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The nature of chromosome segregation errors
in the early mitotic divisions of human
embryos

Emma Ford

Supervisors:

Prof. Geraldine Hartshorne

Prof. Andrew McAinsh

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Declaration

This thesis was submitted for the degree of Doctor of Philosophy in Medical Sciences. It has been composed by myself and has not been submitted in any previous publication for any degree.

The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

- The analysis of micronuclei and multinucleated clinical embryos (Chapter 4.3) was performed by Lucy Benham Whyte.
- The embryos from the patients 3210, 3346 and 3350 were stained and imaged by Cerys Currie (table 1, table 6).

Some of this thesis has been published by the author and can be found in the following preprint:

Ford, E., Currie, C. E., Taylor, D. M., Erent, M., Marston, A. L., Hartshorne, G. M., McAinsh, A. D. (2020) "The First Mitotic Division of the Human Embryo is Highly Error-prone." bioRxiv.

Inclusion of Published work

In the appendix A., I have included a preprint to which I have contributed: “The First Mitotic Division of the Human Embryo is Highly Error-prone” (Ford et al. 2020). The paper is currently under revision.

Covid-19 Impact Statement

Due to Covid-19, the centre for reproductive medicine (CRM) at the University Hospital Coventry and Warwickshire (UHCW) was closed for 4 months, from March to July 2020, meaning that no patients were receiving fertility treatment during that period and that there were no fresh oocytes/embryos available for research during that period of time. I was unable to do any experiments, setting me back towards the end of my PhD, when I should have been working hard to get as much data as possible.

Abstract

Every single one of the ~50 million cells that constitute a human being originates from serial divisions of the zygote – the fertilised egg that brings maternal and paternal genomes together for the first time. Surprisingly, over half of human embryos are aneuploid indicating that chromosomes must be erroneously segregated during the early mitotic divisions. This can also create mosaic embryos, containing karyotypically different cell lineages. However, the types of errors and mechanisms leading to aneuploidies are unknown in early human embryos as these events have never been imaged in live embryos.

In this study I imaged chromosome movements during the first two mitotic divisions of human embryos. I show that mitosis I is extended compared to somatic cells and error-prone with embryos displaying lagging chromosomes, multipolar chromosome segregation and micronuclei. These errors appear to be maternal age independent. This is in contrast to the second mitotic division which is shorter and mostly bipolar, with much fewer lagging chromosomes, putting forward the uniquely erroneous nature of the first mitotic division.

Crucially, this data is based on fresh normally fertilised human embryos thanks to our egg sharer program, as well as on fresh human oocytes and embryos donated to research as they could not be used for IVF or ICSI treatment, which I term deselected. Comparing embryos from different sources as well as clinical embryos showed that deselected embryos are a robust model when it comes to studying early human embryonic development.

I also show that the spindle assembly checkpoint (SAC) is not responsible for setting the timing to anaphase onset, instead it could be an intrinsic timing mechanism which is responsible for triggering anaphase. I thus hypothesise that a

weak SAC, which is normally responsible for holding a mitotic cell in metaphase until all the chromosomes are aligned on the metaphase plate, could be at the origin of the high aneuploidy rates of human embryos.

Abbreviations

APC/C - anaphase promoting complex/cyclosome
CCAN - constitutive centromere associated network
CL - corpus luteum
COC - cumulus-oocyte complex
CPC - chromosomal passenger complex
DSB - double strand break
EGA - embryonic genome activation
ER - endoplasmic reticulum
ESCRT-III - endosomal sorting complex required for transport-III
FSH - follicular stimulating hormone
GnRH - gonadotrophin-release hormone
GV - germinal vesicle
hCG - human chorionic gonadotrophin
ICM - inner cell mass
ICSI - intracytoplasmic sperm injection
IVF - In vitro fertilisation
LH - luteinising hormone
MAPK - mitogen-activated protein kinase
MCC - mitotic checkpoint complex
MPF – maturation-promoting factor
MTOC - microtubule organising centre
PB - polar body
PDH - pyruvate dehydrogenase
PGS – preimplantation genetic screening
PIP2 - phosphatidylinositol 4,5-biphosphate
PKA - protein kinase A
PKC - protein kinase C
PLK - Polo-like kinase
PN - Pronucleus

