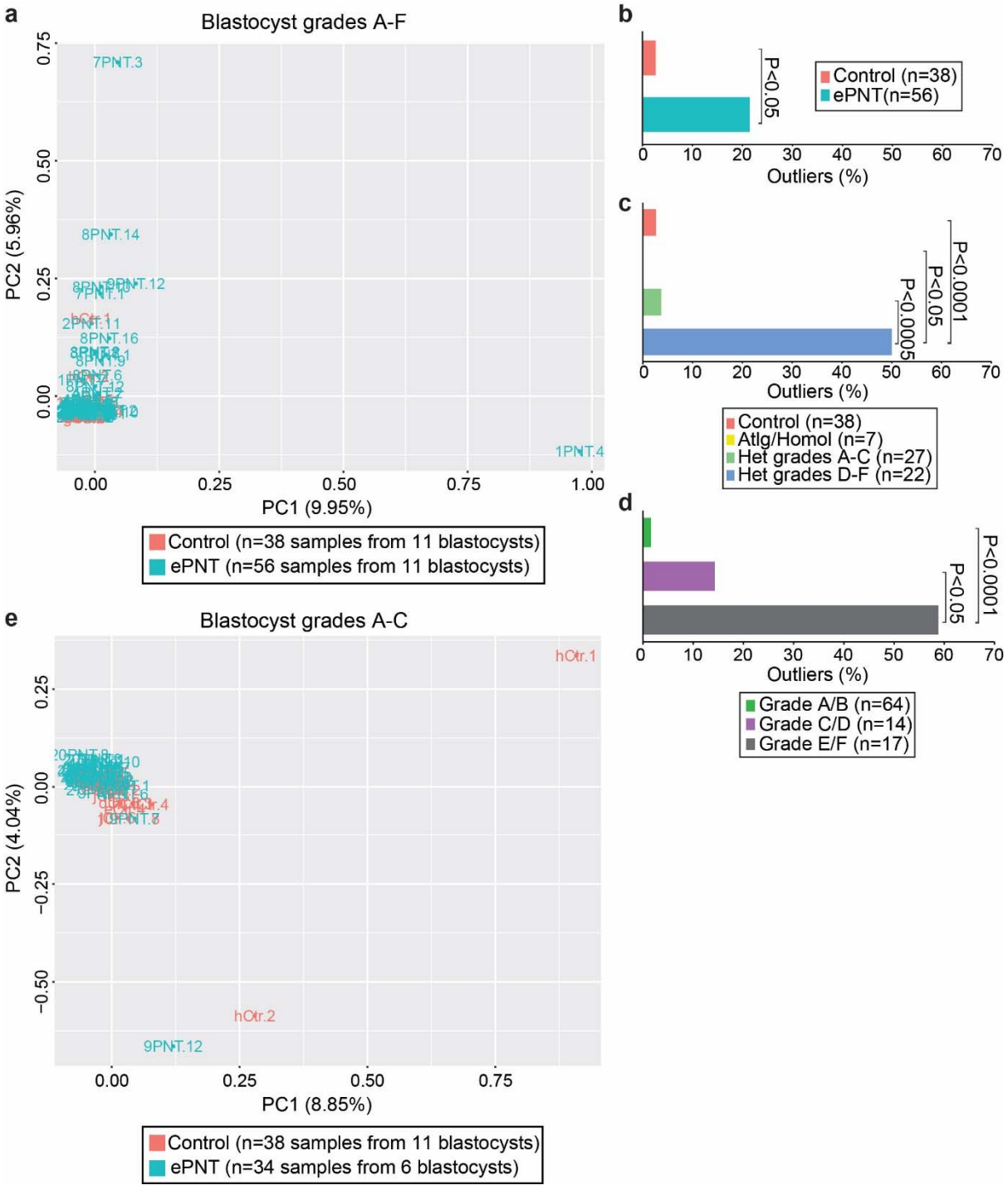


Figure 6.2: Global gene expression patterns in unmanipulated controls and ePNT blastocysts according to DESeq2 normalised counts. a) PCA of scRNA-seq data comparing global gene expression profiles in unmanipulated controls and ePNT blastocysts (grades A-F). **b)** Graph showing percentage of outliers from control and ePNT samples ($P<0.05$; Fisher's exact test). **c)** Graph showing percentage outliers according to origin (P-values are shown; Fisher's exact test). **d)** Graph shows an increased percentage of outliers in samples from poor quality blastocysts (P values are shown; Fisher's exact test). **e)** PCA of scRNA-seq data comparing unmanipulated control samples with samples from good quality ePNT blastocysts.

These results provide reassurance that the PCA data are reproducible by two independent approaches to normalisation of the scRNA-seq data. Moreover, both approaches indicate that the analyses are sufficiently sensitive to detect differences in global gene expression between samples from good and poor quality blastocysts. Importantly, by confining the analysis to blastocysts whose morphological features are compatible with implantation, we found that ePNT samples clustered together with samples from controls. We conclude that global gene expression is indistinguishable between good quality ePNT and control blastocysts.

6.2.2 Global gene expression related to morphological characteristics of blastocysts

While the analysis of poor quality blastocysts has limited relevance from a clinical perspective, analysis of the differences in gene expression between good and poor quality blastocysts has the potential to provide new biological insights into pathways and processes determining embryo viability. To further investigate the gene expression patterns in poor quality blastocysts, we combined samples from good quality (grades A-C) ePNT and control blastocysts for comparison with poor quality (grades D-F) blastocysts. Figure 6.3 shows PCA (RPKM and DESeq2 normalised counts) distinguishing poor quality ePNT samples and grouped controls/ good quality ePNT samples. Good quality samples generally cluster closely together, whereas poor quality samples appear to have high variability in global gene expression. This is demonstrated by the significantly higher number of outlying samples from poor quality blastocysts in the PCA using both RPKM ($P<0.0005$) and DESeq2 ($P<0.0001$) normalised counts (Figure 6.3b, d).

In order to investigate differences in gene expression related to morphological characteristics, I performed differential gene expression analysis comparing blastocysts grades A-C (control and ePNT samples) with blastocysts grades D-F (ePNT samples) using DESeq2. For identification of differentially expressed genes, DESeq2 applies shrinkage estimators for dispersion and logarithmic fold change calculations. I then used the R package GOstats to find Gene Ontologies (GOs) associated with the list of differentially expressed genes from DESeq2. For this analysis I only included genes with an adjusted P value (which accounts for multiple comparisons) of <0.05 and also considered whether the selected genes were upregulated or downregulated in poor quality blastocysts. I found that only a small proportion of differentially expressed genes were downregulated in grade D-F blastocysts (19.1%; 961 out of 5037 genes). Enriched GOs for downregulated genes are shown in Table 6.1 and include several related to ion transport and cell signalling.

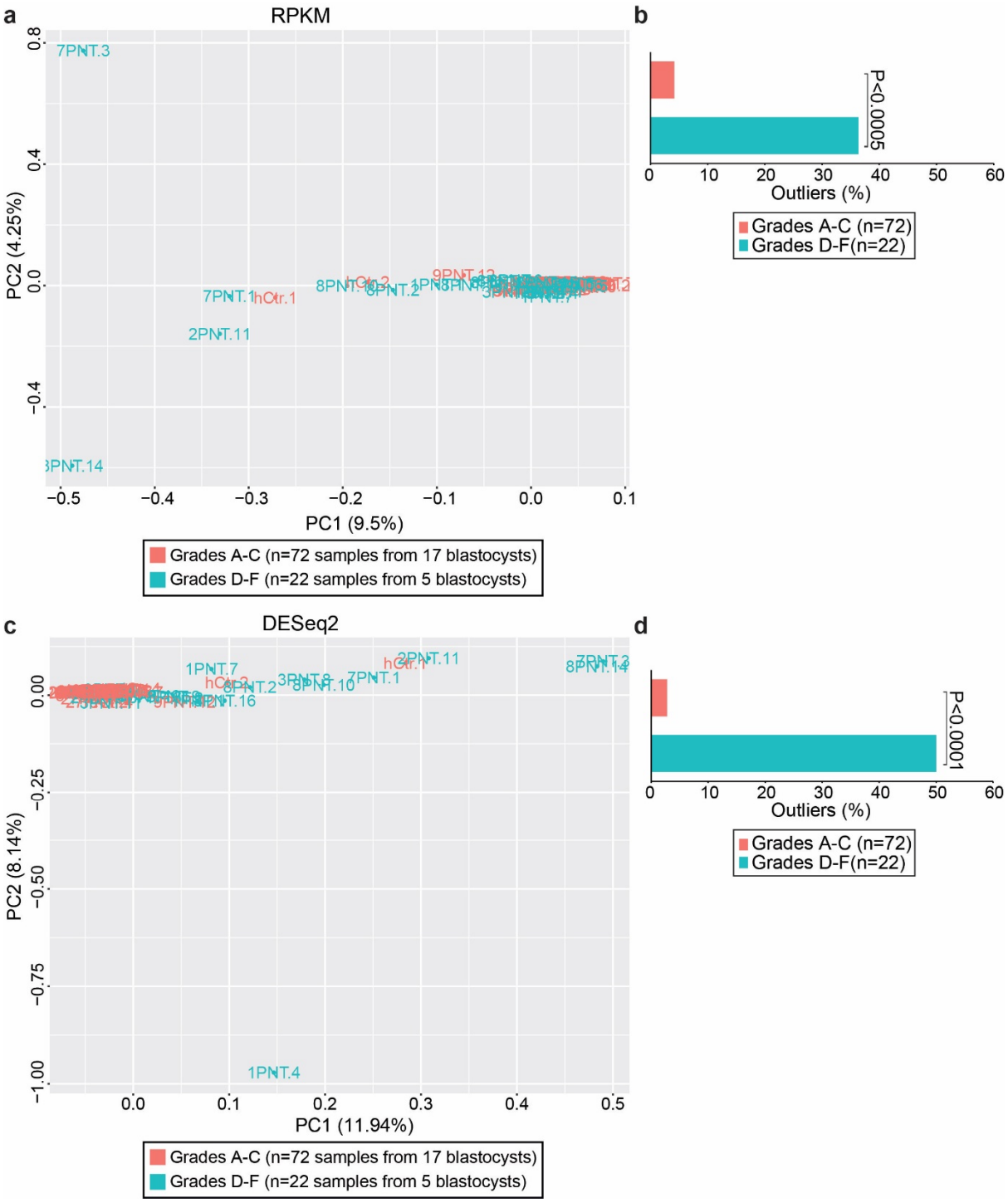


Figure 6.3: Global gene expression patterns in good and poor quality blastocysts. a) PCA of scRNA-seq data comparing samples from good quality blastocysts (grades A-C; controls and ePNT) and poor quality blastocysts (grades D-F; ePNT) using RPKM normalised counts. **b)** Graph showing percentage outliers from blastocysts grades A-C and grades D-F ($P<0.0005$; Fisher's exact test). **c)** PCA comparing samples from good quality blastocysts (grades A-C; controls and ePNT) and poor quality blastocysts (grades D-F; ePNT) using DESeq2 normalised counts. **d)** Graph showing percentage outliers from blastocysts grades A-C and grades D-F ($P<0.0001$; Fisher's exact test).

The majority of differentially expressed genes identified by DESeq2 were upregulated in grade D-F blastocysts (80.9%; 4076 out of 5037 genes). Enriched GOs shown in Table 6.2 are mainly related to metabolism and catabolism. This finding supports the 'quiet embryo hypothesis' (Leese, 2002), which suggests that viable mammalian embryos have a low metabolism, glycolytic rate and amino acid turnover. Interestingly, cellular response to stress and DNA damage stimulus is also included in the enriched GO terms for genes upregulated in poor quality ePNT blastocysts. However, it is surprising that cell death genes are not upregulated in poor quality blastocysts. I also found that upregulated genes included genes associated with the cell cycle. As upregulated genes include both metabolic and cell cycle genes, this may be linked to metabolic control of cell cycle progression (Fajas, 2013; Lee and Finkel, 2013; Kalucka *et al.*, 2015). Alternatively, as discussed in Chapter 4 (Figure 4.16), a high proportion of poor quality samples are aneuploid for multiple chromosomes, which is likely to disrupt the cell cycle.

Downregulated in samples from blastocysts grade D-F

GO ID	Term	P value
GO:0055085	transmembrane transport	2.81E-13
GO:0006811	ion transport	2.88E-13
GO:0022610	biological adhesion	4.62E-12
GO:0007155	cell adhesion	7.31E-12
GO:0030001	metal ion transport	1.56E-11
GO:0006812	cation transport	3.31E-11
GO:0034220	ion transmembrane transport	4.35E-11
GO:0007268	synaptic transmission	9.55E-11
GO:0099536	synaptic signaling	9.55E-11
GO:0099537	trans-synaptic signaling	9.55E-11
GO:0044707	single-multicellular organism process	3.80E-10
GO:0007267	cell-cell signaling	1.22E-09
GO:0070838	divalent metal ion transport	4.79E-09
GO:0072511	divalent inorganic cation transport	7.30E-09
GO:0098660	inorganic ion transmembrane transport	8.07E-09
GO:0032501	multicellular organismal process	2.05E-08
GO:0009605	response to external stimulus	2.07E-08
GO:0042391	regulation of membrane potential	2.37E-08
GO:0006816	calcium ion transport	3.65E-08
GO:0098609	cell-cell adhesion	4.72E-08
GO:0043269	regulation of ion transport	5.90E-08
GO:0007215	glutamate receptor signaling pathway	5.98E-08
GO:0098662	inorganic cation transmembrane transport	6.63E-08
GO:0098655	cation transmembrane transport	6.65E-08
GO:0051239	regulation of multicellular organismal process	9.19E-08
GO:0023052	signaling	5.83E-07
GO:0044700	single organism signaling	5.93E-07
GO:0035637	multicellular organismal signaling	6.34E-07
GO:0016337	single organismal cell-cell adhesion	6.50E-07
GO:0007154	cell communication	7.29E-07

Table 6.1: Gene ontologies enriched in genes downregulated in poor quality blastocysts. Differentially expressed genes were identified using DESeq2 and enriched gene ontologies identified using GOstats. Genes identified by DESeq2 included in this analysis had an adjusted P value of <0.05 and a negative fold change in samples from blastocysts grades D-F. Table shows the gene ontology (GO) ID, term and significance level (P value).

Gene ontologies upregulated in samples from blastocysts grade D-F

GO ID	Term	P value
GO:0008152	metabolic process	1.39E-10
GO:0044260	cellular macromolecule metabolic process	9.58E-09
GO:0033554	cellular response to stress	2.23E-08
GO:0007049	cell cycle	3.93E-08
GO:0071704	organic substance metabolic process	5.73E-08
GO:0044237	cellular metabolic process	6.72E-08
GO:0044238	primary metabolic process	1.41E-07
GO:0043170	macromolecule metabolic process	3.71E-07
GO:0051726	regulation of cell cycle	1.65E-06
GO:0010498	proteasomal protein catabolic process	1.75E-06
GO:0090304	nucleic acid metabolic process	1.91E-06
GO:0031329	regulation of cellular catabolic process	1.98E-06
GO:1901360	organic cyclic compound metabolic process	2.8E-06
GO:0034641	cellular nitrogen compound metabolic process	3.11E-06
GO:0046483	heterocycle metabolic process	3.66E-06
GO:0000278	mitotic cell cycle	4.05E-06
GO:0006725	cellular aromatic compound metabolic process	5.43E-06
GO:0006139	nucleobase-containing compound metabolic process	5.82E-06
GO:0051348	negative regulation of transferase activity	7.92E-06
GO:0043161	proteasome-mediated ubiquitin-dependent protein catabolic process	8.67E-06
GO:0022402	cell cycle process	9.75E-06
GO:0031400	negative regulation of protein modification process	1.48E-05
GO:0009894	regulation of catabolic process	1.54E-05
GO:0032446	protein modification by small protein conjugation	1.62E-05
GO:1903047	mitotic cell cycle process	2.17E-05
GO:0070647	protein modification by small protein conjugation or removal	2.38E-05
GO:0006807	nitrogen compound metabolic process	2.54E-05
GO:0051603	proteolysis involved in cellular protein catabolic process	3.39E-05
GO:0006974	cellular response to DNA damage stimulus	3.51E-05
GO:0044257	cellular protein catabolic process	3.95E-05

Table 6.2: Gene ontologies enriched in genes upregulated in poor quality blastocysts. Differentially expressed genes were identified using DESeq2 and enriched gene ontologies identified using GOstats. Genes identified by DESeq2 included in this analysis had an adjusted P value of <0.05 and a positive fold change in samples from blastocysts grades D-F. Table shows the gene ontology (GO) ID, term and significance level (P value).

6.3 Expression of lineage-associated genes

6.3.1 Lineage-associated gene expression in good quality control and ePNT blastocysts

Having established that global gene expression is similar between good quality ePNT samples and unmanipulated controls, we asked whether there are any detectable differences in lineage-associated genes. As discussed earlier, mammalian blastocysts consist of an outer trophectoderm (TE) layer and inner cell mass (ICM), which segregates to form the epiblast (EPI) and primitive endoderm (PE). Gene expression profiles for these lineages have been described (Blakeley *et al.*, 2015; Petropoulos *et al.*, 2016). Here, we include lineage-associated genes identified as highly differentially expression according to DESeq2.

Analysis of samples from good quality blastocysts using t-SNE (t-distributed stochastic stochastic neighbour embedding), a non-linear method for dimensionality reduction, for the top 6,000 most variable genes from DESeq2 analysis (Figure 6.4), revealed that samples cluster according to lineage (Figure 6.4). Within the TE lineage, control samples are overrepresented. This is likely due to a learning curve effect during the disaggregation of single-cells from blastocysts; analysis of the reference population was performed before our unmanipulated controls and ePNT blastocysts, therefore, more TE samples were collected than ICM samples. We were unable to detect any differences between ePNT and control blastocysts within each of the lineages. Thus, while t-SNE can distinguish samples by lineage, it cannot detect differences between ePNT and control samples within each of the three lineages. This finding was confirmed by unsupervised hierarchical clustering (Figure 6.5) using a subset of 30 differentially expressed well-known lineage-associated genes, in which control and ePNT samples clustered together based on lineage. These findings suggest that ePNT does not disrupt lineage specification in good quality blastocysts.

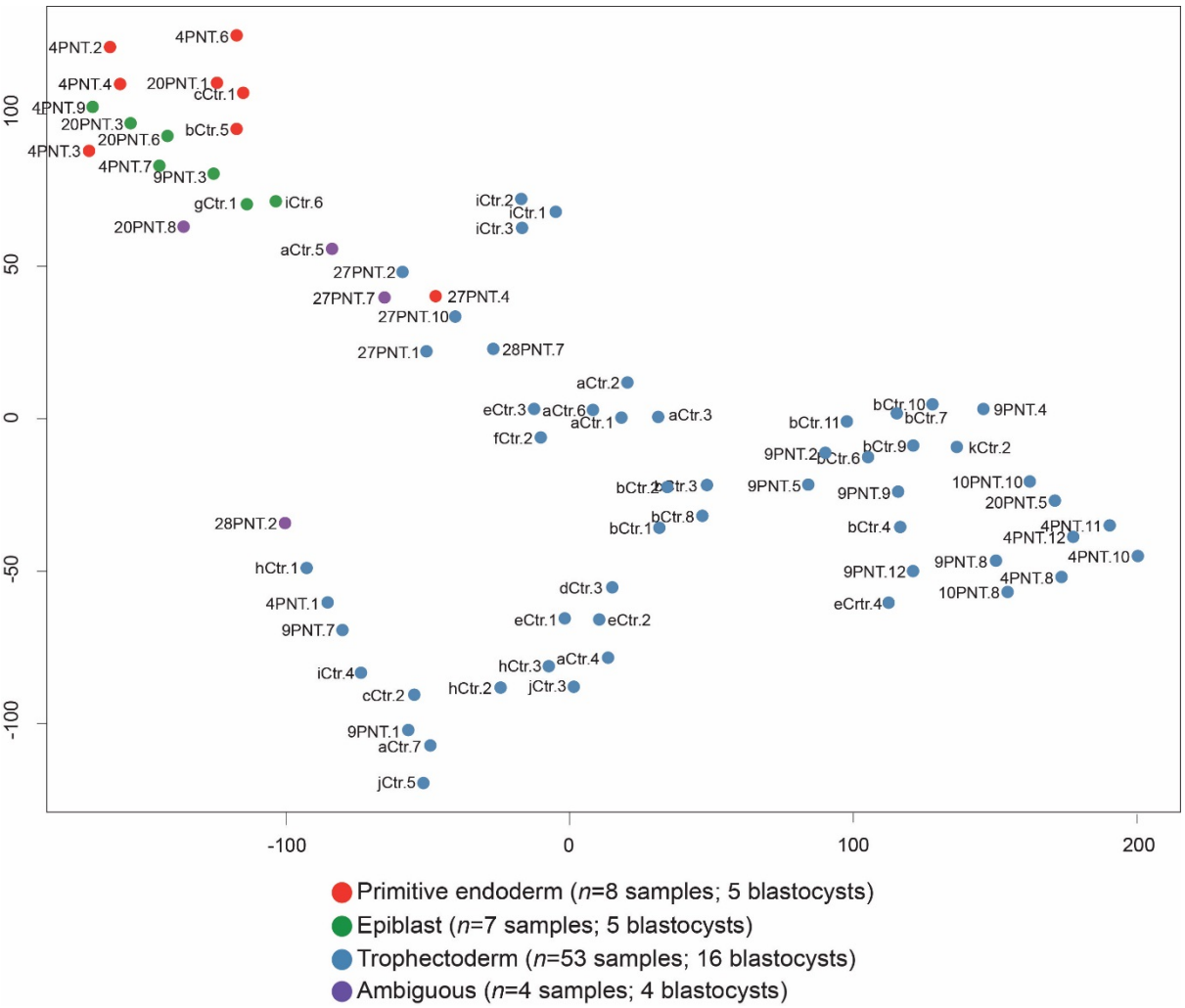


Figure 6.4: Differential gene expression analysis in good quality ePNT and control samples. t-SNE analysis of the top 6,000 most variably expressed genes according to DESeq2, where samples were distinguished by lineage. Lineage is indicated by colour code, sample and blastocyst numbers are shown.

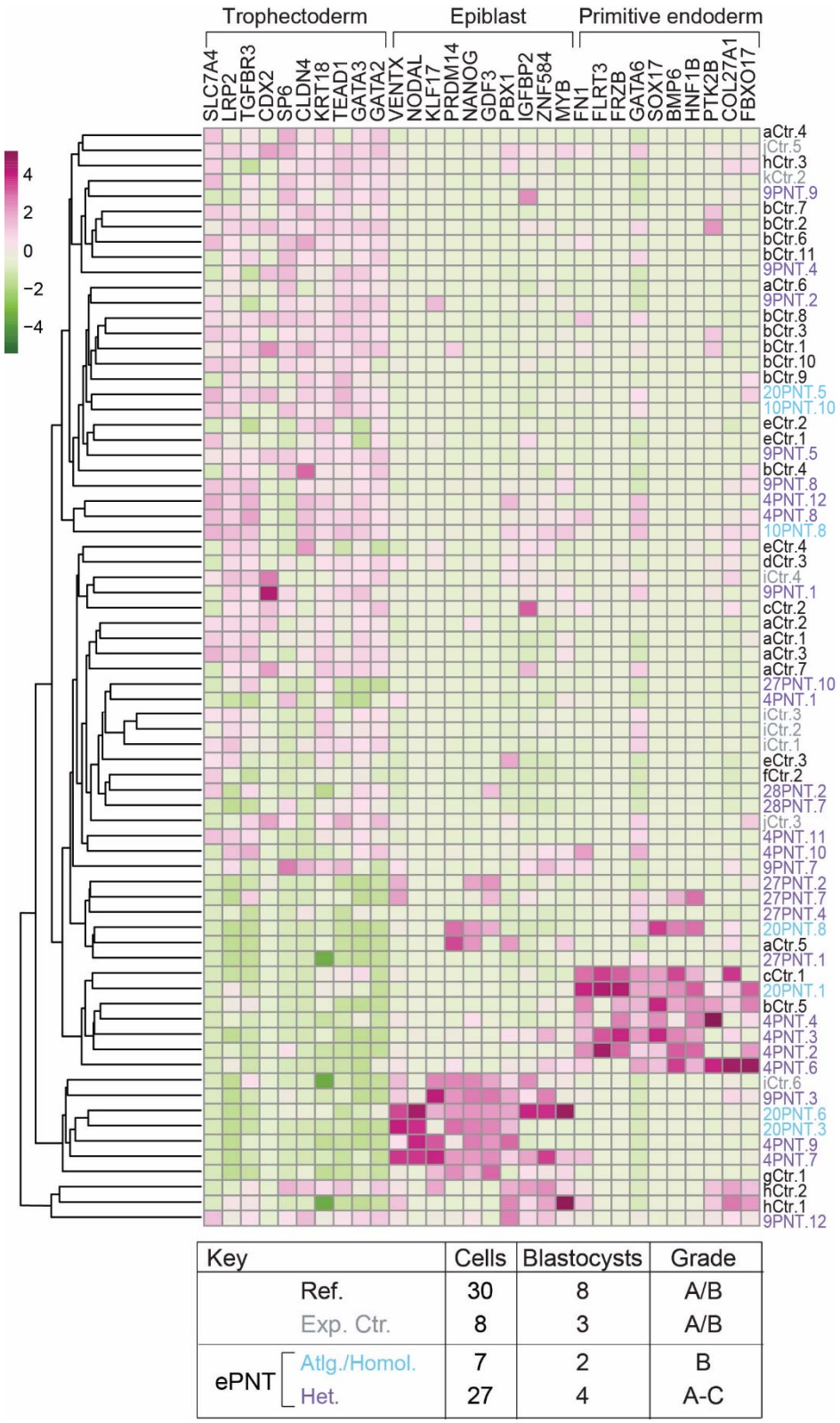


Figure 6.5: Expression of lineage specific genes in controls and samples from good quality ePNT blastocysts. Heatmap showing $\log_2$-transformed RPKM values of a subset of differentially expressed genes, according to DESeq2, in trophectoderm (n=10), epiblast (n=10) and primitive endoderm (n=10) lineages. Expression level is plotted on a high-to-low scale (purple-white-green). The key shows the number of samples (cells), blastocysts and grade. Samples are labelled according to origin using a colour code shown in the key.

6.3.2. Lineage-associated gene expression in good and poor quality blastocysts

In order to determine whether unsupervised hierarchical clustering could detect differences in lineage specification in samples from poor quality blastocysts, we repeated the above analysis including all samples. As before, good quality ePNT samples clustered together with unmanipulated controls according to lineage (Figure 6.6a). However, I did see an increase in the incidence of samples showing mixed expression of lineage-associated genes, or generally low expression of lineage-associated genes (Figure 6.6a). Poor quality ePNT blastocysts had the highest proportion of samples displaying mixed/ low expression of lineage-associated genes (Figure 6.6b). This was significantly higher than control blastocysts ($P<0.0001$) and good quality ePNT blastocysts ($P<0.0001$). Good quality ePNT blastocysts did have a higher percentage of samples with mixed expression than controls but this was not statistically significant (Figure 6.6b). Of samples with mixed expression, I found that most commonly EPI and PE genes were co-expressed. This suggests that poor quality ePNT samples may have delayed development and that cells of the ICM have not yet finally committed to the epiblast or primitive endoderm lineage. On the other hand, mixed expression may reflect a more fundamental problem in the regulation of gene expression affecting lineage specification in poor quality blastocysts.

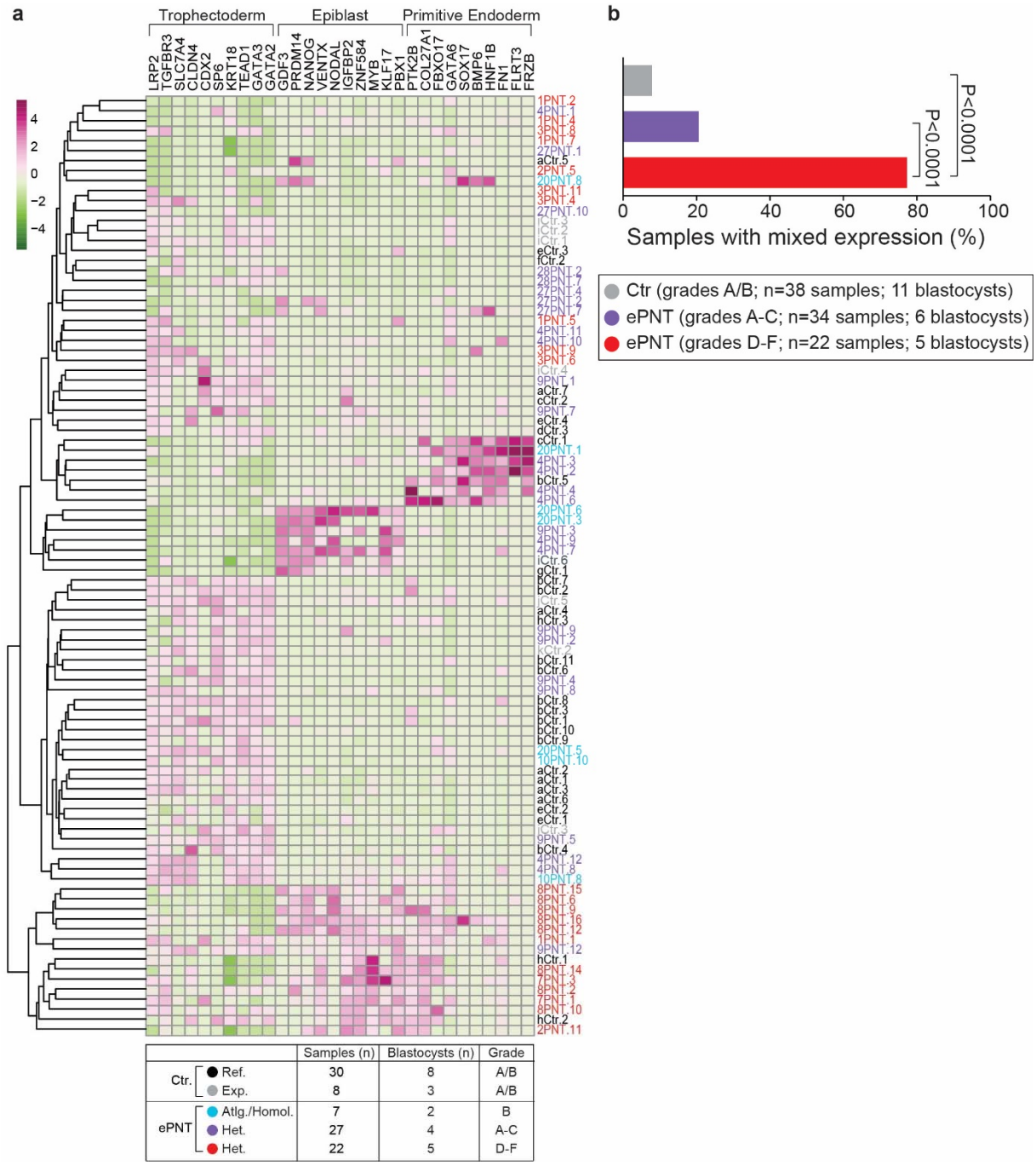


Figure 6.6: Expression of lineage specific genes in controls and samples from ePNT blastocysts. a) Heatmap showing log2-transformed RPKM values of a subset of differentially expressed genes in trophectoderm (n=10), epiblast (n=10) and primitive endoderm (n=10) lineages. Expression level is plotted on a high-to-low scale (purple-white-green). The key shows the number of samples (cells), blastocysts and grade. Samples are labelled using a colour code shown in the key. **b)** Graph shows increased percentage of samples with mixed expression of lineage specific genes in poor quality blastocysts (P values are shown; Fisher's exact test).

6.3.3 Expression of non-lineage-associated genes in the three blastocyst cell lineages

To further explore whether there are differences in gene expression between lineages aside from lineage-associated genes, I performed differential gene expression analysis using DESeq2 grouping samples by lineage, followed by GO analysis including differentially expressed genes with an adjusted P value of <0.05. Comparison of the TE and ICM gave expected results, with genes related to GOs such as gastrulation, embryo development and endoderm development upregulated in the inner cell mass. Interestingly, comparison of the ICM lineages, EPI and PE, revealed differentially expressed genes related to GOs including organelle organisation, cell death and cell cycle, with these upregulated in the primitive endoderm. Following unsupervised hierarchical clustering, samples generally cluster together when taking into consideration all genes within these ontologies (Figure 6.7). This may reflect the behaviours which have been observed leading to the formation of the primitive endoderm, such as selective apoptosis for cell sorting within the ICM (Plusa *et al.*, 2008).

Comparison of samples with mixed/ambiguous lineage to samples which were assigned a lineage revealed the upregulation of genes related to ontologies linked to cellular response to stress and metabolism (Table 6.3). This is unsurprising as a high proportion of poor quality blastocysts provided samples that were not allocated a lineage. As discussed in 6.2.2, samples from poor quality blastocysts had upregulation of genes related to metabolism compared to good quality blastocysts. This is consistent with the finding that viable embryos have a 'quiet' metabolism (Leese, 2002); high amino acid turnover is correlated with DNA damage (Sturmey *et al.*, 2009) and embryos with an 'active' metabolism may be responding to stress and DNA damage (Leese *et al.*, 2007; Leese *et al.*, 2008).

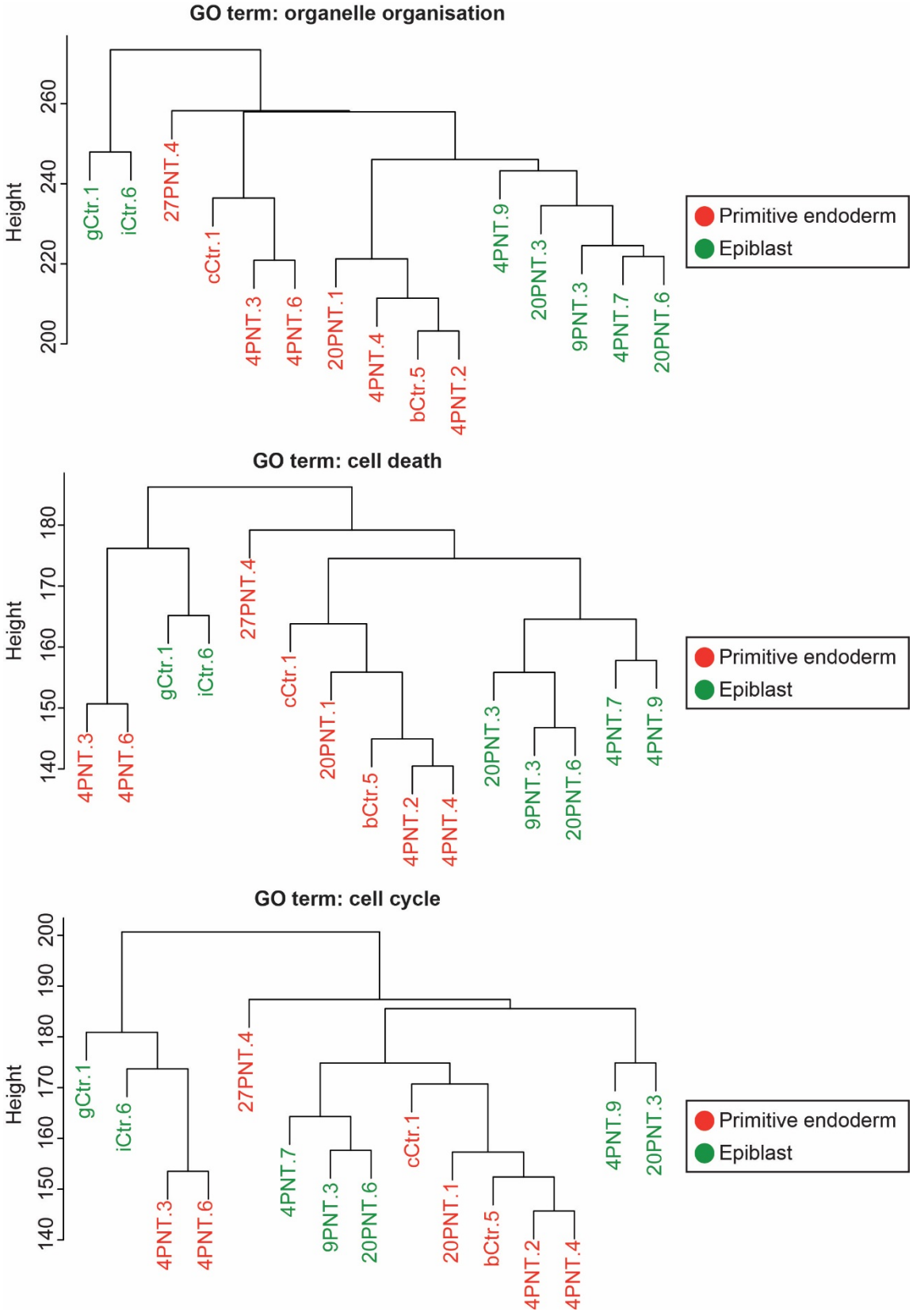


Figure 6.7: Gene ontologies enriched in differentially expressed genes between epiblast and primitive endoderm samples. Dendrograms showing samples clustering according to inner cell mass (ICM) lineage (primitive endoderm (PE), red; epiblast (EPI), green) for expression of genes involved in organelle organisation, cell death and cell cycle.

GO ID	Term	P value
GO:0033554	cellular response to stress	5.04E-16
GO:0044260	cellular macromolecule metabolic process	2.17E-14
GO:0071704	organic substance metabolic process	7.47E-13
GO:0008152	metabolic process	7.56E-13
GO:0030163	protein catabolic process	1.09E-12
GO:0044238	primary metabolic process	1.21E-12
GO:0044237	cellular metabolic process	1.49E-12
GO:0043170	macromolecule metabolic process	3.67E-12
GO:0044257	cellular protein catabolic process	5.50E-12
GO:0051603	proteolysis involved in cellular protein catabolic process	1.42E-11
GO:0034976	response to endoplasmic reticulum stress	1.46E-11
GO:0007049	cell cycle	1.60E-11
GO:0010498	proteasomal protein catabolic process	1.95E-11
GO:0000278	mitotic cell cycle	9.75E-11
GO:0019941	modification-dependent protein catabolic process	1.72E-10
GO:0043161	proteasome-mediated ubiquitin-dependent protein catabolic process	2.36E-10
GO:0006511	ubiquitin-dependent protein catabolic process	2.62E-10
GO:0043632	modification-dependent macromolecule catabolic process	2.90E-10
GO:0009057	macromolecule catabolic process	6.59E-10
GO:0031329	regulation of cellular catabolic process	7.46E-10
GO:0044248	cellular catabolic process	8.82E-10
GO:0044265	cellular macromolecule catabolic process	1.91E-09
GO:1903047	mitotic cell cycle process	2.12E-09
GO:0022402	cell cycle process	4.73E-09
GO:0034641	cellular nitrogen compound metabolic process	5.00E-09
GO:0036503	ERAD pathway	5.04E-09
GO:0006996	organelle organization	5.37E-09
GO:0009056	catabolic process	5.58E-09
GO:0009894	regulation of catabolic process	5.83E-09
GO:0006807	nitrogen compound metabolic process	7.60E-09

Table 6.3: Gene ontologies enriched in genes upregulated in samples showing mixed expression of lineage-associated genes. Differentially expressed genes were identified using DESeq2 and enriched gene ontologies identified using GOstats. Genes identified by DESeq2 included in this analysis had an adjusted P value of <0.05 and a positive fold change in samples showing mixed expression of lineage-associated genes. Table shows the gene ontology (GO) ID, term and significance level (P value).

6.4 Mitochondrial gene expression

6.4.1 Does mitochondrial gene expression differ between unmanipulated controls and ePNT blastocysts?

The purpose of ePNT is to offer a clinical treatment which will reduce the risk of transmission of mtDNA disease from mother to child. This treatment will result in the formation of new combinations of nuclear and mitochondrial genomes in the embryo, which could possibly disrupt mitochondrial gene expression. To investigate the effect of ePNT on genes required for mitochondrial function, I have performed PCA using RPKM and DESeq2 normalised counts, comparing control samples and good quality (grades A-C) ePNT samples. For this analysis, a list of mitochondrial genes (encoded by nuclear and mtDNA) was downloaded from MitoCarta 2.0 (Calvo *et al.*, 2016). I found that there was wide variation between samples, but generally control and ePNT samples overlap (Figure 6.8). Although there are several outliers, these originate from ePNT and control blastocysts.

I next performed PCA of good quality ePNT samples grouped with controls and poor quality ePNT samples; to determine whether poor quality blastocysts display altered mitochondrial gene expression. I found that although there was overlap between good quality and poor quality samples, poor quality samples displayed much higher variation in mitochondrial gene expression (Figure 6.9). This is also apparent when visualised using a heatmap created following unsupervised hierarchical clustering (Figure 6.10). There are clusters visible at the 'top' of the heatmap with a distinct expression profile, showing generally lower expression of mitochondrial genes. The majority of samples in these clusters originate from poor quality ePNT blastocysts (60%). This suggests that poor quality blastocysts have altered expression of mitochondrial genes compared to good quality blastocysts. However, good quality ePNT samples are indistinguishable from controls.