PSSC - premature separation of sister chromatids

SAC - spindle assembly checkpoint

SC - synaptonemal complex

SiR - Silicon Rhodamine

SMC - structural maintenance of chromosomes

SSC - spermatogonial stem cell

TCA tricarboxylic acid cycle

TZP - transzonal projections

UHCW – University Hospital Coventry and Warwickshire

Chapter 1: Introduction

Human Embryonic development

Only one from hundreds of millions of sperm (male gamete) fertilises the oocyte (female gamete), bringing the maternal and paternal genetic material together for the first time, thus creating an embryo with a unique genome. This initial first cell divides to form the 37.2 trillion cells which constitute a human being.

1.1. Sperm entry

Sperm cells have to travel from the cervix to the uterus with only 1% reaching the destination. From the uterus, they travel to the oviduct, a journey which can take from 30 minutes to 6 days. The sperm and oocyte finally meet in the ampulla of the fallopian tube. The sperm accesses the oocyte by releasing the enzyme hyaluronidase, from the acrosome which is the tip of the sperm head. This removes the hyaluronic acid which connects the somatic cells around the oocyte, exposing the zona pellucida, a proteinaceous membrane surrounding the oocyte. The zona pellucida surrounding the human oocyte is composed of the proteins ZP1, ZP2, ZP3 and ZP4. The sperm head specifically binds to these zona pellucida proteins and penetrates the zona by releasing acrosin from the acrosome to digest the zona pellucida. This process is known as the acrosome reaction. The sperm then fuses with the oocyte membrane and is released in the cytoplasm (Khan and Ackerman 2020). This results in the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂) in the oocyte by PLC ζ , which is expressed on the sperm head, triggering calcium release from endoplasmic reticulum (ER) stores (Stewart and Davis 2019). This calcium signalling is essential as it causes key events leading to fertilisation. These events include the cortical reaction, which blocks the entry of

other sperm to prevent polyspermy, and the resumption of the second meiotic division (nuclear division resulting in cells with haploid genome) at the end of which half of the oocyte's genetic material will be extruded in a second polar body (a small cell which is a by-product of meiosis). Ca oscillations also cause the recruitment of maternal mRNA, which will be translated into proteins to drive the fertilisation process and support early embryonic divisions (Whitaker 2006).

1.2. Fertilisation and initial cleavage divisions

Whilst the oocyte is undergoing its second meiotic division, the sperm's genetic material undergoes changes of its own: the paternal chromosomes are decondensed, demethylated and packaged around maternal histones, with a nuclear membrane assembling around the chromosomes, forming the paternal pronucleus (Li, Lu et al. 2013). The maternal pronucleus is formed at the end of the second meiotic division. Once the maternal and paternal pronuclei formed, they move towards each other. At this stage, the fertilised oocyte is known as a zygote with its characteristic two pronuclei (2PN)

The two pronuclei then disappear, marking the start of the first cell division and the start of the cleavage stage of development. Until the 16 cell stage, the embryo divides without the cells increasing in size, resulting in cells with the same size and morphology. These cells are known as blastomeres and are juxtaposed next to one another with gap junctions only starting to form at the 8 cell stage. These initial divisions are relatively slow, the embryo approximately follows these timings: 2 cell stage after one day, 4 cell stage after 2 days, 12 cell stage after 3 days and finally 16 cell stage after 4 days (Fig. 1) (Chamayou, Patrizio et al. 2013, Khan and Ackerman 2020).

1.3. Embryonic genome activation

Until the 8-cell stage, the embryo relies on maternal mRNAs and proteins to support its development. Past that stage, the embryo becomes more self-sufficient, and this requires its DNA to be transcribed in a process known as embryonic genome activity (EGA) (Khan and Ackerman 2020). This is a complex process as the embryonic genome needs to be activated whilst still maintaining basic cellular processes which are controlled by maternal RNAs and proteins. EGA happens in successive waves of transcription, with the major wave taking place between the 4 and 8 cell stage when over a thousand genes are transcribed (Pfeffer 2018).