On the basis that analysis of global and lineage-associated gene expression indicates that results are similar when counts are normalised by RPKM or DESeq2, I performed subsequent analysis of mitochondrial gene expression using only RPKM normalised counts. In further support of this, PCA of genes involved in regulating mitochondrial function revealed similar results between RPKM and DESeq2.

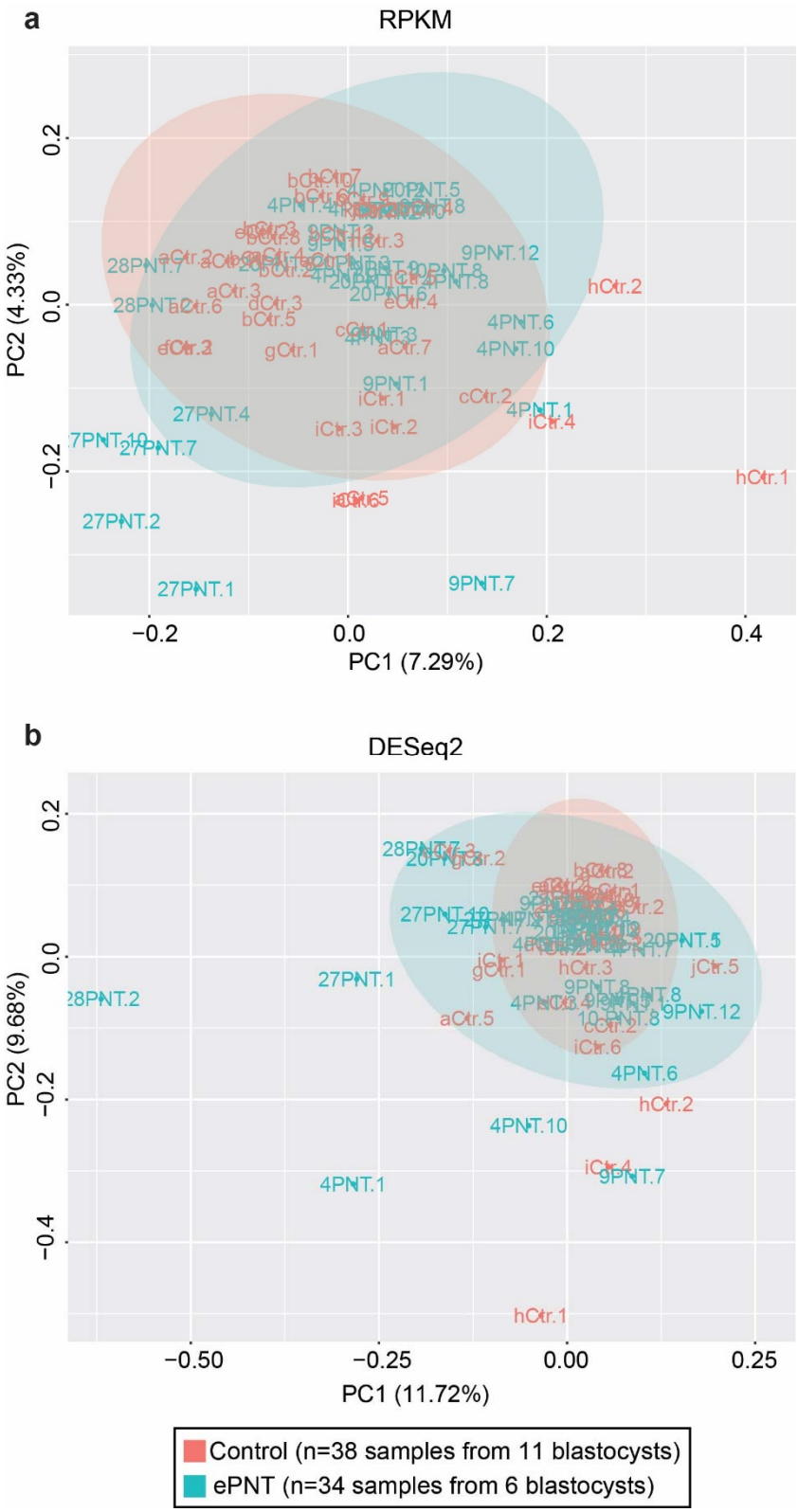


Figure 6.8: Analysis of expression of nuclear and mtDNA encoded mitochondrial gene expression in control and ePNT samples. PCA comparing mitochondrial gene expression in control and good quality ePNT samples, **a)** using RPKM normalised counts and **b)** using DESeq2 normalised counts. A list of mitochondrial genes was downloaded from MitoCarta 2.0 and included genes encoded by the nuclear and mitochondrial genomes. A t-distribution is included to visualise the overlap between control and ePNT samples.

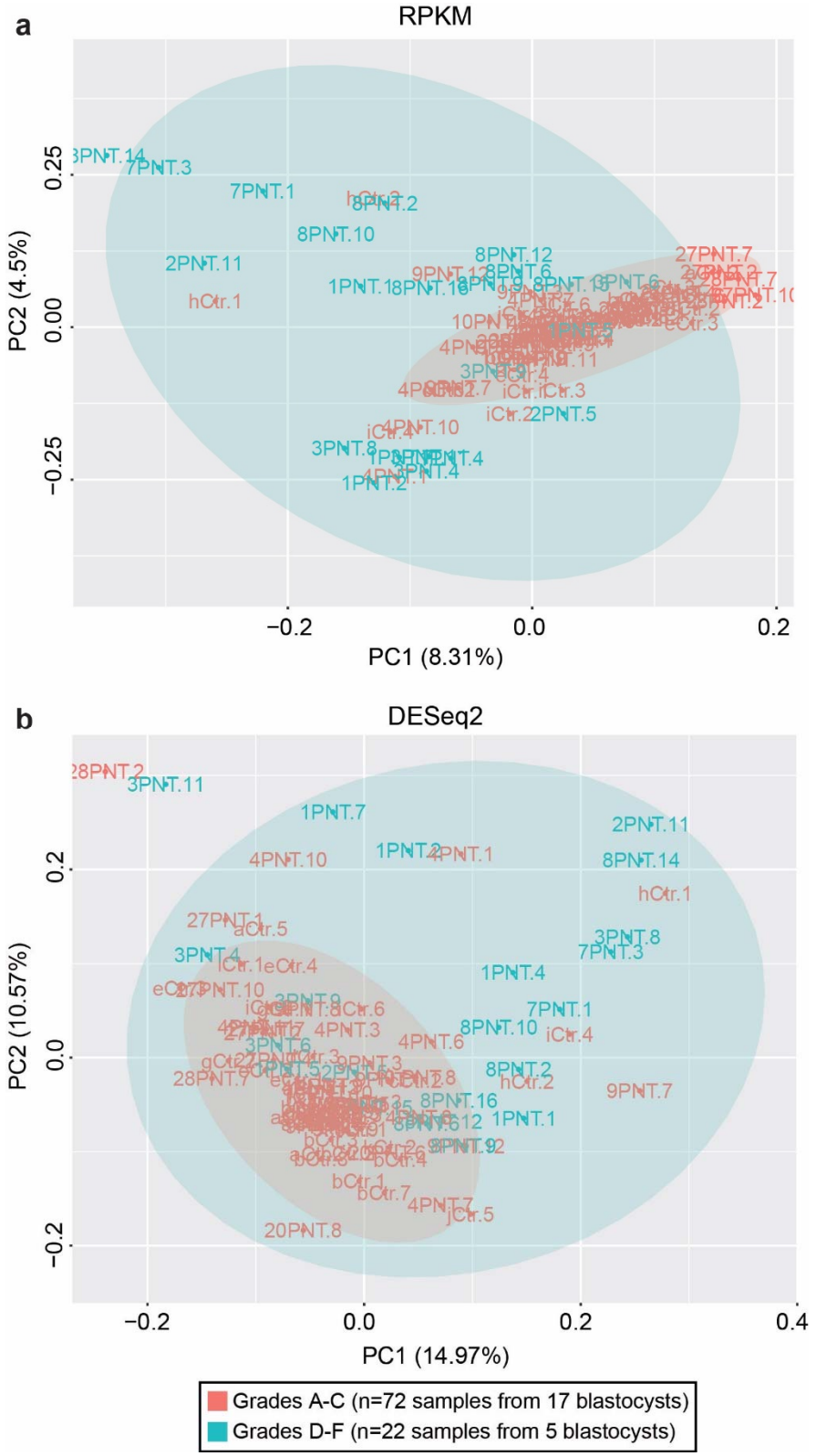
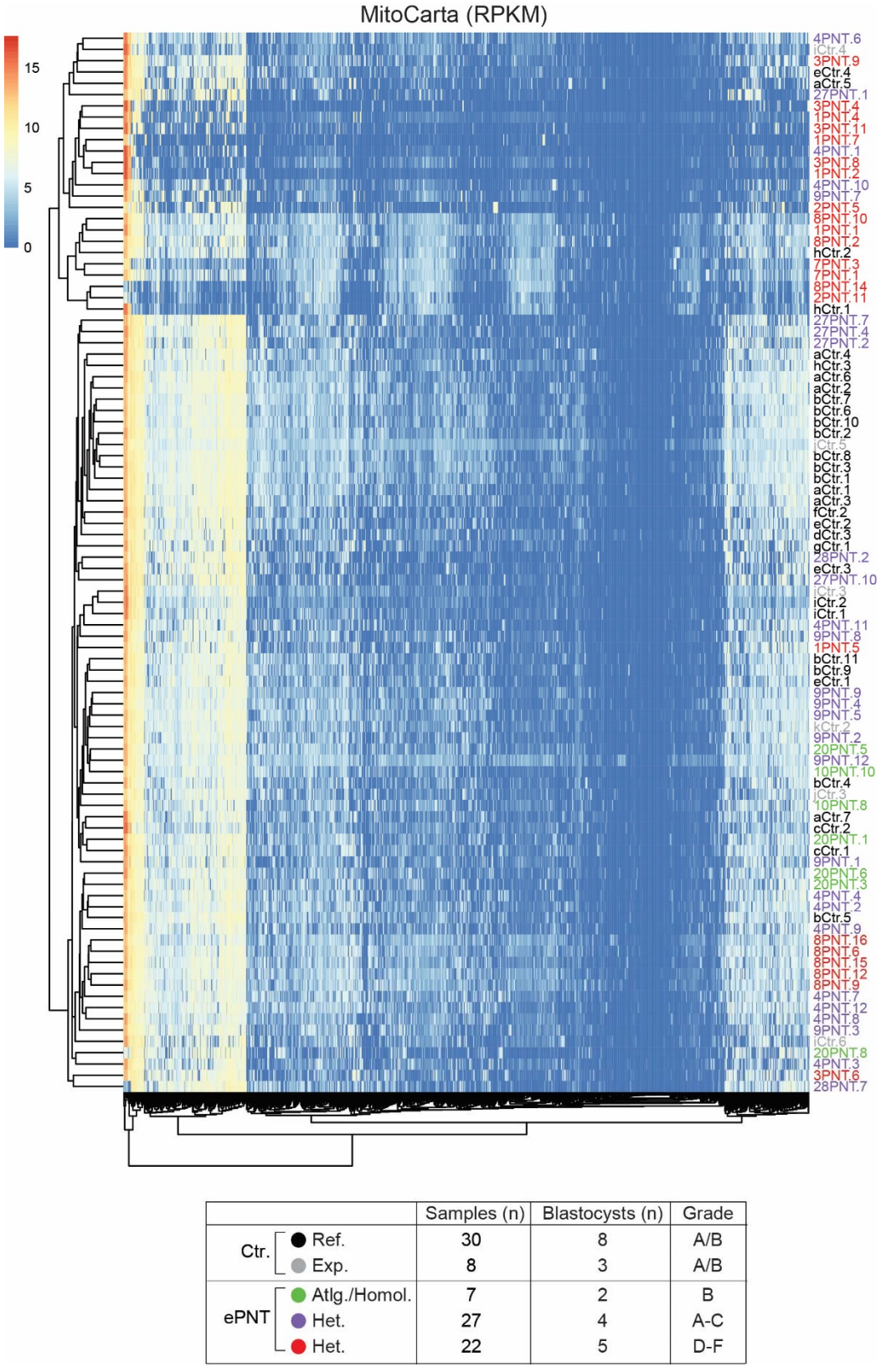


Figure 6.9: Analysis of nuclear and mtDNA encoded mitochondrial gene expression in samples from good and poor quality blastocysts. PCA comparing mitochondrial gene expression in good and poor quality samples, **a)** using RPKM normalised counts and **b)** using DESeq2 normalised counts. A list of mitochondrial genes was downloaded from MitoCarta 2.0 and included genes encoded by the nuclear and mitochondrial genomes. A t-distribution is included to visualise the overlap between control and ePNT samples.



		Samples (n)	Blastocysts (n)	Grade
Ctr.	Ref.	30	8	A/B
	Exp.	8	3	A/B
ePNT	Atlg./Homol.	7	2	B
	Het.	27	4	A-C
	Het.	22	5	D-F

Figure 6.10: Nuclear and mtDNA encoded mitochondrial gene expression. Heatmap showing the expression of mitochondrial genes from MitoCarta 2.0 gene list (nuclear and mtDNA encoded), using RPKM normalised counts. Expression level is plotted on a high-to-low scale (red to blue). Key shows blastocyst grade and the number of samples and blastocysts. Samples are labelled using a colour code indicated in the key.

6.4.2 Expression of OXPHOS genes

mtDNA encoded OXPHOS genes

Next, the expression of mitochondrial oxidative phosphorylation (OXPHOS) genes encoded by mtDNA was examined more closely. A heatmap created following unsupervised hierarchical clustering is shown in Figure 6.11a. The input was RPKM normalised counts from unmanipulated controls and all ePNT samples. There is wide variation in the level of expression of mtDNA encoded OXPHOS genes, but on the whole control and ePNT samples tend to cluster together. A notable exception is four samples with very low expression across all mtDNA OXPHOS genes. These four samples are all from ePNT blastocysts; 3 from heterologous ePNT blastocysts (1 grade A-C, 2 grades D-F), which therefore have new combinations of nuclear and mitochondrial genomes and one sample from a homologous ePNT blastocyst. As this cluster includes a technical control (homologous ePNT), it suggests that this low expression is not caused by new combinations of nuclear and mitochondrial genomes. Furthermore, there are other samples originating from the same blastocysts (28PNT, 2PNT, 20PNT and 8PNT) that cluster together with controls.

In Figure 6.11b, PCA of mtDNA encoded OXPHOS gene expression in control and ePNT samples (grades A-C and grades D-F) is shown. There is high variation in expression, with PC1 accounting for 81.59% of the variation. However, clusters of controls and ePNT samples of all grades overlap. The wide variation in mtDNA encoded OXPHOS gene expression, regardless of origin or blastocyst quality, is also highlighted in the graph shown in Figure 6.11c. Interestingly, in addition to wide variation in expression between blastocysts, there is also variation between samples from the same blastocyst.

Nuclear and mtDNA encoded OXPHOS genes

I next investigated the relationship between nuclear and mitochondrial genes encoding subunits of the OXPHOS complexes. Firstly, I created a heatmap including controls and all ePNT samples (Figure 6.12). I found the expression of mtDNA encoded OXPHOS genes to be higher than that of nuclear encoded OXPHOS genes, which could be due to the increased copy number of the mitochondrial genome. However, a number of samples appear to have unusual expression of mtDNA and nuclear encoded OXPHOS genes compared to the general pattern shown by other samples. This can be seen in the cluster towards the 'top' of the heatmap, and includes samples from both control and ePNT blastocysts. The majority

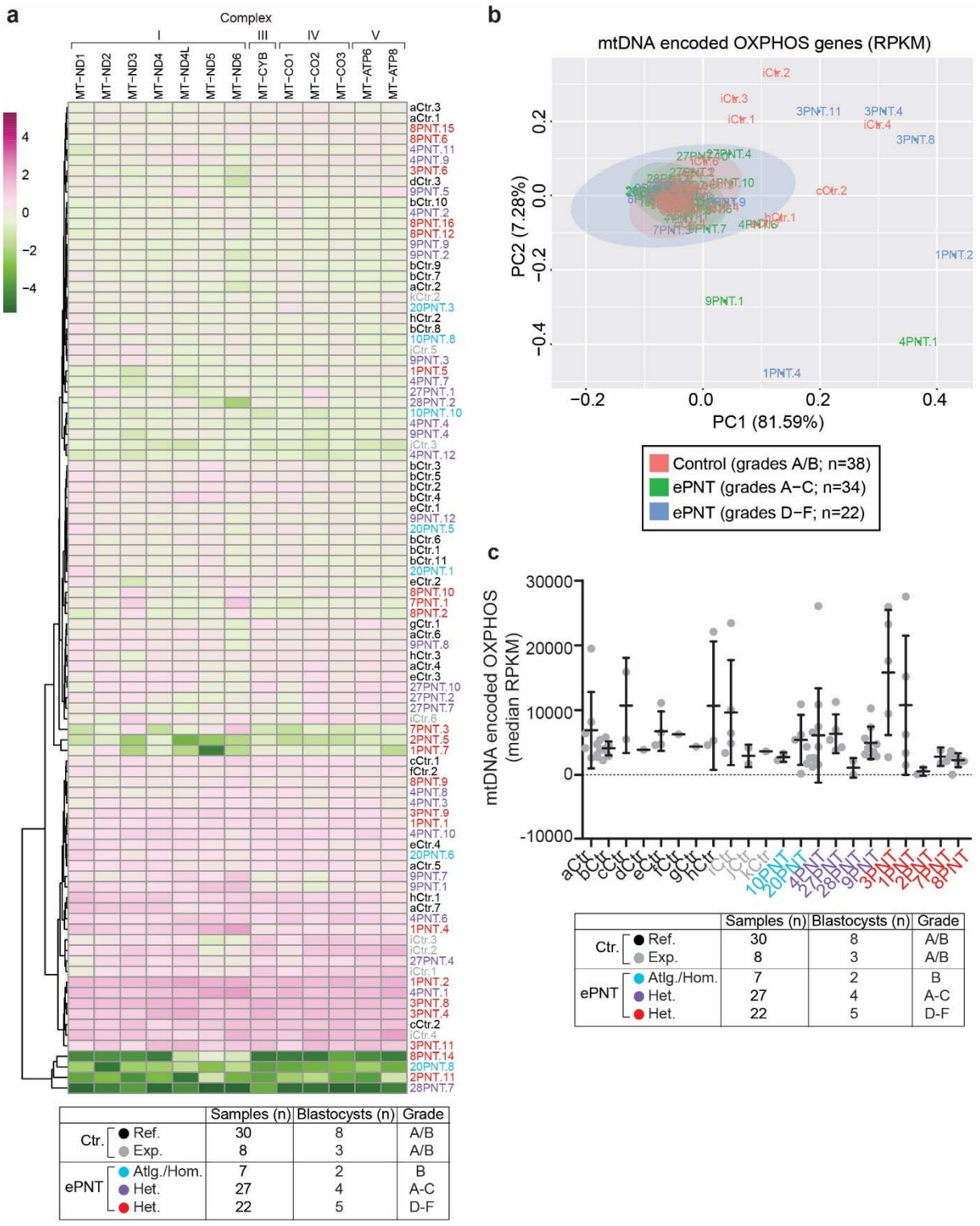


Figure 6.11: Expression of mtDNA encoded OXPHOS genes. a) Heatmap showing expression of mtDNA encoded OXPHOS genes (RPKM) plotted on a high-to-low scale (purple-white-green). **b)** PCA of mtDNA encoded OXPHOS genes, comparing control, good and poor quality ePNT samples. **c)** Graph showing median mtDNA encoded OXPHOS gene expression (RPKM) per sample. Blastocysts are ordered according to grade (A-F) and labelled using a colour code indicated in the key. Bars show the mean ± SD. Numbers of samples and blastocysts are shown.

(64.3%) of samples in this cluster originate from poor quality ePNT blastocysts (grades D-F). The expression of mtDNA encoded OXPHOS genes appears to be generally increased, whereas the expression of nuclear encoded OXPHOS genes is decreased compared to the remaining samples.

Another cluster, which branches close to the cluster mentioned above, has visibly decreased expression across all OXPHOS complex genes includes the samples 8PNT.14 and 2PNT.11, which are from poor quality blastocysts and were observed in Figure 6.11 to have very low expression of mtDNA encoded OXPHOS genes. Exploring previous analysis revealed that these samples were also outliers in the PCA of global gene expression (Figures 6.2 and 6.3) and had mixed expression of lineage-associated genes (Figure 6.6).

It is possible that samples displaying a reduced level of expression of nuclear encoded OXPHOS genes may have generally decreased expression of nuclear genes. To test this, I created a heatmap using a list of housekeeping genes suggested to be suitable for use as reference genes in experiments measuring RNA expression ((Eisenberg and Levanon, 2013), Figure 6.13). Although the expression levels of the selected housekeeping genes were not as consistent as expected, I identified a cluster of 12 samples with decreased expression across the list of 11 housekeeping genes. Of these 12 samples, 11 (91.7%) were present in the clusters showing reduced expression of nuclear encoded OXPHOS genes (Figure 6.11). This accounts for 78.6% of samples displaying reduced expression of nuclear encoded OXPHOS genes, suggesting that these samples may have generally decreased expression of nuclear genes that is not specific to those involved in mitochondrial function.

Removal of samples from poor quality blastocysts from this analysis results in more consistent expression of nuclear and mtDNA encoded OXPHOS genes across and within samples (Figure 6.14). A number of nuclear encoded OXPHOS genes have low expression across all samples; it may be interesting to look at these genes in more detail in the future.

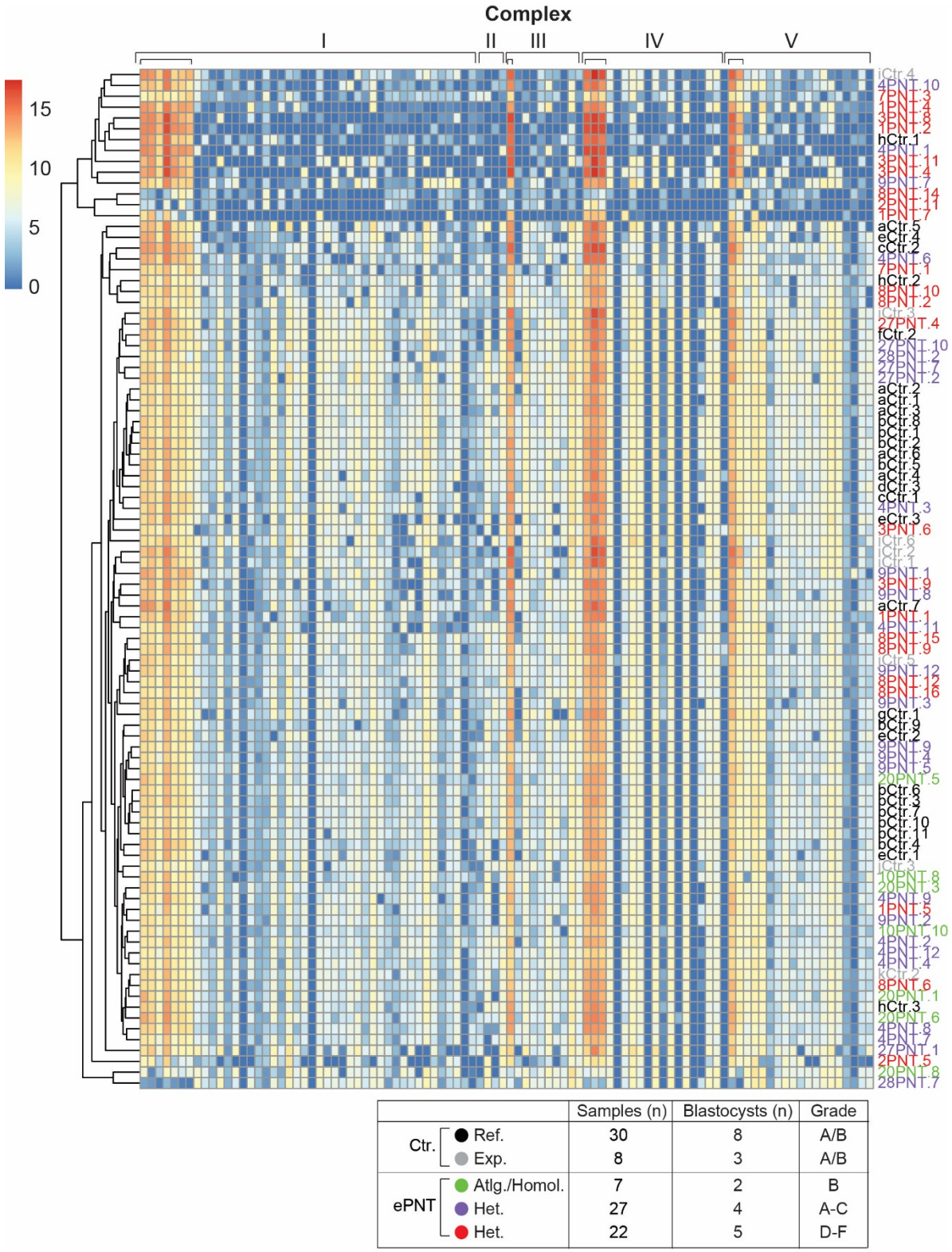


Figure 6.12: Expression of OXPHOS genes. Heatmap showing the expression of nuclear and mtDNA encoded OXPHOS genes (RPKM) according to complex, plotted on a high-to-low scale (red to blue). Brackets within those showing each complex indicate mtDNA encoded genes. Samples are labelled using a colour code indicated in the key. This analysis includes all ePNT samples (blastocysts grade A-F).

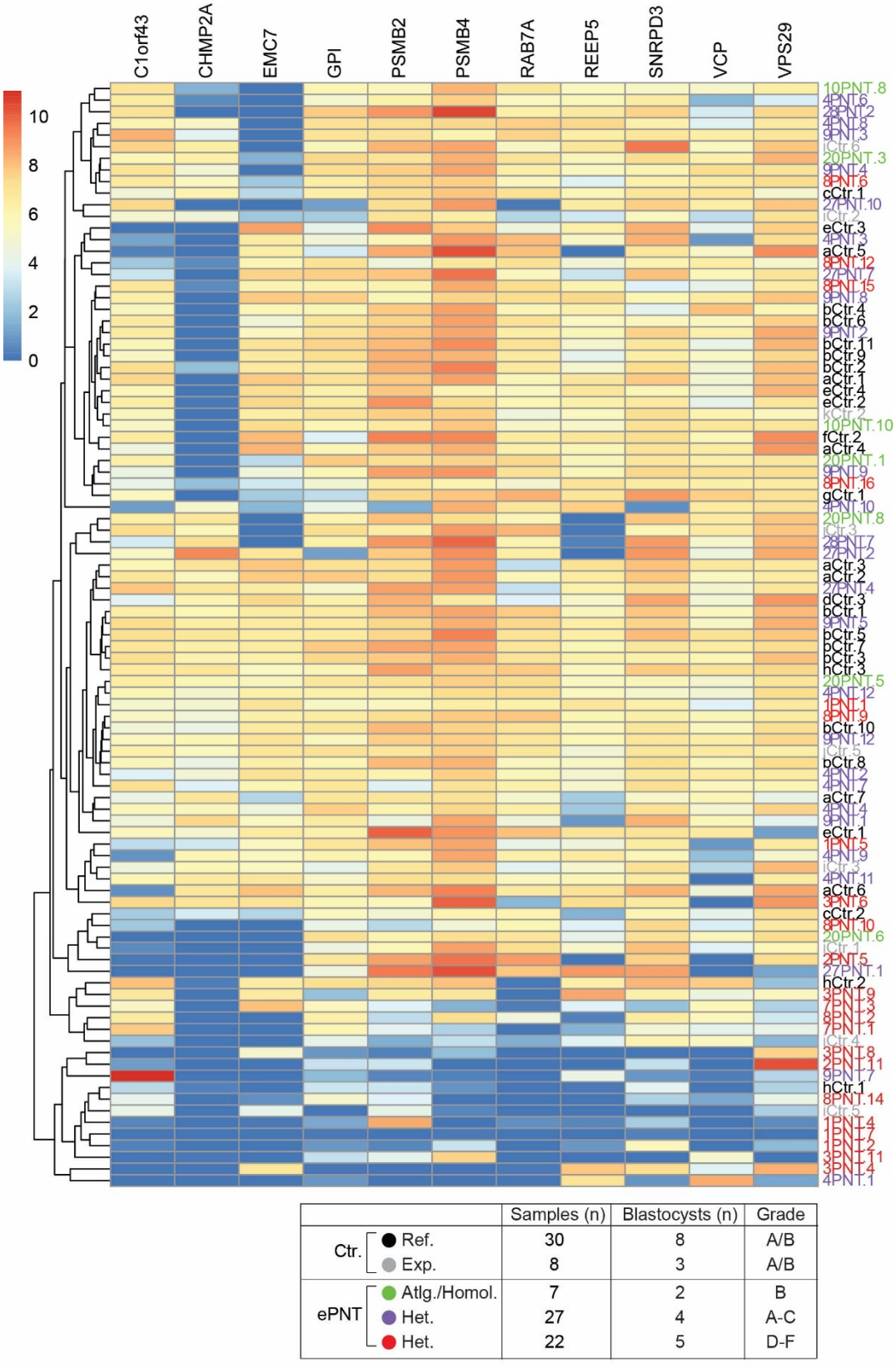
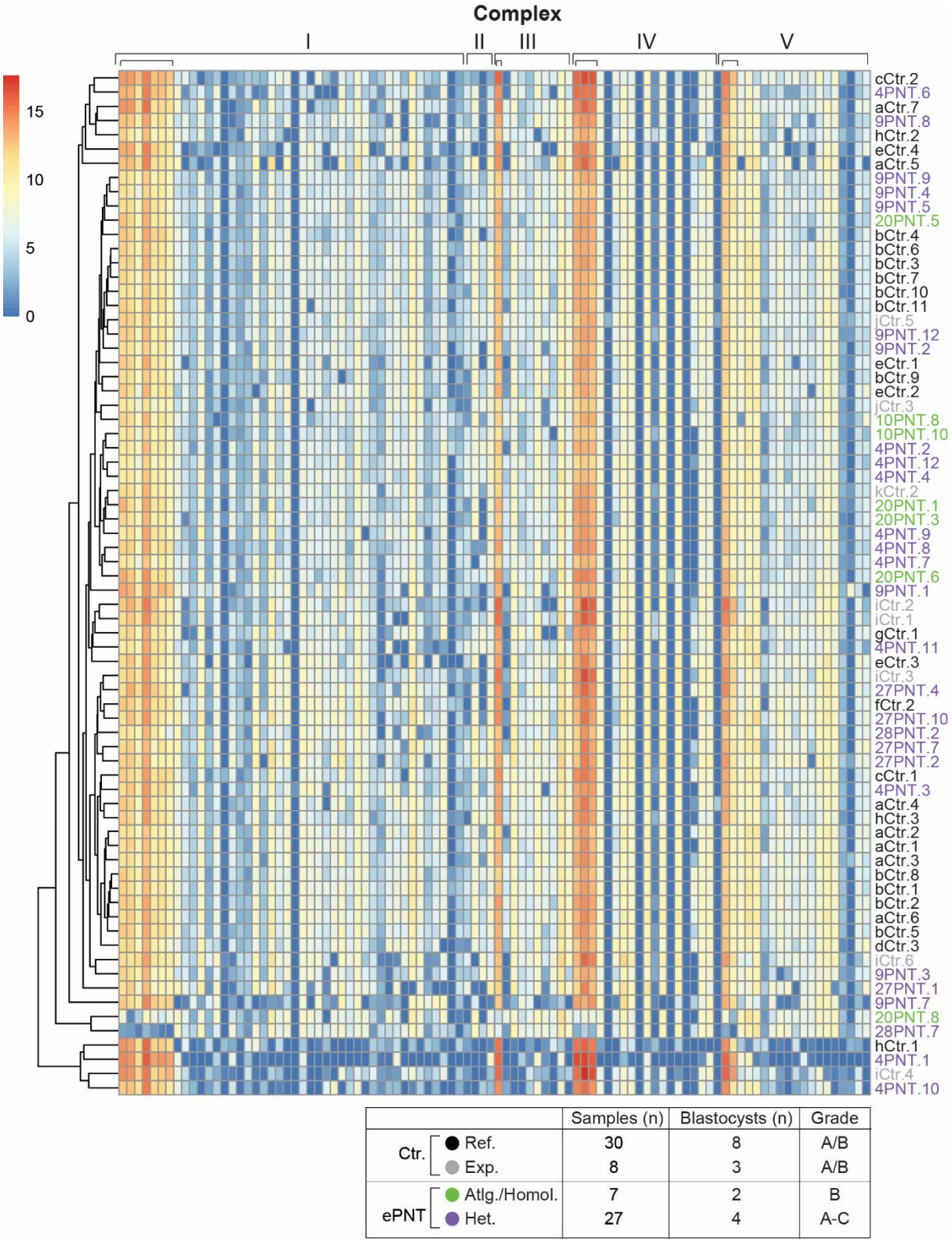


Figure 6.13: Expression of housekeeping genes. Heatmap showing the expression of nuclear encoded housekeeping genes (RPKM) plotted on a high-to-low scale (red to blue). Samples are labelled using a colour code indicated in the key.



		Samples (n)	Blastocysts (n)	Grade
Ctr.	Ref.	30	8	A/B
	Exp.	8	3	A/B
ePNT	Atlg./Homol.	7	2	B
	Het.	27	4	A-C

Figure 6.14: Expression of OXPHOS genes. Heatmap showing the expression of nuclear and mtDNA encoded OXPHOS genes (RPKM) according to complex, plotted on a high-to-low scale (red to blue). Brackets within those showing each complex indicate mtDNA encoded genes. Samples are labelled using a colour code indicated in the key. This analysis excludes poor quality ePNT samples.

6.4.3 Mitochondrial gene expression according to lineage

Finally, it is possible that mitochondrial gene expression could be related to lineage. This could be a contributing factor towards the high variation of mtDNA gene expression within blastocysts. To investigate this I calculated the median level of expression (RPKM) across all mtDNA encoded OXPHOS genes per sample and plotted the values, grouping samples according to lineage (Figure 6.15). I found that the average level of expression was similar between lineages; there was no statistically significant difference between groups. However, there was a trend towards increased expression of mtDNA encoded OXPHOS genes in TE and samples of mixed lineage. Upregulation of mitochondrial activity is thought to be a characteristic of TE differentiation (Hewitson and Leese, 1993; Houghton, 2006); a number of TE samples have increased median mtDNA encoded OXPHOS gene expression and the mean of the TE group is increased compared to the ICM lineages. Increased mtDNA encoded OXPHOS expression in samples of mixed lineage may be linked to the upregulation of genes associated with metabolism in these samples (Table 6.3). Furthermore, there is a trend towards reduced mtDNA encoded OPXHOS expression in the EPI lineage; this may be a mechanism to reduce the risk of acquired mtDNA mutations in the population destined for transmission to the next generation.

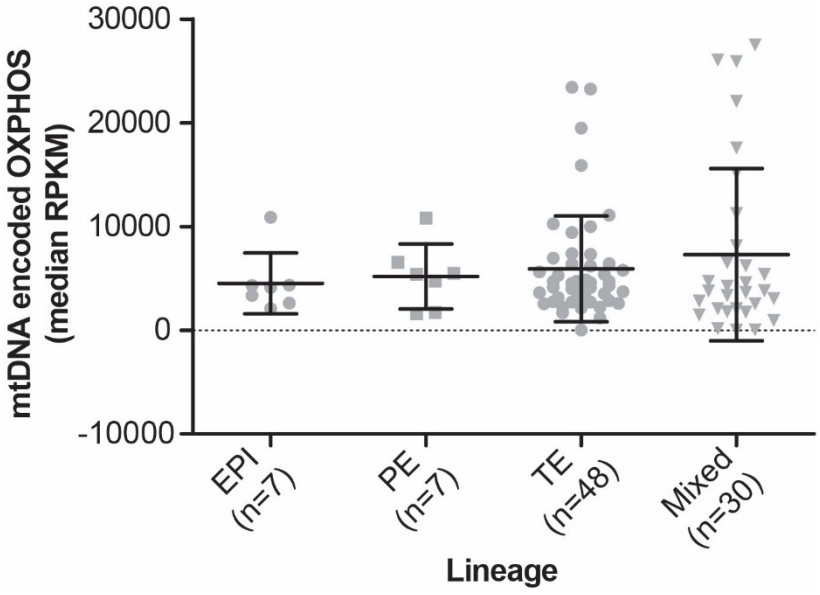


Figure 6.15: Expression of mtDNA encoded OXPHOS genes according to lineage. Graph showing median expression of mtDNA encoded OXPHOS genes (RPKM) in samples grouped according to lineage: epiblast (EPI), primitive endoderm (PE), trophectoderm (TE) and mixed. Bars show the mean ± SD. The number of samples within each group is shown.

6.5 Discussion

In this chapter I have examined global, lineage-associated and mitochondrial gene expression between single-cell samples from unmanipulated control and ePNT blastocysts. Overall, global gene expression patterns in the blastocyst do not appear to be disrupted by the ePNT procedure, and the techniques we have used for analysis are sensitive enough to detect differences in gene expression between samples from good and poor quality blastocysts and to distinguish cells by lineage.

Gene ontology (GO) analysis of differentially expressed genes between good and poor quality blastocysts revealed enrichment of GOs related to metabolism in genes upregulated in poor quality blastocysts, this provides support to the 'quiet embryo hypothesis' (Leese, 2002). The molecular characteristics throughout embryonic development which may contribute to a 'quiet' metabolism and embryo viability have been discussed (Baumann *et al.*, 2007; Leese *et al.*, 2007) and include protein synthesis and recycling. In further support of this hypothesis, a relationship has been demonstrated between amino acid consumption and DNA damage in porcine and bovine embryos (Sturmey *et al.*, 2009), and amino acid turnover used as a marker of blastocyst developmental potential in ICSI (Brison *et al.*, 2004) and cryopreserved human embryos (Stokes *et al.*, 2007). The 'quiet embryo hypothesis' has recently been extended and describes a 'Goldilocks zone' of metabolic activity within which embryos with maximum developmental potential are located (Leese *et al.*, 2016). Embryos with a 'quiet' metabolism display reduced DNA damage and increased capability to respond to damage effectively, as resources are not used for continuous repair (Leese *et al.*, 2007). Whereas embryos with an 'active' metabolism may have increased metabolic processes linked to DNA damage and stress response, which could cause increased levels of Reactive Oxygen Species (ROS) within the blastocyst and subsequent negative consequences for development (Leese *et al.*, 2008). Furthermore, it could be argued that increased metabolism can cause increased ROS production, which in turn increases the risk of DNA damage.

Genes linked to the cell cycle were also found to be upregulated in poor quality blastocysts. As discussed in Chapter 4.5, a high proportion of poor quality blastocysts were aneuploid for multiple chromosomes (Figure 4.16), this includes blastocysts submitted to scRNA-seq (7PNT and 8PNT). Progression of the cell cycle is delayed if all chromosomes are not correctly attached to the spindle via kinetochores; this is regulated by the spindle assembly checkpoint (SAC) and maintains genome stability by preventing cell division when chromosomes cannot be segregated accurately. It is surprising that blastocysts with such high

incidence of aneuploidy could continue to develop to the blastocyst stage. However, results from a study into the origin and impact of embryonic aneuploidy indicate that it is possible for aneuploid embryos to develop to the blastocyst stage (Fragouli *et al.*, 2013). The authors suggest that aneuploid embryos may be selected against at around the time of implantation or shortly after, as they are not detected in clinical pregnancies (Fragouli *et al.*, 2013). The upregulation of cell cycle genes in poor quality blastocysts could indicate disruption in the regulation of cell cycle progression in samples from embryos which had a high incidence of aneuploidy.

Enrichment of GOs related to cell signalling and ion transport were identified in genes downregulated in poor quality blastocysts. Formation of the fluid filled cavity known as the blastocoel requires transport of Na^+ to cause osmotic fluid accumulation, which contributes to the expansion of the blastocyst. The fact that GOs relating to ion transport are downregulated in poor quality blastocysts may indicate a problem with blastocyst expansion which could be specifically related to blastocyst which were grade E (early) on day 6 of development, at the time of disaggregation for RNA-seq analysis. Alternatively, there is possibly a general mis-regulation of cell signalling within poor quality blastocysts.

Analysis of lineage-associated gene expression showed that it is possible to assign lineages to samples from both control and ePNT blastocysts. The high proportion of samples from poor quality blastocysts that had mixed expression of lineage-associated genes, often from the EPI and PE, could be due to mis-regulation of gene expression, or a developmental delay meaning that cell fate is undecided at the point of analysis. Interestingly, genes that were upregulated in PE samples were linked to GOs including organelle organisation, cell death and cell cycle. This could be associated with the cell sorting which occurs in the inner cell mass prior to lineage specification (Plusa *et al.*, 2008). It is known that apoptosis may play a role in human preimplantation development, for example by eliminating abnormal cells and those with inappropriate developmental potential (reviewed in (Hardy (1999)). Furthermore, in Chapter 4 TUNEL staining revealed a trend towards increased cell death in the ICM compared to the TE (Figure 4.7).