This raises the question: what are the mechanisms triggering these timely waves of transcription? (Nagpal and Fukagawa 2016, Pfeffer 2018). The fact that embryos can mature *in vitro* and that oocytes can be activated without sperm (a process called parthenogenesis) argues against the need to for an external stimulus for EGA (Pfeffer 2018). However, pyruvate, which is abundantly present in the mother's oviduct, has been shown to be essential for EGA in mice as embryos deprived of pyruvate halted their development at the time when EGA would have happened. These arrested embryos were lacking histone modifications associated with the opening up of chromatin for gene transcription. Pyruvate is a key player in providing cells with the large amount of energy they require. Indeed, it is converted to acetyl-CoA by pyruvate dehydrogenase (PDH), allowing the tricarboxylic acid cycle (TCA) cycle to produce large amounts of ATP. Moreover, the fact that active PDH localised to the nucleus in human and mice embryos from the time of EGA, suggests that this dependency on pyruvate is also conserved in humans (Nagaraj, Sharpley et al. 2017)

1.4. Morula

The cleavage divisions end with the formation of a 16-cell embryo known as the morula. The subsequent morula stage of development is characterised by the process of compaction, during which the cells become more and more tightly bound together until their boundaries progressively disappear (Khan and Ackerman 2020). This process depends on calcium signalling, E-cadherin and filopodia (Shahbazi 2020).

During the late stages of compaction, the boundaries between cells start to reappear in between cells and the 16 cells divide again into 32 (Fig. 1). The blastomeres become polarised, resulting in the apex becoming enriched in microvilli, F-actin, protein kinase C (PKC) and the Par protein complex (Pfeffer 2018). In mice, this process is believed to be dependent on PKC which drives actomyosin polarisation by Rho kinases, and is driven by the transcription factors such as Tfap2c and Tead4, which were synthesised following EGA (Shahbazi 2020). Cell polarisation appears to be cell-intrinsic as blastomeres that were kept apart from 4-cell stage onwards could still become polarised. However, the cell-cell adhesion molecule E-cadherin accelerates and synchronises this process across the blastomeres and establishes the axis of polarisation, making sure the apex is on the edge of the embryo, where there are no cell-cell contacts. Because the apex attracts one of the spindle poles (the cytoskeletal structure which drives cell division), it establishes the axis of cell division. Indeed, suppressing E-cadherin in mice embryo causes a delayed polarisation and random polarisation axes (Watson, Natale et al. 2004). The division will result in two very different cell types: polar cells on the edge of the embryo which contain the apex and apolar cells in the middle of the embryo. These cell types have different gene expression patterns, allowing them to differentiate into different cell lineages (Pfeffer 2018). The outer cells will further polarise and form cell-cell junctions between them, eventually forming a sealed epithelial layer on the outside of the morula, known as the trophectoderm (Pfeffer 2018, Khan and Ackerman 2020).

1.5. Blastocyst

Once the epithelial layer is sealed, the outer cells that have an increased number of Na⁺/K⁺ ATPase dependent pumps, create an ion gradient by pumping Na⁺ into the embryo and pumping K⁺ out. This ion gradient causes water and solutes to enter inside the embryo through osmosis (Watson, Natale et al. 2004, Pfeffer 2018). This causes a fluid-filled cavity, known as the blastocoele to form, which is characteristic of an embryo at the blastocyst stage (Fig. 1). As the cavity expands, being filled with more fluid, the cells that were present in the centre of the embryo get grouped to one side. These are stem cells with unrestricted differentiation potential that together form the inner cell mass (ICM), which will give rise to the embryo itself and some extraembryonic tissues. During this stage, the trophoctoderm will differentiate into trophoblasts, which will later give rise to the placenta (Pfeffer 2018, Khan and Ackerman 2020).