Comparison of mitochondrial gene expression between controls and ePNT samples revealed high variation in gene expression but overlap of clusters of control and ePNT samples of all grades. However, samples from poor quality ePNT blastocysts displayed increased variation and a distinct gene expression profile to other samples. This could be due to the increased

metabolism of poor quality ePNT blastocysts causing increased levels of ROS and possibly prompting apoptosis, which are all processes linked to mitochondria.

I have shown that there is high variation of mtDNA encoded OXPHOS gene expression within and between blastocysts, which is not dependent on grade. I hypothesised that this could be related to the lineage of samples; with some lineages having higher mtDNA gene expression than others. Although I found no statistically significant difference in expression of mtDNA encoded OXPHOS genes between lineages, there was a trend towards increased expression in TE and samples with mixed lineage. The number of EPI and PE samples is very low compared to TE and mixed samples. It will be particularly interesting to further explore the trend towards reduced expression of mitochondrial genes in EPI cells. This may act to minimise the risk of mtDNA mutations in the founder population destined for transmission to the next generation.

The observation of higher expression of mtDNA encoded OXPHOS genes compared to nuclear DNA encoded OXPHOS genes could simply be due to the increased copy number of mtDNA; there are 2 copies of the nuclear genome per cell compared with approximately 1,500 copies of mtDNA. Alternatively, a recent publication studied mitochondrial and nuclear gene expression in *Saccharomyces cerevisiae* during mitochondrial biogenesis (Couvillion *et al.*, 2016). The authors found that transcript levels of nuclear and mtDNA encoded subunits of OXPHOS complexes did not increase/decrease synchronously. However, it should be noted that a number of poor quality samples with decreased expression of nuclear encoded OXPHOS genes also displayed reduced expression of housekeeping genes, suggesting this was not specifically related to mis-regulated mitochondrial gene expression. It is surprising that not all these samples were outliers in PCA of global gene expression, as would be expected if gene expression was generally reduced.

Overall, these findings suggest that expression of mitochondrial genes is highly variable within and between blastocysts. The ePNT procedure and production of new combinations of nuclear and mitochondrial genomes does not appear to disrupt mitochondrial gene expression in good quality blastocysts compared to unmanipulated controls.

As scRNA-seq is a relatively new technology, there are many computational challenges relating to the analysis of scRNA-seq data. There is currently no standard pipeline for computational analysis. In this chapter I have used two different normalisation methods (RPKM and DESeq2) and demonstrated that results are not influenced by the chosen method. Furthermore, I have used several techniques for the clustering of the data. However, it would

be interesting to reanalyse the data using the updated human genome to align the reads. The data presented above are based on reads aligned to hg19 whereas the most recently available is hg38, which contains the updated mitochondrial genome.

It would also be interesting to analyse expression of mitochondrial genes throughout preimplantation development, and further investigate the expression of mitochondrial genes between lineages. This would be possible by extending our data to analyse more samples from blastocysts and embryos at different stages throughout development, including the cleavage divisions. There is also publicly available scRNA-seq data from numerous publications that have investigated gene expression throughout preimplantation development.

To conclude, samples from good quality ePNT blastocysts are indistinguishable from unmanipulated controls regarding global, lineage-associated and mitochondrial gene expression. This analysis indicates that the gene expression profile is not disrupted in ePNT blastocysts with the potential for implantation. The analysis of poor quality blastocysts has allowed us to gain new biological insights into pathways and processes which may contribute to determining embryo viability. Results suggest that blastocysts of poorer quality show an increased level of metabolic activity, developmental delay and/or mis-regulation of gene expression. Thus, these blastocysts should not be selected for use in treatment if ePNT is to be offered clinically.

Chapter 7. Discussion

The central aim of this project was to perform preclinical studies testing the safety and efficiency of PNT. Survival and formation of good quality blastocysts has improved by making numerous modifications to the PNT procedure, including altering the timing of PNT and modifications to the enucleation and embryo culture conditions. While blastocyst formation was slightly reduced following heterologous ePNT, the overall proportion of good quality blastocysts offers a reasonable chance of a successful treatment outcome. Testing several parameters to investigate the safety of ePNT, our findings indicate that good quality ePNT blastocysts are similar to unmanipulated controls, suggesting that PNT is compatible with the establishment of a normal pregnancy. Therefore, the main aim of this research has been successfully addressed.

Proof of concept studies performed using abnormally fertilised human zygotes revealed that PNT has the potential to reduce the risk of transmission of mtDNA disease (Craven *et al.*, 2010). However, the limited developmental potential of abnormally fertilised zygotes hindered further studies of PNT. Therefore, preclinical studies were performed using normally fertilised human zygotes. Surprisingly, techniques used in proof of concept studies were not well tolerated by normally fertilised zygotes. We hypothesised that this was due to the relatively accelerated development of normally fertilised zygotes and modified the timing of PNT to be performed ~8 hours post-fertilisation (ePNT) opposed to ~16-20 hours post fertilisation (LtPNT). At this stage, the zygote is expected to be in the G1 phase of the cell cycle rather than G2. We found that allowing more time for recovery between the manipulations and first mitotic division improved survival of reconstituted zygotes.

Although altering the timing of manipulations increased survival, blastocyst quality remained poor with a reduced cell number. We therefore performed a second series of ePNT experiments (series II ePNT) to test further modifications in order to promote the development of good quality blastocysts. Modifications included using calcium-free manipulation medium, reducing the concentration of HVJ-E and switching to a single-step embryo culture medium. To determine which modification improved the development of good quality blastocysts would require changing each element separately, which is not possible due to the limited number of donated human oocytes. Interestingly, mouse PNT using the same conditions as series II ePNT apart from the reduced HVJ-E concentration did not alleviate the issue of reduced blastocyst cell number in PNT blastocysts, although this problem was resolved in human embryos by the modifications introduced in series II ePNT.

This raises the possibility that the reduced concentration of HVJ-E resulted in improved blastocyst cell number and quality during series II ePNT. It is possible that high concentrations of HVJ-E used during manipulations may have residual membrane fusion effects that contribute to reduced blastocyst cell number and quality. To further investigate this, the concentration of HVJ-E used in mouse PNT should be reduced to the lowest possible concentration compatible with fusion and the effect on cell number determined. However, it is difficult to assess the effect on mouse blastocyst quality, as there is limited variability between embryos.

Use of calcium-free medium during manipulations could facilitate improved development of good quality blastocysts by reducing interference with calcium signalling. For example, it is known that sperm-induced calcium oscillations continue until pronuclei formation (Marangos *et al.*, 2003) and amplitude and frequency of oscillations can influence blastocyst development (Ozil *et al.*, 2006), and membrane damage during the manipulations could have resulted in an influx of calcium, which may have disrupted intracellular calcium homeostasis. Indeed, this problem may have been exacerbated by increased membrane porosity in the higher concentrations of HVJ-E.

The single-step culture medium G-TL was initially tested using mouse embryos in our laboratory and showed increased blastocyst formation compared with sequential medium, which required moving the embryos to a different medium on day 3. Following these findings, G-TL was introduced into the clinical laboratory at Newcastle Fertility Centre and results show increased blastocyst formation and quality (unpublished data). Therefore, it is likely that this modification also contributed to improved blastocyst formation and quality of unmanipulated and technical controls during series II ePNT. One possible explanation for the improved development in single step medium is that it reduced environmental insults due to fluctuations in temperature and pH. However, because embryos are handled in environmentally controlled isolators in our lab, this is unlikely to be the only explanation. Another possibility is that transferring embryos to a different medium deprives the embryos of any embryonic factors that may be secreted into the surrounding environment.

In series II ePNT, blastocyst formation and quality following autologous ePNT was equivalent to unmanipulated controls, suggesting that the technical procedures and reagents do not disrupt preimplantation development. However, there is a reduction in blastocyst formation following heterologous ePNT. As heterologous ePNT involves reciprocal transfers between zygotes originating from fresh and vitrified oocytes, it is likely that this reduction in

blastocyst formation is at least partially caused by an effect of vitrification. There was a reduction in blastocyst formation of MII vitrified heterologous ePNT embryos compared to autologous ePNT embryos, which occurred when the embryo was composed of fresh cytoplast and vitrified karyoplast (FreshCy) or vitrified cytoplast and fresh karyoplast (VitCy).

To test whether development was compromised by subjecting MII oocytes to multiple manipulations (warming, ICSI and ePNT) in quick succession, we performed a series of experiments in which oocytes were injected with sperm before being vitrified. Vitrification was therefore performed after the second polar body appeared. However, we found that this had a negative effect on blastocyst formation, particularly when the karyoplast was vitrified (FreshCy). This finding suggests that the nuclear component is sensitive to the effects of vitrification immediately post-fertilisation. If possible, the effect of vitrification could be investigated by performing transfers between freshly harvested oocytes, but synchronisation of egg donors is an obstacle and it is most likely that in clinical treatment vitrification of oocytes will be required.

It has also been suggested that incompatibilities between mitochondrial and nuclear genomes may contribute to reduced blastocyst development (Morrow, 2016). While this is plausible in the case of inbred laboratory species, it is less likely in humans as new combinations of nuclear and mitochondrial genomes are created in each round of meiosis and fertilisation. If incompatibility is a problem in humans, one would expect to observe reproductive barriers between people with divergent mitochondrial genotypes.

Future work will aim to investigate possible causes of reduced blastocyst formation following heterologous ePNT. A concern is that asynchrony between zygote pairs from different donors may be contributing to reduced blastocyst formation. At this early stage in the zygote, several events are occurring but are generally not well characterised in the human. Importantly, the paternal genome is demethylated and maternal and paternal DNA are replicated. It is possible that transferring pronuclei containing DNA undergoing demethylation or replication to cytoplasm which had contained pronuclei at a different stage would disrupt these processes, as the recipient cytoplast may not be able to support them. However, research in the mouse suggests fusion of G1 phase pronuclei with S phase cytoplast does not induce premature DNA replication (Yamauchi *et al.*, 2009). Research using mouse zygotes has shed light on the mechanistic basis for paternal genome demethylation, which involves oxidative demethylation catalysed by Tet3 (Gu *et al.*, 2011; Wossidlo *et al.*, 2011). The maternal

genome and imprinted regions of paternal DNA are protected by Stella (PGC7/ Dppa3) (Wossidlo *et al.*, 2011). The functional significance of global demethylation of the paternal genome is unclear, but research suggests that disruption of oxidative demethylation of the paternal genome involving Tet3 has downstream effects that can result in disrupted embryonic development (Gu *et al.*, 2011). Thus, it is possible that disruption of this process following asynchronous transfers contributed to reduced blastocyst formation following heterologous ePNT. The mechanisms and timing of centriole duplication in human zygotes is also unknown and it is not possible to study this event using mouse zygotes, as centrosomes are maternally inherited in the mouse. Characterisation of these processes in human zygotes may enable us to determine morphological correlates and tolerable limits of asynchrony compatible with blastocyst formation, which will enable improvement of blastocyst formation in future treatment.

To determine whether ePNT was associated with an increased incidence of chromosome segregation errors, we tested samples of cells, mostly taken from the trophectoderm of unmanipulated and ePNT blastocysts. This may also give an indication as to whether centriole inheritance is disrupted by ePNT, as this may cause chromosomal segregation errors. Analysis by array-CGH revealed a slight increase in aneuploidy in our control and ePNT blastocysts compared to a reference population of IVF blastocysts. Further investigation resulted in the discovery of a relationship between blastocyst quality and incidence of aneuploidy. This is also reported in the literature, however it is noted that although poor quality blastocysts, graded according to morphological characteristics, have a higher incidence of aneuploidy compared to top quality blastocysts, aneuploidy does still occur in top quality blastocysts (Fragouli *et al.*, 2014; Minasi *et al.*, 2016). Of unmanipulated control blastocysts for which 2 or more samples were analysed, all observed cases of aneuploidy were mosaic euploid/aneuploid, and the majority of aneuploid good quality ePNT blastocysts were also mosaic euploid/aneuploid. The analysis of more than 1 sample in multiple embryos may have contributed to our finding of increased aneuploidy compared to the reference population, as only 1 sample is tested in clinical IVF. Our findings question the reliability of TE analysis for pre-implantation genetic screening. In support of this, it has been reported that chromosome mosaicism, specifically mosaic euploid/aneuploid, is common in human preimplantation embryos (van Echten-Arends *et al.*, 2011). This type of aneuploidy occurs during the early mitotic cleavage divisions of the embryo. Recent research using a mouse model revealed a progressive depletion of aneuploid cells at the blastocyst stage and beyond (Bolton *et al.*, 2016). The mechanism of depletion was found to be dependent on lineage, with cells in the

ICM eliminated by apoptosis and cells in the TE encountering proliferative defects (Bolton *et al.*, 2016). The authors conclude that mosaic embryos with sufficient numbers of euploid cells have full developmental potential (Bolton *et al.*, 2016).

Nuclear genome transplantation techniques require the use of cytoskeletal inhibitors in order to relax the cytoskeleton and facilitate manipulations. I have investigated the effect and reversibility of cytoskeletal inhibitors, in order to determine which inhibitors are effective for use in PNT and do not have a detrimental effect on subsequent preimplantation development. These experiments are important in order to gain regulatory approval to allow the use of these drugs in the clinic. It is particularly important to demonstrate that the effect of the inhibitors is reversible.

When testing inhibitors of actin polymerisation for use in PNT, I found that despite latrunculin B being less potent than latrunculin A (Spector *et al.*, 1989) and zygotes showing a rapid recovery period of 30 minutes, latrunculin B had a negative effect on subsequent development. A limitation of these experiments is the use of abnormally fertilised human zygotes, which are allocated to research from the clinical lab at approximately 20 hours post-fertilisation and therefore at a late stage. This may not be comparable to zygotes that are at an early stage (~8 hours post-fertilisation) when exposed to cytoskeletal inhibitors during ePNT. Moreover, there may be additional differences between abnormal and normally fertilised human zygotes. For example, polyspermy may cause differences in cytoskeletal organisation, such as additional microtubule asters from the sperm in zygotes with >2 pronuclei, which may increase microtubule polymerisation. Although I have repeated experiments using mouse zygotes, my results indicate that there may be differences between mouse zygotes and abnormally fertilised human zygotes. This was observed during treatment of zygotes with nocodazole, which targets microtubule polymerisation and cytochalasin C, an inhibitor of actin polymerisation. There are numerous factors that could account for differences, including the different sizes, the earlier stage of mouse zygotes and/or additional microtubule asters in abnormally fertilised human zygotes. However, the findings using mouse and abnormally fertilised human zygotes were supported by comparison of the effect of nocodazole and latrunculin A or latrunculin B on survival and preimplantation development following human autologous ePNT, and confirming the results by mouse PNT. Results show that blastocyst quality was reduced following ePNT using latrunculin B. However, blastocyst formation and quality are comparable between autologous ePNT embryos using nocodazole and latrunculin A and unmanipulated control embryos. This suggests that reagents used during ePNT, including the specified cytoskeletal inhibitors, do not disrupt preimplantation development.

Analysis of gene expression patterns revealed that samples from good quality ePNT blastocysts are indistinguishable from samples from unmanipulated controls. Furthermore, the inclusion of poor quality blastocysts in scRNA-seq analysis enabled us to gain new biological insights into pathways and processes which may contribute to the developmental potential of embryos. Gene Ontology (GO) analysis of differentially expressed genes in samples from good and poor quality blastocysts revealed the upregulation of genes related to metabolic processes. This is consistent with the 'quiet embryo hypothesis' (Leese, 2002), which was recently modified to describe an optimal range of metabolic activity compatible with onward development, referred to as the 'Goldilocks zone'; metabolism that is not too 'quiet' or too 'active' (Leese *et al.*, 2016).

While I found a correlation between blastocyst morphology and altered gene expression profiles compared to good quality blastocysts, including upregulation of genes involved in metabolic processes, this may not be the case for cleavage stage embryos. For example, it has been reported that amino acid turnover, but not morphological score, was correlated with DNA damage (Sturmey *et al.*, 2009). This is supported by previous research indicating that amino acid profiling may offer the ability to predict embryo viability independent of embryo grade (Brison *et al.*, 2004), and that metabolic data to predict viability does not correlate with blastomere number or fragmentation (Vergouw *et al.*, 2008). These findings gave ground to the suggestion that morphological assessment is an unreliable method for the assessment of embryo quality (Sturmey *et al.*, 2009). However, my findings suggest that the quality score assigned to ePNT blastocysts, which takes into account blastocyst expansion and morphology of the ICM and TE (Cutting *et al.*, 2008) correlates with metabolic activity. Moreover, the grading scheme used in this thesis showed a strong correlation with implantation potential of IVF blastocysts. In addition, poor quality blastocysts showed a considerably higher incidence of aneuploidy.

Samples from blastocysts used for scRNA-seq analysis were also analysed using array-CGH to detect aneuploidy. All blastocysts grades A-C included in gene expression analysis were either euploid or mosaic euploid/aneuploid. A number of blastocysts grades D-F were also euploid and mosaic euploid/aneuploid, but 7PNT and 8PNT (both grade F) were mosaic aneuploid and uniform aneuploid, respectively. It is likely that this influenced gene expression, and may have contributed to the upregulation of genes involved in the cell cycle in poor quality blastocysts, as cell cycle progression would be disrupted in aneuploid cells. It is possible that aneuploid cells may be delayed in progression through M phase by the spindle

assembly checkpoint. Further gene expression analysis should account for aneuploidy and possibly investigate the extent to which this affects gene expression.

Gene expression analysis showed that lineages can be assigned to samples from control and ePNT blastocysts. Interestingly, GO analysis revealed upregulation of genes involved in organelle organisation, cell cycle and cell death in the primitive endoderm compared to the epiblast. Regarding cell cycle and organelle organisation, it is possible that these are linked. For example, it is known that mitochondrial morphology and dynamics change throughout the cell cycle. In mitosis, mitochondria are individual organelles able to segregate to daughter cells, whereas during other cell cycle phases, mitochondria can be observed as a fused network (reviewed in Detmer and Chan (2007)). Increased cell death was also observed in the ICM compared to the TE following TUNEL staining of control and ePNT blastocysts. It is possible that cell death contributes to cell sorting in the ICM prior to lineage specification (Plusa *et al.*, 2008), or eliminates abnormal cells (Hardy, 1999). Moreover, it has been reported that apoptosis occurs in the ICM to eliminate aneuploid cells (Bolton *et al.*, 2016).

Analysis of mitochondrial gene expression revealed variability within and between blastocysts, which could be linked to lineage and/or the involvement of mitochondria in many cellular processes. With regards to lineage, a trend towards reduced expression of mtDNA encoded OXPHOS genes in the epiblast may act to minimise risk of mtDNA mutations occurring in cells destined to become the foetus. It will be interesting to investigate this further, using an increased sample size. Importantly, these results indicate that ePNT does not disrupt mitochondrial gene expression.

I have performed robust analysis of scRNA-seq data, using two normalisation methods and several techniques for clustering of the data. A recent publication presented a model for analysis of scRNA-seq data using a beta-Poisson mixture model and suggest this offers the opportunity for improved analysis of scRNA-seq data (Vu *et al.*, 2016). However, this has not been tested in cases in which multiple confounding factors may drive variation in gene expression. It would be interesting to reanalyse the data using the updated human genome (hg38) to align the reads, as this contains the updated mitochondrial genome. Also, extending the data to include more samples from the ICM lineages and possibly blastomeres from the earlier cleavage stage divisions would enable the possibility to gain new biological insights into mitochondrial gene expression throughout development and between different lineages of the blastocyst.

To conclude, here I have presented the first preclinical studies testing the safety of PNT in normally fertilised zygotes. The findings indicate that ePNT does not have a detrimental effect on preimplantation development by all tested parameters. Modifications to the procedure resulted in the production of good quality blastocysts, whose morphological features are compatible with the establishment of normal pregnancies. Testing of cytoskeletal inhibitors revealed that they are reversible within 2 hours. Of the actin polymerisation inhibitors tested, latrunculin A was found to be the most suitable for use in ePNT. Finally, analysis of gene expression patterns in good quality blastocysts did not detect any disruption to global, lineage-associated or mitochondrial gene expression in ePNT blastocysts. Furthermore, analysis of scRNA-seq data revealed that genes involved in the cell cycle and metabolism are upregulated in poor quality blastocysts, providing new insights into pathways and processes which may contribute to embryo viability.

Moving towards clinical treatment, the choice of technique for nuclear genome transplantation will depend on a balance between the level of mtDNA carryover and developmental competence. It has been reported that PNT is associated with increased levels of mtDNA carryover compared to polar body transfer and metaphase II spindle transfer (Wang *et al.*, 2014). Although I have not presented data on mtDNA carryover as it is beyond the scope of this thesis, this data was included in our recent publication (Hyslop *et al.*, 2016). We found that mtDNA carryover was <2% in the majority of blastocysts following ePNT. However, a progressive increase in heteroplasmy was observed in one stem cell line derived from an ePNT blastocyst with a low level of carryover (<4%). Therefore, although ePNT may reduce the risk of mtDNA disease transmission, prevention cannot be guaranteed. It will be important to test metaphase II spindle transfer and polar body transfer using human oocytes, and compare mtDNA carryover and blastocyst development between the techniques.