In summary, the two cell lineages that emerged as a result of polarisation in the morula become very apparent and morphologically different in the blastocyst, in the form of the ICM and the trophoblasts, with an extended fluid cavity in the centre of the embryo. The blastocyst stage is reached at day 5 after fertilisation, when the embryo contains between 50 and 150 cells (Khan and Ackerman 2020).

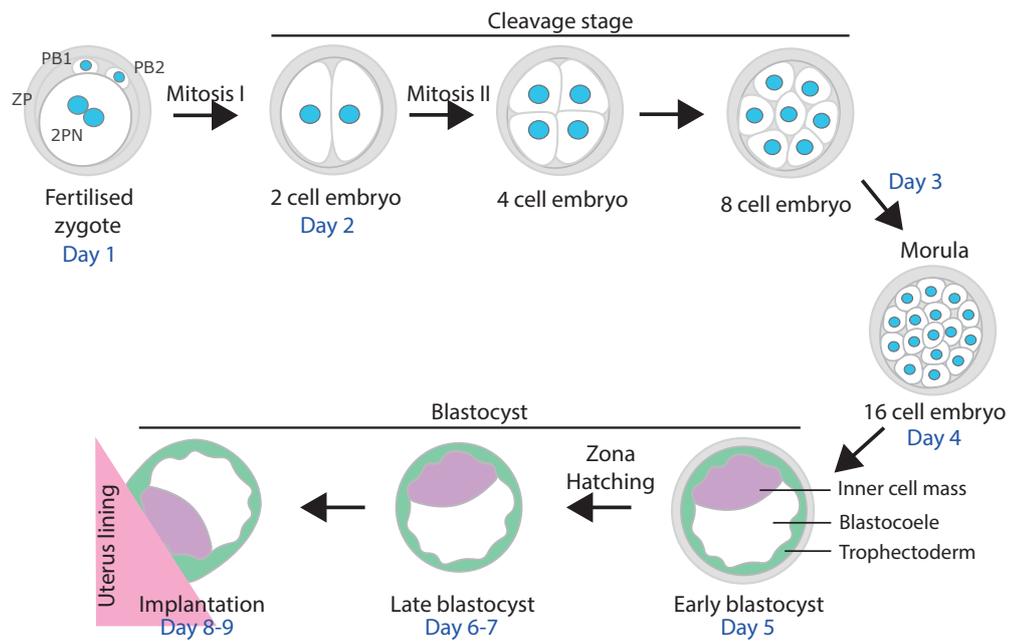


Figure 1: Human preimplantation embryonic development. The fertilised zygote has 2 pronuclei (PN), 2 polar bodies (PB) and is surrounded by the zona pellucida (ZP). It then undergoes a series of mitotic divisions which are not followed by cell growth (cleavage divisions), giving rise to daughter cells with identical morphology, to form a 16 cell embryo (morula). The morula will become compacted, causing the embryo to become polarised, leading the formation of an epithelial layer on the outside of the embryos (the trophoctoderm), which will form the placenta. Ion channels will establish an ion gradient, leading the formation of fluid-filled cavity (blastocoele) by osmosis: the embryo is now at the blastocyst stage. The cells in the centre of the embryo will group together to one side and start to differentiate, forming the inner cell mass which will form the embryo and some extra-embryonic tissue. The blastocyst then squeezes out of the zona pellucida (hatching) to implant, with the trophoctoderm orientated towards the uterine epithelium.

1.6. Implantation

At this stage, the blastocyst has reached the uterus and is ready to implant, a process during which the embryo adheres to the endometrial lining of the uterus and embeds itself, receiving oxygen and nutrient from the mother. Until this stage the embryo is still surrounded by the zona pellucida, but to implant, it needs to be released from the zona, through a process known as hatching. The blastocyst

hatches and orientates itself in order to have its ICM facing the uterine epithelium (Fig. 1) (Salamonsen, Evans et al. 2016).

Importantly, for embryo implantation to happen, the endometrium and the blastocyst need to develop synchronously. There is only a small window of time (between 6 and 10 days post ovulation), when the uterus is receptive to implantation. During that time, endometrial cells undergo rapid proliferation and differentiation to accommodate the embryo, in response to progesterone signalling (Salamonsen, Evans et al. 2016).