Abbreviations

ADP	Adenosine diphosphate
APC	Anaphase promoting complex
ATP	Adenosine triphosphate
CO_2	Carbon dioxide
DAPI	4',6-Diamidino-2-Phenylindole
DIC	Differential interference contrast
D-loop	Displacement loop
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EGA	Embryonic genome activation
EPI	Epiblast
ER	Endoplasmic reticulum
ESCs	Embryonic stem cells
ETC	Electron transport chain
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GFP	Green fluorescent protein
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
hCG	Human chorionic gonadotrophin
HFEA	Human Fertilisation and Embryology Authority
HSP	Heavy stand promoter
H-strand	Heavy stand
HVJ	Hemagglutinating virus of Japan
HVJ-E	Hemagglutinating virus of Japan envelope
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection

IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
kb	Kilobase
LH	Luteinising hormone
LSP	Light strand promoter
L-strand	Light strand
M	Molar
MELAS	Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes
MERRF	Myoclonic epilepsy and ragged red fibres
MI	Metaphase I
MII	Metaphase II
Mfn	Mitofusin
ml	Millilitre
mM	Millimolar
mm	Millimetre
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
mtEF	Mitochondrial elongation factor
mtIF	Mitochondrial initiation factor
MST	Metaphase II spindle transfer
mtSSB	Mitochondrial single-stranded binding protein
nM	Nanomolar
O_H	Origin of heavy strand replication
O_L	Origin of light strand replication
OXPHOS	Oxidative phosphorylation
PB	Polar body
PBS	Phostphate buffered saline
PE	Primitive endoderm

PFA	Paraformaldehyde
PGC	Primordial germ cell
PGD	Preimplantation genetic diagnosis
PMSG	Pregnant mare's serum gonadotrophin
PNT	Pronuclear transfer
POLG	Polymerase gamma
POLRMT	Mitochondrial RNA polymerase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SD	Standard deviation
TE	Trophectoderm
TFAM	Mitochondrial transcription factor A
TFB	Transcription factor B
tRNA	Transfer RNA
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UV	Ultraviolet

References

Ahlstrom, A., Westin, C., Reismer, E., Wikland, M. and Hardarson, T. (2011) 'Trophectoderm morphology: an important parameter for predicting live birth after single blastocyst transfer', *Hum Reprod*, 26(12), pp. 3289-96.

Al Rawi, S., Louvet-Vallee, S., Djeddi, A., Sachse, M., Culetto, E., Hajjar, C., Boyd, L., Legouis, R. and Galy, V. (2011) 'Postfertilization autophagy of sperm organelles prevents paternal mitochondrial DNA transmission', *Science*, 334(6059), pp. 1144-7.

Alberts, B., Johnson, A., Lewis, J., Morgan, D., Raff, M., Roberts, K. and Walter, P. (2015) *Molecular Biology of the Cell*. Sixth edn. New York: Garland Science, Taylor and Francis Group.

Alizadeh, Z., Kageyama, S. and Aoki, F. (2005) 'Degradation of maternal mRNA in mouse embryos: selective degradation of specific mRNAs after fertilization', *Mol Reprod Dev*, 72(3), pp. 281-90.

Anders, S., Pyl, P.T. and Huber, W. (2015) 'HTSeq--a Python framework to work with high-throughput sequencing data', *Bioinformatics*, 31(2), pp. 166-9.

Aoki, F., Worrad, D.M. and Schultz, R.M. (1997) 'Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo', *Dev Biol*, 181(2), pp. 296-307.

Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N. and Lovell-Badge, R. (2003) 'Multipotent cell lineages in early mouse development depend on SOX2 function', *Genes Dev*, 17(1), pp. 126-40.

Babcock, D.F., Herrington, J., Goodwin, P.C., Park, Y.B. and Hille, B. (1997) 'Mitochondrial participation in the intracellular Ca2+ network', *J Cell Biol*, 136(4), pp. 833-44.

Balakier, H., MacLusky, N.J. and Casper, R.F. (1993) 'Characterization of the first cell cycle in human zygotes: implications for cryopreservation', *Fertil Steril*, 59(2), pp. 359-65.

Barrell, B.G., Bankier, A.T. and Drouin, J. (1979) 'A different genetic code in human mitochondria', *Nature*, 282(5735), pp. 189-94.

Barrett, S.L. and Albertini, D.F. (2010) 'Cumulus cell contact during oocyte maturation in mice regulates meiotic spindle positioning and enhances developmental competence', *J Assist Reprod Genet*, 27(1), pp. 29-39.

Barritt, J.A., Brenner, C.A., Malter, H.E. and Cohen, J. (2001) 'Mitochondria in human offspring derived from ooplasmic transplantation', *Hum Reprod*, 16(3), pp. 513-6.

Barritt, J.A., Kokot, M., Cohen, J., Steuerwald, N. and Brenner, C.A. (2002) 'Quantification of human ooplasmic mitochondria', *Reprod Biomed Online*, 4(3), pp. 243-7.

Barros, C. and Yanagimachi, R. (1971) 'Induction of zona reaction in golden hamster eggs by cortical granule material', *Nature*, 233(5317), pp. 268-9.

Battaglia, D.E., Goodwin, P., Klein, N.A. and Soules, M.R. (1996) 'Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women', *Hum Reprod*, 11(10), pp. 2217-22.

Baughman, J.M., Perocchi, F., Girgis, H.S., Plovanich, M., Belcher-Timme, C.A., Sancak, Y., Bao, X.R., Strittmatter, L., Goldberger, O., Bogorad, R.L., Koteliansky, V. and Mootha, V.K. (2011) 'Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter', *Nature*, 476(7360), pp. 341-5.

Baumann, C.G., Morris, D.G., Sreenan, J.M. and Leese, H.J. (2007) 'The quiet embryo hypothesis: molecular characteristics favoring viability', *Mol Reprod Dev*, 74(10), pp. 1345-53.

Bianchi, E., Doe, B., Goulding, D. and Wright, G.J. (2014) 'Juno is the egg Izumo receptor and is essential for mammalian fertilization', *Nature*, 508(7497), pp. 483-7.

Bianchi, E. and Wright, G.J. (2014) 'Izumo meets Juno: preventing polyspermy in fertilization', *Cell Cycle*, 13(13), pp. 2019-20.

Blakeley, P., Fogarty, N.M., del Valle, I., Wamaitha, S.E., Hu, T.X., Elder, K., Snell, P., Christie, L., Robson, P. and Niakan, K.K. (2015) 'Defining the three cell lineages of the human blastocyst by single-cell RNA-seq', *Development*, 142(18), pp. 3151-65.

Bloor, D.J., Metcalfe, A.D., Rutherford, A., Brison, D.R. and Kimber, S.J. (2002) 'Expression of cell adhesion molecules during human preimplantation embryo development', *Mol Hum Reprod*, 8(3), pp. 237-45.

Bolton, H., Graham, S.J., Van der Aa, N., Kumar, P., Theunis, K., Fernandez Gallardo, E., Voet, T. and Zernicka-Goetz, M. (2016) 'Mouse model of chromosome mosaicism reveals lineage-specific depletion of aneuploid cells and normal developmental potential', *Nat Commun*, 7, p. 11165.

Boulet, L., Karpati, G. and Shoubridge, E.A. (1992) 'Distribution and threshold expression of the tRNA(Lys) mutation in skeletal muscle of patients with myoclonic epilepsy and ragged-red fibers (MERRF)', *Am J Hum Genet*, 51(6), pp. 1187-200.

Boveris, A., Oshino, N. and Chance, B. (1972) 'The cellular production of hydrogen peroxide', *Biochem J*, 128(3), pp. 617-30.

Braude, P., Bolton, V. and Moore, S. (1988) 'Human gene expression first occurs between the four- and eight-cell stages of preimplantation development', *Nature*, 332(6163), pp. 459-61.

Bredenoord, A.L. and Braude, P. (2010) 'Ethics of mitochondrial gene replacement: from bench to bedside', *Bmj*, 341, p. c6021.

Bredenoord, A.L., Pennings, G. and de Wert, G. (2008) 'Ooplasmic and nuclear transfer to prevent mitochondrial DNA disorders: conceptual and normative issues', *Hum Reprod Update*, 14(6), pp. 669-78.

Brennecke, P., Anders, S., Kim, J.K., Kolodziejczyk, A.A., Zhang, X., Proserpio, V., Baying, B., Benes, V., Teichmann, S.A., Marioni, J.C. and Heisler, M.G. (2013) 'Accounting for technical noise in single-cell RNA-seq experiments', *Nat Methods*, 10(11), pp. 1093-5.

Briggs, R. and King, T.J. (1952) 'Transplantation of living nuclei from blastula cells into enucleated frog's eggs', *Proc Natl Acad Sci USA,* 38, pp. 455-61.

Brison, D.R., Houghton, F.D., Falconer, D., Roberts, S.A., Hawkhead, J., Humpherson, P.G., Lieberman, B.A. and Leese, H.J. (2004) 'Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover', *Hum Reprod*, 19(10), pp. 2319-24.

Brison, D.R., Sturmey, R.G. and Leese, H.J. (2014) 'Metabolic heterogeneity during preimplantation development: the missing link?', *Hum Reprod Update*, 20(5), pp. 632-40.

Bromhall, J.D. (1975) 'Nuclear transplantation in the rabbit egg', *Nature,* 258(5537), pp. 719-22.

Brouhard, G.J. (2015) 'Dynamic instability 30 years later: complexities in microtubule growth and catastrophe', *Mol Biol Cell*, 26(7), pp. 1207-10.

Brown, D.T., Herbert, M., Lamb, V.K., Chinnery, P.F., Taylor, R.W., Lightowlers, R.N., Craven, L., Cree, L., Gardner, J.L. and Turnbull, D.M. (2006) 'Transmission of mitochondrial DNA disorders: possibilities for the future', *Lancet*, 368(9529), pp. 87-9.

Burkart, A.D., Xiong, B., Baibakov, B., Jimenez-Movilla, M. and Dean, J. (2012) 'Ovastacin, a cortical granule protease, cleaves ZP2 in the zona pellucida to prevent polyspermy', *J Cell Biol*, 197(1), pp. 37-44.

Calarco, P.G. (2005) 'The role of microfilaments in early meiotic maturation of mouse oocytes', *Microsc Microanal*, 11(2), pp. 146-53.

Calvo, S.E., Clauser, K.R. and Mootha, V.K. (2016) 'MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins', *Nucleic Acids Res*, 44(D1), pp. D1251-7.

Calvo, S.E. and Mootha, V.K. (2010) 'The mitochondrial proteome and human disease', *Annu Rev Genomics Hum Genet*, 11, pp. 25-44.

Cao, L., Shitara, H., Horii, T., Nagao, Y., Imai, H., Abe, K., Hara, T., Hayashi, J. and Yonekawa, H. (2007) 'The mitochondrial bottleneck occurs without reduction of mtDNA content in female mouse germ cells', *Nat Genet*, 39(3), pp. 386-90.

Cao, L., Shitara, H., Sugimoto, M., Hayashi, J., Abe, K. and Yonekawa, H. (2009) 'New evidence confirms that the mitochondrial bottleneck is generated without reduction of mitochondrial DNA content in early primordial germ cells of mice', *PLoS Genet*, 5(12), p. e1000756.

Capmany, G., Taylor, A., Braude, P.R. and Bolton, V.N. (1996) 'The timing of pronuclear formation, DNA synthesis and cleavage in the human 1-cell embryo', *Mol Hum Reprod*, 2(5), pp. 299-306.

Carafoli, E., Tiozzo, R., Lugli, G., Crovetti, F. and Kratzing, C. (1974) 'The release of calcium from heart mitochondria by sodium', *J Mol Cell Cardiol*, 6(4), pp. 361-71.

Chaigne, A., Campillo, C., Voituriez, R., Gov, N.S., Sykes, C., Verlhac, M.H. and Terret, M.E. (2016) 'F-actin mechanics control spindle centring in the mouse zygote', *Nat Commun*, 7, p. 10253.

Chazaud, C., Yamanaka, Y., Pawson, T. and Rossant, J. (2006) 'Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway', *Dev Cell*, 10(5), pp. 615-24.

Chiang, T., Duncan, F.E., Schindler, K., Schultz, R.M. and Lampson, M.A. (2010) 'Evidence that weakened centromere cohesion is a leading cause of age-related aneuploidy in oocytes', *Curr Biol*, 20(17), pp. 1522-8.

Chinnery, P.F., Elliott, H.R., Hudson, G., Samuels, D.C. and Relton, C.L. (2012) 'Epigenetics, epidemiology and mitochondrial DNA diseases', *Int J Epidemiol*, 41(1), pp. 177-87.

Claiborne, A.B., English, R.A. and Kahn, J.P. (2016) 'ETHICS OF NEW TECHNOLOGIES. Finding an ethical path forward for mitochondrial replacement', *Science*, 351(6274), pp. 668-70.

Clayton, D.A. (1982) 'Replication of animal mitochondrial DNA', *Cell*, 28(4), pp. 693-705.

Clift, D. and Schuh, M. (2013) 'Restarting life: fertilization and the transition from meiosis to mitosis', *Nat Rev Mol Cell Biol*, 14(9), pp. 549-62.

Cockburn, K. and Rossant, J. (2010) 'Making the blastocyst: lessons from the mouse', *J Clin Invest*, 120(4), pp. 995-1003.

Cohen, J., Scott, R., Alikani, M., Schimmel, T., Munne, S., Levron, J., Wu, L., Brenner, C., Warner, C. and Willadsen, S. (1998) 'Ooplasmic transfer in mature human oocytes', *Mol Hum Reprod*, 4(3), pp. 269-80.

Couvillion, M.T., Soto, I.C., Shipkovenska, G. and Churchman, L.S. (2016) 'Synchronized mitochondrial and cytosolic translation programs', *Nature*, 533(7604), pp. 499-503.

Craven, L., Tuppen, H.A., Greggains, G.D., Harbottle, S.J., Murphy, J.L., Cree, L.M., Murdoch, A.P., Chinnery, P.F., Taylor, R.W., Lightowlers, R.N., Herbert, M. and Turnbull, D.M. (2010) 'Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA disease', *Nature*, 465(7294), pp. 82-5.

Cree, L.M., Samuels, D.C., de Sousa Lopes, S.C., Rajasimha, H.K., Wonnapinij, P., Mann, J.R., Dahl, H.H. and Chinnery, P.F. (2008) 'A reduction of mitochondrial DNA molecules during embryogenesis explains the rapid segregation of genotypes', *Nat Genet*, 40(2), pp. 249-54.

Cutting, R., Morroll, D., Roberts, S.A., Pickering, S. and Rutherford, A. (2008) 'Elective single embryo transfer: guidelines for practice British Fertility Society and Association of Clinical Embryologists', *Hum Fertil (Camb)*, 11(3), pp. 131-46.

Dalton, C.M. and Carroll, J. (2013) 'Biased inheritance of mitochondria during asymmetric cell division in the mouse oocyte', *J Cell Sci*, 126(Pt 13), pp. 2955-64.

Dean, N.L., Battersby, B.J., Ao, A., Gosden, R.G., Tan, S.L., Shoubridge, E.A. and Molnar, M.J. (2003) 'Prospect of preimplantation genetic diagnosis for heritable mitochondrial DNA diseases', *Mol Hum Reprod*, 9(10), pp. 631-8.

Deng, M., Suraneni, P., Schultz, R.M. and Li, R. (2007) 'The Ran GTPase mediates chromatin signaling to control cortical polarity during polar body extrusion in mouse oocytes', *Dev Cell*, 12(2), pp. 301-8.

Desai, A. and Mitchison, T.J. (1997) 'Microtubule polymerization dynamics', *Annu Rev Cell Dev Biol*, 13, pp. 83-117.

Detmer, S.A. and Chan, D.C. (2007) 'Functions and dysfunctions of mitochondrial dynamics', *Nat Rev Mol Cell Biol*, 8(11), pp. 870-9.

Dimauro, S. and Davidzon, G. (2005) 'Mitochondrial DNA and disease', *Ann Med*, 37(3), pp. 222-32.

Dobson, A.T., Raja, R., Abeyta, M.J., Taylor, T., Shen, S., Haqq, C. and Pera, R.A. (2004) 'The unique transcriptome through day 3 of human preimplantation development', *Hum Mol Genet*, 13(14), pp. 1461-70.

Ducibella, T. and Anderson, E. (1975) 'Cell shape and membrane changes in the eight-cell mouse embryo: prerequisites for morphogenesis of the blastocyst', *Dev Biol*, 47(1), pp. 45-58.

Dumollard, R., Duchen, M. and Carroll, J. (2007) 'The role of mitochondrial function in the oocyte and embryo', *Curr Top Dev Biol*, 77, pp. 21-49.

Eisenberg, E. and Levanon, E.Y. (2013) 'Human housekeeping genes, revisited', *Trends Genet*, 29(10), pp. 569-74.

Embley, T.M. and Martin, W. (2006) 'Eukaryotic evolution, changes and challenges', *Nature*, 440(7084), pp. 623-30.

Estes, J.E., Selden, L.A. and Gershman, L.C. (1981) 'Mechanism of action of phalloidin on the polymerization of muscle actin', *Biochemistry*, 20(4), pp. 708-12.

Fajas, L. (2013) 'Re-thinking cell cycle regulators: the cross-talk with metabolism', *Front Oncol*, 3, p. 4.

Falkenberg, M., Larsson, N.G. and Gustafsson, C.M. (2007) 'DNA replication and transcription in mammalian mitochondria', *Annu Rev Biochem*, 76, pp. 679-99.

Feenan, K. and Herbert, M. (2006) 'Can 'abnormally' fertilized zygotes give rise to viable embryos?', *Hum Fertil (Camb)*, 9(3), pp. 157-69.

Fenwick, J., Platteau, P., Murdoch, A.P. and Herbert, M. (2002) 'Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos in vitro', *Hum Reprod*, 17(2), pp. 407-12.

Fleming, T.P., Sheth, B. and Fesenko, I. (2001) 'Cell adhesion in the preimplantation mammalian embryo and its role in trophectoderm differentiation and blastocyst morphogenesis', *Front Biosci*, 6, pp. D1000-7.

Fragouli, E., Alfarawati, S., Spath, K., Jaroudi, S., Sarasa, J., Enciso, M. and Wells, D. (2013) 'The origin and impact of embryonic aneuploidy', *Hum Genet*, 132(9), pp. 1001-13.

Fragouli, E., Alfarawati, S., Spath, K. and Wells, D. (2014) 'Morphological and cytogenetic assessment of cleavage and blastocyst stage embryos', *Mol Hum Reprod*, 20(2), pp. 117-26.

Friedman, J.R., Lackner, L.L., West, M., DiBenedetto, J.R., Nunnari, J. and Voeltz, G.K. (2011) 'ER tubules mark sites of mitochondrial division', *Science*, 334(6054), pp. 358-62.

Gerards, M., Sallevelt, S.C. and Smeets, H.J. (2016) 'Leigh syndrome: Resolving the clinical and genetic heterogeneity paves the way for treatment options', *Mol Genet Metab*, 117(3), pp. 300-12.

Gigant, B., Wang, C., Ravelli, R.B., Roussi, F., Steinmetz, M.O., Curmi, P.A., Sobel, A. and Knossow, M. (2005) 'Structural basis for the regulation of tubulin by vinblastine', *Nature*, 435(7041), pp. 519-22.

Gigarel, N., Hesters, L., Samuels, D.C., Monnot, S., Burlet, P., Kerbrat, V., Lamazou, F., Benachi, A., Frydman, R., Feingold, J., Rotig, A., Munnich, A., Bonnefont, J.P., Frydman, N.

and Steffann, J. (2011) 'Poor correlations in the levels of pathogenic mitochondrial DNA mutations in polar bodies versus oocytes and blastomeres in humans', *Am J Hum Genet*, 88(4), pp. 494-8.

Goto, Y., Nonaka, I. and Horai, S. (1990) 'A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies', *Nature*, 348(6302), pp. 651-3.

Gray, M.W., Burger, G. and Lang, B.F. (1999) 'Mitochondrial evolution', *Science*, 283(5407), pp. 1476-81.

Greaves, L.C., Reeve, A.K., Taylor, R.W. and Turnbull, D.M. (2012) 'Mitochondrial DNA and disease', *J Pathol*, 226(2), pp. 274-86.

Greggains, G.D., Lister, L.M., Tuppen, H.A., Zhang, Q., Needham, L.H., Prathalingam, N., Hyslop, L.A., Craven, L., Polanski, Z., Murdoch, A.P., Turnbull, D.M. and Herbert, M. (2014) 'Therapeutic potential of somatic cell nuclear transfer for degenerative disease caused by mitochondrial DNA mutations', *Sci Rep*, 4, p. 3844.

Gu, T.P., Guo, F., Yang, H., Wu, H.P., Xu, G.F., Liu, W., Xie, Z.G., Shi, L., He, X., Jin, S.G., Iqbal, K., Shi, Y.G., Deng, Z., Szabo, P.E., Pfeifer, G.P., Li, J. and Xu, G.L. (2011) 'The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes', *Nature*, 477(7366), pp. 606-10.

Gumbiner, B.M. (2005) 'Regulation of cadherin-mediated adhesion in morphogenesis', *Nat Rev Mol Cell Biol*, 6(8), pp. 622-34.

Guo, G., Huss, M., Tong, G.Q., Wang, C., Li Sun, L., Clarke, N.D. and Robson, P. (2010) 'Resolution of cell fate decisions revealed by single-cell gene expression analysis from zygote to blastocyst', *Dev Cell*, 18(4), pp. 675-85.

Gurdon, J.B., Elsdale, T.R., and Fischberg M. (1958) 'Sexually mature individuals of Xenopus laevis from the transplantation of single somatic nuclei', *Nature*, 182(4627), pp. 64-65.

Halet, G. and Carroll, J. (2007) 'Rac activity is polarized and regulates meiotic spindle stability and anchoring in mammalian oocytes', *Dev Cell*, 12(2), pp. 309-17.

Hamatani, T., Carter, M.G., Sharov, A.A. and Ko, M.S. (2004) 'Dynamics of global gene expression changes during mouse preimplantation development', *Dev Cell*, 6(1), pp. 117-31.

Hardarson, T., Caisander, G., Sjogren, A., Hanson, C., Hamberger, L. and Lundin, K. (2003) 'A morphological and chromosomal study of blastocysts developing from morphologically suboptimal human pre-embryos compared with control blastocysts', *Hum Reprod*, 18(2), pp. 399-407.

Harding, A.E., Holt, I.J., Sweeney, M.G., Brockington, M. and Davis, M.B. (1992) 'Prenatal diagnosis of mitochondrial DNA8993 T----G disease', *Am J Hum Genet*, 50(3), pp. 629-33.

Hardy, K. (1999) 'Apoptosis in the human embryo', *Rev Reprod*, 4(3), pp. 125-34.

Hardy, K. and Handyside, A.H. (1996) 'Metabolism and cell allocation during parthenogenetic preimplantation mouse development', *Mol Reprod Dev*, 43(3), pp. 313-22.

Hassold, T. and Hunt, P. (2001) 'To err (meiotically) is human: the genesis of human aneuploidy', *Nat Rev Genet*, 2(4), pp. 280-91.

Hellebrekers, D.M., Wolfe, R., Hendrickx, A.T., de Coo, I.F., de Die, C.E., Geraedts, J.P., Chinnery, P.F. and Smeets, H.J. (2012a) 'PGD and heteroplasmic mitochondrial DNA point mutations: a systematic review estimating the chance of healthy offspring', *Hum Reprod Update*, 18(4), pp. 341-9.

Hellebrekers, D.M.E.I., Wolfe, R., Hendrickx, A.T.M., de Coo, I.F.M., de Die, C.E., Geraedts, J.P.M., Chinnery, P.F. and Smeets, H.J.M. (2012b) 'PGD and heteroplasmic mitochondrial DNA point mutations: a systematic review estimating the chance of healthy offspring', *Human Reproduction Update*, 18(4), pp. 341-349.

Heng, Y.W. and Koh, C.G. (2010) 'Actin cytoskeleton dynamics and the cell division cycle', *Int J Biochem Cell Biol*, 42(10), pp. 1622-33.

Herbert, M., Kalleas, D., Cooney, D., Lamb, M. and Lister, L. (2015) 'Meiosis and maternal aging: insights from aneuploid oocytes and trisomy births', *Cold Spring Harb Perspect Biol*, 7(4), p. a017970.

Hewitson, L.C. and Leese, H.J. (1993) 'Energy metabolism of the trophectoderm and inner cell mass of the mouse blastocyst', *J Exp Zool*, 267(3), pp. 337-43.

HFEA (2014) *Third scientific review of the safety and efficacy of methods to avoid mitochondrial disease through assisted conception: 2014 update*. [Online]. Available at: http://www.hfea.gov.uk/docs/Third_Mitochondrial_replacement_scientific_review.pdf.

Ho, P.C., Yeung, W.S., Chan, Y.F., So, W.W. and Chan, S.T. (1994) 'Factors affecting the incidence of polyploidy in a human in vitro fertilization program', *Int J Fertil Menopausal Stud*, 39(1), pp. 14-9.

Holt, I.J., Harding, A.E. and Morgan-Hughes, J.A. (1988) 'Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies', *Nature*, 331(6158), pp. 717-9.

Holt, I.J., Lorimer, H.E. and Jacobs, H.T. (2000) 'Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA', *Cell*, 100(5), pp. 515-24.

Houghton, F.D. (2006) 'Energy metabolism of the inner cell mass and trophectoderm of the mouse blastocyst', *Differentiation*, 74(1), pp. 11-8.

Hyslop, L.A., Blakeley, P., Craven, L., Richardson, J., Fogarty, N.M., Fragouli, E., Lamb, M., Wamaitha, S.E., Prathalingam, N., Zhang, Q., O'Keefe, H., Takeda, Y., Arizzi, L., Alfarawati, S., Tuppen, H.A., Irving, L., Kalleas, D., Choudhary, M., Wells, D., Murdoch, A.P., Turnbull, D.M., Niakan, K.K. and Herbert, M. (2016) 'Towards clinical application of pronuclear transfer to prevent mitochondrial DNA disease', *Nature*, 534(7607), pp. 383-6.

Jacobs, L.J., de Coo, I.F., Nijland, J.G., Galjaard, R.J., Los, F.J., Schoonderwoerd, K., Niermeijer, M.F., Geraedts, J.P., Scholte, H.R. and Smeets, H.J. (2005) 'Transmission and prenatal diagnosis of the T9176C mitochondrial DNA mutation', *Mol Hum Reprod*, 11(3), pp. 223-8.

Jiang, D., Zhao, L. and Clapham, D.E. (2009) 'Genome-wide RNAi screen identifies Letm1 as a mitochondrial Ca2+/H+ antiporter', *Science*, 326(5949), pp. 144-7.

Kagawa, Y. and Hayashi, J.I. (1997) 'Gene therapy of mitochondrial diseases using human cytoplasts', *Gene Ther*, 4(1), pp. 6-10.

Kalucka, J., Missiaen, R., Georgiadou, M., Schoors, S., Lange, C., De Bock, K., Dewerchin, M. and Carmeliet, P. (2015) 'Metabolic control of the cell cycle', *Cell Cycle*, 14(21), pp. 3379-88.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R. and Salzberg, S.L. (2013) 'TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions', *Genome Biol*, 14(4), p. R36.

Kim, N.H., Chung, H.M., Cha, K.Y. and Chung, K.S. (1998) 'Microtubule and microfilament organization in maturing human oocytes', *Hum Reprod*, 13(8), pp. 2217-22.

Kirschner, M. and Mitchison, T. (1986) 'Beyond self-assembly: from microtubules to morphogenesis', *Cell*, 45(3), pp. 329-42.

Kleckner, N. (2006) 'Chiasma formation: chromatin/axis interplay and the role(s) of the synaptonemal complex', *Chromosoma*, 115(3), pp. 175-94.

Korhonen, J.A., Pham, X.H., Pellegrini, M. and Falkenberg, M. (2004) 'Reconstitution of a minimal mtDNA replisome in vitro', *Embo j*, 23(12), pp. 2423-9.

Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R. and Grosveld, F. (1999) 'The transcription factor GATA6 is essential for early extraembryonic development', *Development*, 126(9), pp. 723-32.

Kukat, C., Wurm, C.A., Spahr, H., Falkenberg, M., Larsson, N.G. and Jakobs, S. (2011) 'Super-resolution microscopy reveals that mammalian mitochondrial nucleoids have a uniform size and frequently contain a single copy of mtDNA', *Proc Natl Acad Sci U S A*, 108(33), pp. 13534-9.

Lazarou, M., Sliter, D.A., Kane, L.A., Sarraf, S.A., Wang, C., Burman, J.L., Sideris, D.P., Fogel, A.I. and Youle, R.J. (2015) 'The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy', *Nature*, 524(7565), pp. 309-14.

Lebon, S., Chol, M., Benit, P., Mugnier, C., Chretien, D., Giurgea, I., Kern, I., Girardin, E., Hertz-Pannier, L., de Lonlay, P., Rotig, A., Rustin, P. and Munnich, A. (2003) 'Recurrent de novo mitochondrial DNA mutations in respiratory chain deficiency', *J Med Genet*, 40(12), pp. 896-9.

Lee, H.S., Ma, H., Juanes, R.C., Tachibana, M., Sparman, M., Woodward, J., Ramsey, C., Xu, J., Kang, E.J., Amato, P., Mair, G., Steinborn, R. and Mitalipov, S. (2012) 'Rapid mitochondrial DNA segregation in primate preimplantation embryos precedes somatic and germline bottleneck', *Cell Rep*, 1(5), pp. 506-15.

Lee, I.H. and Finkel, T. (2013) 'Metabolic regulation of the cell cycle', *Curr Opin Cell Biol*, 25(6), pp. 724-9.

Leese, H.J. (2002) 'Quiet please, do not disturb: a hypothesis of embryo metabolism and viability', *Bioessays*, 24(9), pp. 845-9.

Leese, H.J., Baumann, C.G., Brison, D.R., McEvoy, T.G. and Sturmey, R.G. (2008) 'Metabolism of the viable mammalian embryo: quietness revisited', *Mol Hum Reprod*, 14(12), pp. 667-72.

Leese, H.J., Guerif, F., Allgar, V., Brison, D., Lundin, K. and Sturmey, R.G. (2016) 'Biological optimization, the Goldilocks principle, and how much is lagom in the preimplantation embryo', *Mol Reprod Dev*.

Leese, H.J., Sturmey, R.G., Baumann, C.G. and McEvoy, T.G. (2007) 'Embryo viability and metabolism: obeying the quiet rules', *Hum Reprod*, 22(12), pp. 3047-50.

Leshinsky-Silver, E., Perach, M., Basilevsky, E., Hershkovitz, E., Yanoov-Sharav, M., Lerman-Sagie, T. and Lev, D. (2003) 'Prenatal exclusion of Leigh syndrome due to T8993C mutation in the mitochondrial DNA', *Prenat Diagn*, 23(1), pp. 31-3.

Lin, D.C., Tobin, K.D., Grumet, M. and Lin, S. (1980) 'Cytochalasins inhibit nuclei-induced actin polymerization by blocking filament elongation', *J Cell Biol*, 84(2), pp. 455-60.

Lister, L.M., Kouznetsova, A., Hyslop, L.A., Kalleas, D., Pace, S.L., Barel, J.C., Nathan, A., Floros, V., Adelfalk, C., Watanabe, Y., Jessberger, R., Kirkwood, T.B., Hoog, C. and Herbert, M. (2010) 'Age-related meiotic segregation errors in mammalian oocytes are preceded by depletion of cohesin and Sgo2', *Curr Biol*, 20(17), pp. 1511-21.

Liu, J.L., Sung, L.Y., Tian, X.C. and Yang, X. (2002) 'Hypertonicity-induced projections reflect cell polarity in mouse metaphase II oocytes: involvement of microtubules, microfilaments, and chromosomes', *Biol Reprod*, 67(6), pp. 1853-63.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R. and Wang, X. (1996) 'Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c', *Cell*, 86(1), pp. 147-57.

Loane, M., Morris, J.K., Addor, M.C., Arriola, L., Budd, J., Doray, B., Garne, E., Gatt, M., Haeusler, M., Khoshnood, B., Klungsoyr Melve, K., Latos-Bielenska, A., McDonnell, B.,

Mullaney, C., O'Mahony, M., Queisser-Wahrendorf, A., Rankin, J., Rissmann, A., Rounding, C., Salvador, J., Tucker, D., Wellesley, D., Yevtushok, L. and Dolk, H. (2013) 'Twenty-year trends in the prevalence of Down syndrome and other trisomies in Europe: impact of maternal age and prenatal screening', *Eur J Hum Genet*, 21(1), pp. 27-33.

Lodish, H., Berk, A., Zipursky, S. and al., e. (2000) *Molecular cell biology. 4th edition.* . New York: W. H. Freeman.

Longo, F.J. and Chen, D.Y. (1985) 'Development of cortical polarity in mouse eggs: involvement of the meiotic apparatus', *Dev Biol*, 107(2), pp. 382-94.

Lorenzoni, P.J., Scola, R.H., Kay, C.S., Silvado, C.E. and Werneck, L.C. (2014) 'When should MERRF (myoclonus epilepsy associated with ragged-red fibers) be the diagnosis?', *Arq Neuropsiquiatr*, 72(10), pp. 803-11.

Love, M.I., Huber, W. and Anders, S. (2014) 'Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2', *Genome Biol*, 15(12), p. 550.

Mai, N., Chrzanowska-Lightowlers, Z.M. and Lightowlers, R.N. (2016) 'The process of mammalian mitochondrial protein synthesis', *Cell Tissue Res*.

Mandavilli, B.S., Santos, J.H. and Van Houten, B. (2002) 'Mitochondrial DNA repair and aging', *Mutat Res*, 509(1-2), pp. 127-51.

Marangos, P., FitzHarris, G. and Carroll, J. (2003) 'Ca2+ oscillations at fertilization in mammals are regulated by the formation of pronuclei', *Development*, 130(7), pp. 1461-72.

Martin, W. (2010) 'Evolutionary origins of metabolic compartmentalization in eukaryotes', *Philos Trans R Soc Lond B Biol Sci*, 365(1541), pp. 847-55.

Martin, W. and Schnarrenberger, C. (1997) 'The evolution of the Calvin cycle from prokaryotic to eukaryotic chromosomes: a case study of functional redundancy in ancient pathways through endosymbiosis', *Curr Genet*, 32(1), pp. 1-18.

McConnell, J.M. and Petrie, L. (2004) 'Mitochondrial DNA turnover occurs during preimplantation development and can be modulated by environmental factors', *Reprod Biomed Online*, 9(4), pp. 418-24.

McGrath, J. and Solter, D. (1983) 'Nuclear transplantation in the mouse embryo by microsurgery and cell fusion', *Science*, 220(4603), pp. 1300-2.

Meirelles, F.V. and Smith, L.C. (1997) 'Mitochondrial genotype segregation in a mouse heteroplasmic lineage produced by embryonic karyoplast transplantation', *Genetics*, 145(2), pp. 445-51.

Meirelles, F.V. and Smith, L.C. (1998) 'Mitochondrial genotype segregation during preimplantation development in mouse heteroplasmic embryos', *Genetics*, 148(2), pp. 877-83.

Minasi, M.G., Colasante, A., Riccio, T., Ruberti, A., Casciani, V., Scarselli, F., Spinella, F., Fiorentino, F., Varricchio, M.T. and Greco, E. (2016) 'Correlation between aneuploidy, standard morphology evaluation and morphokinetic development in 1730 biopsied blastocysts: a consecutive case series study', *Hum Reprod*.

Mitalipov, S., Amato, P., Parry, S. and Falk, M.J. (2014) 'Limitations of preimplantation genetic diagnosis for mitochondrial DNA diseases', *Cell Rep*, 7(4), pp. 935-7.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M. and Yamanaka, S. (2003) 'The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells', *Cell*, 113(5), pp. 631-42.

Miyazaki, S., Hashimoto, N., Yoshimoto, Y., Kishimoto, T., Igusa, Y. and Hiramoto, Y. (1986) 'Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization of golden hamster eggs', *Dev Biol*, 118(1), pp. 259-67.

Moore, D.P. and Orr-Weaver, T.L. (1998) 'Chromosome segregation during meiosis: building an unambivalent bivalent', *Curr Top Dev Biol*, 37, pp. 263-99.

Moraes, C.T., Ricci, E., Petruzzella, V., Shanske, S., DiMauro, S., Schon, E.A. and Bonilla, E. (1992) 'Molecular analysis of the muscle pathology associated with mitochondrial DNA deletions', *Nat Genet*, 1(5), pp. 359-67.

Morris, J.K. and Alberman, E. (2009) 'Trends in Down's syndrome live births and antenatal diagnoses in England and Wales from 1989 to 2008: analysis of data from the National Down Syndrome Cytogenetic Register', *Bmj*, 339, p. b3794.

Morrisey, E.E., Tang, Z., Sigrist, K., Lu, M.M., Jiang, F., Ip, H.S. and Parmacek, M.S. (1998) 'GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo', *Genes Dev*, 12(22), pp. 3579-90.

Morrow, T. (2016) "Safe' call? My thoughts on the latest mitochondrial replacement paper', *Ted's blog* [Blog]. Available at: https://tedmorrow.wordpress.com/2016/06/14/safe-call-my-thoughts-on-the-latest-mitochondrial-replacement-paper/.

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L. and Wold, B. (2008) 'Mapping and quantifying mammalian transcriptomes by RNA-Seq', *Nat Methods*, 5(7), pp. 621-8.

Nagaoka, S.I., Hassold, T.J. and Hunt, P.A. (2012) 'Human aneuploidy: mechanisms and new insights into an age-old problem', *Nat Rev Genet*, 13(7), pp. 493-504.

Nakamura, T., Liu, Y.J., Nakashima, H., Umehara, H., Inoue, K., Matoba, S., Tachibana, M., Ogura, A., Shinkai, Y. and Nakano, T. (2012) 'PGC7 binds histone H3K9me2 to protect against conversion of 5mC to 5hmC in early embryos', *Nature*, 486(7403), pp. 415-9.

Neupane, J., Vandewoestyne, M., Heindryckx, B., Ghimire, S., Lu, Y., Qian, C., Lierman, S., Van Coster, R., Gerris, J., Deroo, T., Deforce, D. and De Sutter, P. (2014) 'A systematic analysis of the suitability of preimplantation genetic diagnosis for mitochondrial diseases in a heteroplasmic mitochondrial mouse model', *Hum Reprod*, 29(4), pp. 852-9.

Niakan, K.K. and Eggan, K. (2013) 'Analysis of human embryos from zygote to blastocyst reveals distinct gene expression patterns relative to the mouse', *Dev Biol*, 375(1), pp. 54-64.

Niakan, K.K., Han, J., Pedersen, R.A., Simon, C. and Pera, R.A. (2012) 'Human pre-implantation embryo development', *Development*, 139(5), pp. 829-41.

Niakan, K.K., Ji, H., Maehr, R., Vokes, S.A., Rodolfa, K.T., Sherwood, R.I., Yamaki, M., Dimos, J.T., Chen, A.E., Melton, D.A., McMahon, A.P. and Eggan, K. (2010) 'Sox17 promotes differentiation in mouse embryonic stem cells by directly regulating extraembryonic gene expression and indirectly antagonizing self-renewal', *Genes Dev*, 24(3), pp. 312-26.

Nishioka, N., Inoue, K., Adachi, K., Kiyonari, H., Ota, M., Ralston, A., Yabuta, N., Hirahara, S., Stephenson, R.O., Ogonuki, N., Makita, R., Kurihara, H., Morin-Kensicki, E.M., Nojima, H., Rossant, J., Nakao, K., Niwa, H. and Sasaki, H. (2009) 'The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass', *Dev Cell*, 16(3), pp. 398-410.

Olivo, P.D., Van de Walle, M.J., Laipis, P.J. and Hauswirth, W.W. (1983) 'Nucleotide sequence evidence for rapid genotypic shifts in the bovine mitochondrial DNA D-loop', *Nature*, 306(5941), pp. 400-2.

Ota, M. and Sasaki, H. (2008) 'Mammalian Tead proteins regulate cell proliferation and contact inhibition as transcriptional mediators of Hippo signaling', *Development*, 135(24), pp. 4059-69.

Ozil, J.P., Banrezes, B., Toth, S., Pan, H. and Schultz, R.M. (2006) 'Ca2+ oscillatory pattern in fertilized mouse eggs affects gene expression and development to term', *Dev Biol*, 300(2), pp. 534-44.

Palmieri, S.L., Peter, W., Hess, H. and Scholer, H.R. (1994) 'Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation', *Dev Biol*, 166(1), pp. 259-67.

Paull, D., Emmanuele, V., Weiss, K.A., Treff, N., Stewart, L., Hua, H., Zimmer, M., Kahler, D.J., Goland, R.S., Noggle, S.A., Prosser, R., Hirano, M., Sauer, M.V. and Egli, D. (2013) 'Nuclear genome transfer in human oocytes eliminates mitochondrial DNA variants', *Nature*, 493(7434), pp. 632-7.

Petronczki, M., Siomos, M.F. and Nasmyth, K. (2003) 'Un menage a quatre: the molecular biology of chromosome segregation in meiosis', *Cell*, 112(4), pp. 423-40.

Petropoulos, S., Edsgard, D., Reinius, B., Deng, Q., Panula, S.P., Codeluppi, S., Plaza Reyes, A., Linnarsson, S., Sandberg, R. and Lanner, F. (2016) 'Single-Cell RNA-Seq Reveals Lineage and X Chromosome Dynamics in Human Preimplantation Embryos', *Cell*, 165(4), pp. 1012-26.

Piko, L. and Taylor, K.D. (1987) 'Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos', *Dev Biol*, 123(2), pp. 364-74.

Piras, V., Tomita, M. and Selvarajoo, K. (2014) 'Transcriptome-wide variability in single embryonic development cells', *Sci Rep*, 4, p. 7137.

Plusa, B., Piliszek, A., Frankenberg, S., Artus, J. and Hadjantonakis, A.K. (2008) 'Distinct sequential cell behaviours direct primitive endoderm formation in the mouse blastocyst', *Development*, 135(18), pp. 3081-91.

Popot, J.L. and de Vitry, C. (1990) 'On the microassembly of integral membrane proteins', *Annu Rev Biophys Biophys Chem*, 19, pp. 369-403.

Pyle, A., Hudson, G., Wilson, I.J., Coxhead, J., Smertenko, T., Herbert, M., Santibanez-Koref, M. and Chinnery, P.F. (2015) 'Extreme-Depth Re-sequencing of Mitochondrial DNA Finds No Evidence of Paternal Transmission in Humans', *PLoS Genet*, 11(5), p. e1005040.

Rasmussen, C.A., Kaufman, P.L., Ritch, R., Haque, R., Brazzell, R.K. and Vittitow, J.L. (2014) 'Latrunculin B Reduces Intraocular Pressure in Human Ocular Hypertension and Primary Open-Angle Glaucoma', *Transl Vis Sci Technol*, 3(5), p. 1.

Reinsch, S. and Gonczy, P. (1998) 'Mechanisms of nuclear positioning', *J Cell Sci*, 111 (Pt 16), pp. 2283-95.

Reinsch, S. and Karsenti, E. (1997) 'Movement of nuclei along microtubules in Xenopus egg extracts', *Curr Biol*, 7(3), pp. 211-4.

Richardson, J., Irving, L., Hyslop, L.A., Choudhary, M., Murdoch, A., Turnbull, D.M. and Herbert, M. (2015) 'Concise reviews: Assisted reproductive technologies to prevent transmission of mitochondrial DNA disease', *Stem Cells*, 33(3), pp. 639-45.

Ridley, M. (2001) *The cooperative gene: how Mendel's demon explains the evolution of complex beings*. New York, US: The Free Press.

Rizzuto, R., De Stefani, D., Raffaello, A. and Mammucari, C. (2012) 'Mitochondria as sensors and regulators of calcium signalling', *Nat Rev Mol Cell Biol*, 13(9), pp. 566-78.

Rizzuto, R., Pinton, P., Carrington, W., Fay, F.S., Fogarty, K.E., Lifshitz, L.M., Tuft, R.A. and Pozzan, T. (1998) 'Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses', *Science*, 280(5370), pp. 1763-6.

Robinson, M.D., McCarthy, D.J. and Smyth, G.K. (2010) 'edgeR: a Bioconductor package for differential expression analysis of digital gene expression data', *Bioinformatics*, 26(1), pp. 139-40.

Rocheleau, J.V. and Petersen, N.O. (2001) 'The Sendai virus membrane fusion mechanism studied using image correlation spectroscopy', *Eur J Biochem*, 268(10), pp. 2924-30.

Roger, A.J. (1999) 'Reconstructing Early Events in Eukaryotic Evolution', *Am Nat*, 154(S4), pp. S146-s163.

Roode, M., Blair, K., Snell, P., Elder, K., Marchant, S., Smith, A. and Nichols, J. (2012) 'Human hypoblast formation is not dependent on FGF signalling', *Dev Biol*, 361(2), pp. 358-63.

Rossignol, R., Faustin, B., Rocher, C., Malgat, M., Mazat, J.P. and Letellier, T. (2003) 'Mitochondrial threshold effects', *Biochem J*, 370(Pt 3), pp. 751-62.

Rossignol, R., Gilkerson, R., Aggeler, R., Yamagata, K., Remington, S.J. and Capaldi, R.A. (2004) 'Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells', *Cancer Res*, 64(3), pp. 985-93.

Rubenstein, D.S., Thomasma, D.C., Schon, E.A. and Zinaman, M.J. (1995) 'Germ-line therapy to cure mitochondrial disease: protocol and ethics of in vitro ovum nuclear transplantation', *Camb Q Healthc Ethics*, 4(3), pp. 316-39.

Sallevelt, S.C., Dreesen, J.C., Drusedau, M., Spierts, S., Coonen, E., van Tienen, F.H., van Golde, R.J., de Coo, I.F., Geraedts, J.P., de Die-Smulders, C.E. and Smeets, H.J. (2013) 'Preimplantation genetic diagnosis in mitochondrial DNA disorders: challenge and success', *J Med Genet*, 50(2), pp. 125-32.

Sasaki, H. (2010) 'Mechanisms of trophectoderm fate specification in preimplantation mouse development', *Dev Growth Differ*, 52(3), pp. 263-73.

Sato, A., Kono, T., Nakada, K., Ishikawa, K., Inoue, S., Yonekawa, H. and Hayashi, J. (2005) 'Gene therapy for progeny of mito-mice carrying pathogenic mtDNA by nuclear transplantation', *Proc Natl Acad Sci U S A*, 102(46), pp. 16765-70.

Sato, M. and Sato, K. (2011) 'Degradation of paternal mitochondria by fertilization-triggered autophagy in C. elegans embryos', *Science*, 334(6059), pp. 1141-4.

Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K. and Lai, F.A. (2002) 'PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development', *Development*, 129(15), pp. 3533-44.

Schuh, M. and Ellenberg, J. (2008) 'A new model for asymmetric spindle positioning in mouse oocytes', *Curr Biol*, 18(24), pp. 1986-92.

Shoubridge, E.A. (1994) 'Mitochondrial DNA diseases: histological and cellular studies', *J Bioenerg Biomembr*, 26(3), pp. 301-10.

Shoubridge, E.A. and Wai, T. (2007) 'Mitochondrial DNA and the mammalian oocyte', *Curr Top Dev Biol*, 77, pp. 87-111.

Smirnova, E., Griparic, L., Shurland, D.L. and van der Bliek, A.M. (2001) 'Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells', *Mol Biol Cell*, 12(8), pp. 2245-56.

Smits, P., Smeitink, J. and van den Heuvel, L. (2010) 'Mitochondrial translation and beyond: processes implicated in combined oxidative phosphorylation deficiencies', *J Biomed Biotechnol*, 2010, p. 737385.

Spector, I., Shochet, N.R., Blasberger, D. and Kashman, Y. (1989) 'Latrunculins--novel marine macrolides that disrupt microfilament organization and affect cell growth: I. Comparison with cytochalasin D', *Cell Motil Cytoskeleton*, 13(3), pp. 127-44.

Spector, I., Shochet, N.R., Kashman, Y. and Groweiss, A. (1983) 'Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells', *Science*, 219(4584), pp. 493-5.

Spemann, H. (1938) *Embryonic Development and Induction.* New York: Hafner Publishing Co.

Srinivasula, S.M., Datta, P., Fan, X.J., Fernandes-Alnemri, T., Huang, Z. and Alnemri, E.S. (2000) 'Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway', *J Biol Chem*, 275(46), pp. 36152-7.

Steffann, J., Frydman, N., Gigarel, N., Burlet, P., Ray, P.F., Fanchin, R., Feyereisen, E., Kerbrat, V., Tachdjian, G., Bonnefont, J.P., Frydman, R. and Munnich, A. (2006) 'Analysis of mtDNA variant segregation during early human embryonic development: a tool for successful NARP preimplantation diagnosis', *J Med Genet*, 43(3), pp. 244-7.

Steffann, J., Gigarel, N., Samuels, D.C., Monnot, S., Borghese, R., Hesters, L., Frydman, N., Burlet, P., Frydman, R., Benachi, A., Rotig, A., Munnich, A. and Bonnefont, J.P. (2014) 'Data from artificial models of mitochondrial DNA disorders are not always applicable to humans', *Cell Rep*, 7(4), pp. 933-4.

Steffann, J., Monnot, S. and Bonnefont, J.P. (2015) 'mtDNA mutations variously impact mtDNA maintenance throughout the human embryofetal development', *Clin Genet*, 88(5), pp. 416-24.

Stegle, O., Teichmann, S.A. and Marioni, J.C. (2015) 'Computational and analytical challenges in single-cell transcriptomics', *Nat Rev Genet*, 16(3), pp. 133-45.

Stephenson, E.L., Braude, P.R. and Mason, C. (2007) 'International community consensus standard for reporting derivation of human embryonic stem cell lines', *Regen Med*, 2(4), pp. 349-62.

Stokes, P.J., Hawkhead, J.A., Fawthrop, R.K., Picton, H.M., Sharma, V., Leese, H.J. and Houghton, F.D. (2007) 'Metabolism of human embryos following cryopreservation: implications for the safety and selection of embryos for transfer in clinical IVF', *Hum Reprod*, 22(3), pp. 829-35.

Sturmey, R.G., Hawkhead, J.A., Barker, E.A. and Leese, H.J. (2009) 'DNA damage and metabolic activity in the preimplantation embryo', *Hum Reprod*, 24(1), pp. 81-91.

Sun, Q.Y. and Schatten, H. (2006) 'Regulation of dynamic events by microfilaments during oocyte maturation and fertilization', *Reproduction*, 131(2), pp. 193-205.

Tachibana, M., Amato, P., Sparman, M., Woodward, J., Sanchis, D.M., Ma, H., Gutierrez, N.M., Tippner-Hedges, R., Kang, E., Lee, H.S., Ramsey, C., Masterson, K., Battaglia, D., Lee, D., Wu, D., Jensen, J., Patton, P., Gokhale, S., Stouffer, R. and Mitalipov, S. (2013) 'Towards germline gene therapy of inherited mitochondrial diseases', *Nature*, 493(7434), pp. 627-31.

Tachibana, M., Sparman, M., Sritanaudomchai, H., Ma, H., Clepper, L., Woodward, J., Li, Y., Ramsey, C., Kolotushkina, O. and Mitalipov, S. (2009) 'Mitochondrial gene replacement in primate offspring and embryonic stem cells', *Nature*, 461(7262), pp. 367-72.

Tajima, H., Sueoka, K., Moon, S.Y., Nakabayashi, A., Sakurai, T., Murakoshi, Y., Watanabe, H., Iwata, S., Hashiba, T., Kato, S., Goto, Y. and Yoshimura, Y. (2007) 'The development of novel quantification assay for mitochondrial DNA heteroplasmy aimed at preimplantation genetic diagnosis of Leigh encephalopathy', *J Assist Reprod Genet*, 24(6), pp. 227-32.

Tatuch, Y., Christodoulou, J., Feigenbaum, A., Clarke, J.T., Wherret, J., Smith, C., Rudd, N., Petrova-Benedict, R. and Robinson, B.H. (1992) 'Heteroplasmic mtDNA mutation (T----G) at

8993 can cause Leigh disease when the percentage of abnormal mtDNA is high', *Am J Hum Genet*, 50(4), pp. 852-8.

Taylor, R.W. and Turnbull, D.M. (2005) 'Mitochondrial DNA mutations in human disease', *Nat Rev Genet*, 6(5), pp. 389-402.

Treff, N.R., Campos, J., Tao, X., Levy, B., Ferry, K.M. and Scott, R.T., Jr. (2012) 'Blastocyst preimplantation genetic diagnosis (PGD) of a mitochondrial DNA disorder', *Fertil Steril*, 98(5), pp. 1236-40.

Upholt, W.B. and Dawid, I.B. (1977) 'Mapping of mitochondrial DNA of individual sheep and goats: rapid evolution in the D loop region', *Cell*, 11(3), pp. 571-83.

Van Blerkom, J. (1991) 'Microtubule mediation of cytoplasmic and nuclear maturation during the early stages of resumed meiosis in cultured mouse oocytes', *Proc Natl Acad Sci U S A*, 88(11), pp. 5031-5.

Van Blerkom, J. (2009) 'Mitochondria in early mammalian development', *Semin Cell Dev Biol*, 20(3), pp. 354-64.

van den Ouweland, J.M., Lemkes, H.H., Ruitenbeek, W., Sandkuijl, L.A., de Vijlder, M.F., Struyvenberg, P.A., van de Kamp, J.J. and Maassen, J.A. (1992) 'Mutation in mitochondrial tRNA(Leu)(UUR) gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness', *Nat Genet*, 1(5), pp. 368-71.

van der Maaten, L. and Hinton, G. (2008) 'Visualizing high-dimensional data using t-SNE', *J. Mach. Learn. Res.*, 9, pp. 2579-2605.

van Echten-Arends, J., Mastenbroek, S., Sikkema-Raddatz, B., Korevaar, J.C., Heineman, M.J., van der Veen, F. and Repping, S. (2011) 'Chromosomal mosaicism in human preimplantation embryos: a systematic review', *Hum Reprod Update*, 17(5), pp. 620-7.

van Loo, G., van Gurp, M., Depuydt, B., Srinivasula, S.M., Rodriguez, I., Alnemri, E.S., Gevaert, K., Vandekerckhove, J., Declercq, W. and Vandenabeele, P. (2002) 'The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity', *Cell Death Differ*, 9(1), pp. 20-6.

Vandewoestyne, M., Heindryckx, B., De Gheselle, S., Lepez, T., Neupane, J., Gerris, J., Van Coster, R., De Sutter, P. and Deforce, D. (2012) 'Poor correlation between polar bodies and blastomere mutation load in a patient with m.3243A>G tRNALeu(UUR) point mutation', *Mitochondrion*, 12(4), pp. 477-9.

Veatch, J.R., McMurray, M.A., Nelson, Z.W. and Gottschling, D.E. (2009) 'Mitochondrial dysfunction leads to nuclear genome instability via an iron-sulfur cluster defect', *Cell*, 137(7), pp. 1247-58.

Vergouw, C.G., Botros, L.L., Roos, P., Lens, J.W., Schats, R., Hompes, P.G., Burns, D.H. and Lambalk, C.B. (2008) 'Metabolomic profiling by near-infrared spectroscopy as a tool to assess embryo viability: a novel, non-invasive method for embryo selection', *Hum Reprod*, 23(7), pp. 1499-504.

Verlhac, M.H., Lefebvre, C., Guillaud, P., Rassinier, P. and Maro, B. (2000) 'Asymmetric division in mouse oocytes: with or without Mos', *Curr Biol*, 10(20), pp. 1303-6.

von Heijne, G. (1986) 'Why mitochondria need a genome', *FEBS Lett*, 198(1), pp. 1-4.

Vu, T.N., Wills, Q.F., Kalari, K.R., Niu, N., Wang, L., Rantalainen, M. and Pawitan, Y. (2016) 'Beta-Poisson model for single-cell RNA-seq data analyses', *Bioinformatics*, 32(14), pp. 2128-35.

Wai, T., Ao, A., Zhang, X., Cyr, D., Dufort, D. and Shoubridge, E.A. (2010) 'The role of mitochondrial DNA copy number in mammalian fertility', *Biol Reprod*, 83(1), pp. 52-62.

Wai, T., Teoli, D. and Shoubridge, E.A. (2008) 'The mitochondrial DNA genetic bottleneck results from replication of a subpopulation of genomes', *Nat Genet*, 40(12), pp. 1484-8.

Wallace, D.C., Brown, M.D. and Lott, M.T. (1999) 'Mitochondrial DNA variation in human evolution and disease', *Gene*, 238(1), pp. 211-30.

Wallace, D.C., Singh, G., Lott, M.T., Hodge, J.A., Schurr, T.G., Lezza, A.M., Elsas, L.J., 2nd and Nikoskelainen, E.K. (1988a) 'Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy', *Science*, 242(4884), pp. 1427-30.

Wallace, D.C., Zheng, X.X., Lott, M.T., Shoffner, J.M., Hodge, J.A., Kelley, R.I., Epstein, C.M. and Hopkins, L.C. (1988b) 'Familial mitochondrial encephalomyopathy (MERRF):

genetic, pathophysiological, and biochemical characterization of a mitochondrial DNA disease', *Cell*, 55(4), pp. 601-10.

Walling, E.A., Krafft, G.A. and Ware, B.R. (1988) 'Actin assembly activity of cytochalasins and cytochalasin analogs assayed using fluorescence photobleaching recovery', *Arch Biochem Biophys*, 264(1), pp. 321-32.

Wang, C. and Youle, R.J. (2009) 'The role of mitochondria in apoptosis*', *Annu Rev Genet*, 43, pp. 95-118.

Wang, T., Sha, H., Ji, D., Zhang, H.L., Chen, D., Cao, Y. and Zhu, J. (2014) 'Polar body genome transfer for preventing the transmission of inherited mitochondrial diseases', *Cell*, 157(7), pp. 1591-604.

Wassarman, P.M. and Litscher, E.S. (2008) 'Mammalian fertilization: the egg's multifunctional zona pellucida', *Int J Dev Biol*, 52(5-6), pp. 665-76.

Watson, A.J. and Barcroft, L.C. (2001) 'Regulation of blastocyst formation', *Front Biosci*, 6, pp. D708-30.

White, S.L., Shanske, S., Biros, I., Warwick, L., Dahl, H.M., Thorburn, D.R. and Di Mauro, S. (1999) 'Two cases of prenatal analysis for the pathogenic T to G substitution at nucleotide 8993 in mitochondrial DNA', *Prenat Diagn*, 19(12), pp. 1165-8.

Wieland, T. and Faulstich, H. (1978) 'Amatoxins, phallotoxins, phallolysin, and antamanide: the biologically active components of poisonous Amanita mushrooms', *CRC Crit Rev Biochem*, 5(3), pp. 185-260.

Willadsen, S.M. (1986) 'Nuclear transplantation in sheep embryos', *Nature,* 320(6057), pp. 63-65

Wossidlo, M., Nakamura, T., Lepikhov, K., Marques, C.J., Zakhartchenko, V., Boiani, M., Arand, J., Nakano, T., Reik, W. and Walter, J. (2011) '5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming', *Nat Commun*, 2, p. 241.

Wuhr, M., Dumont, S., Groen, A.C., Needleman, D.J. and Mitchison, T.J. (2009) 'How does a millimeter-sized cell find its center?', *Cell Cycle*, 8(8), pp. 1115-21.

Xue, Z., Huang, K., Cai, C., Cai, L., Jiang, C.Y., Feng, Y., Liu, Z., Zeng, Q., Cheng, L., Sun, Y.E., Liu, J.Y., Horvath, S. and Fan, G. (2013) 'Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing', *Nature*, 500(7464), pp. 593-7.

Yamauchi, Y., Ward, M.A. and Ward, W.S. (2009) 'Asynchronous DNA replication and origin licensing in the mouse one-cell embryo', *J Cell Biochem*, 107(2), pp. 214-23.

Yan, L., Yang, M., Guo, H., Yang, L., Wu, J., Li, R., Liu, P., Lian, Y., Zheng, X., Yan, J., Huang, J., Li, M., Wu, X., Wen, L., Lao, K., Li, R., Qiao, J. and Tang, F. (2013) 'Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells', *Nat Struct Mol Biol*, 20(9), pp. 1131-9.

Yarmola, E.G., Somasundaram, T., Boring, T.A., Spector, I. and Bubb, M.R. (2000) 'Actin-latrunculin A structure and function. Differential modulation of actin-binding protein function by latrunculin A', *J Biol Chem*, 275(36), pp. 28120-7.

Yasukawa, T., Reyes, A., Cluett, T.J., Yang, M.Y., Bowmaker, M., Jacobs, H.T. and Holt, I.J. (2006) 'Replication of vertebrate mitochondrial DNA entails transient ribonucleotide incorporation throughout the lagging strand', *Embo j*, 25(22), pp. 5358-71.

Yi, K., Unruh, J.R., Deng, M., Slaughter, B.D., Rubinstein, B. and Li, R. (2011) 'Dynamic maintenance of asymmetric meiotic spindle position through Arp2/3-complex-driven cytoplasmic streaming in mouse oocytes', *Nat Cell Biol*, 13(10), pp. 1252-8.

Youle, R.J. and Narendra, D.P. (2011) 'Mechanisms of mitophagy', *Nat Rev Mol Cell Biol*, 12(1), pp. 9-14.

Youle, R.J. and van der Bliek, A.M. (2012) 'Mitochondrial fission, fusion, and stress', *Science*, 337(6098), pp. 1062-5.