Upon binding of the blastocyst's trophoctoderm to the endometrium, the trophoctoderm differentiates into syncytial cells, which are large multinucleated cells to initiate invasion into the endometrium. The embryo establishes itself in the stroma and a layer of epithelial cells form on top of it (Aplin and Ruane 2017).

The embryo carries on developing and differentiating, supported by the mother through the placenta, until a fully formed baby is born, about 40 weeks after fertilisation. The placenta, which form early in development from the trophoctoderm, is an interface between the maternal and fetal vasculatures, allowing gases, nutrients and waste exchanges.

2. Origin of eggs and sperm in humans

In section 1, I outlined how the sperm and the oocyte meet and the divisions which follow. In the following section, I will answer the question: how are the female and male gametes generated?

2.1. Oocyte maturation

2.1.1. From primordial germ cells to primordial follicles

Primordial germ cells, which are the precursors of the female and male gametes (oocytes and spermatozoa), originate very early in embryonic development in the yolk sac endoderm. They start to migrate by 4 to 5 weeks of gestation and undergo many rounds of mitosis (nuclear division maintaining a diploid genome) with an incomplete cytokinesis (cell division) – see section 3 for details. This gives rise to many oogonia connected by bridges. These then aggregate, due to cytoplasmic bridges and cell adhesion molecules, into germ cell cysts (Grive and Freiman 2015) where the meiotic cell divisions (results in cells with haploid genome) are initiated – see section 4 for details of meiosis (Jamnongjit and Hammes 2005).

At around 16 weeks of gestation in humans, the cysts break down, releasing primordial follicles, which are composed of oocytes, surrounded by flat somatic cells known as pre-granulosa cells. This process is accompanied by the apoptosis of many oocytes (Grive and Freiman 2015, Rimon-Dahari, Yerushalmi-Heinemann et al. 2016). I note that these oocytes are arrested at the diplotene stage of meiotic prophase I (see section 4) by inhibitory signals from the ovary, mainly by signalling pathways involving cAMP (Jamnongjit and Hammes 2005).

These primordial follicles remain quiescent and form the ovarian reserve for a women's lifetime. It was believed that a woman was born with a set number of oocytes that could not be replenished but this was challenged by the discovery of primordial germ cells in adult mice oocytes, with the potential to differentiate into new primordial follicles (Johnson, Canning et al. 2004).

2.1.2. Bi-directional signalling between oocyte and somatic cells supports follicle maturation

During every menstrual cycle, primordial follicles can either remain quiescent or be activated in response to paracrine signals (Rimon-Dahari, Yerushalmi-Heinemann et al. 2016). They receive these early activation signals from surrounding somatic cells. They have F-actin and microtubule-rich extensions, known as transzonal projections (TZPs), which are connected to the oocyte via gap junctions and adherens junctions (Li and Albertini 2013). This allows for bidirectional communications between the oocyte and surrounding somatic cells, which are essential for oocyte growth and maturation (Carabatsos, Sellitto et al. 2000). This communication between the oocyte and its surrounding relies on paracrine signalling pathways, many of which involve signalling molecules of the transforming growth factor β superfamily (Li and Albertini 2013). They cause morphological changes in the granulosa cells, from flat to cuboidal, changes to their proliferation and support the growth of the oocyte itself. Moreover, the zona pellucida, a glycoprotein membrane which plays a major role in catalysing sperm entry, forms between the oocyte and granulosa cells. As the oocyte continues to increase in size and granulosa cells to proliferate, a new cell type emerges: the theca cells, which form a layer on the outside of granulosa cells.

2.1.3. Follicular stimulating hormone and luteinising hormone signalling

As the follicle grows, many morphological changes happen in the cells surrounding the oocyte. A fluid-filled cavity known as the antrum starts to form within the layer of granulosa cells, separating different types of granulosa cells: mural cells, which are responsible for steroid synthesis, and cumulus cells, which are adjacent to the oocyte (Rimon-Dahari, Yerushalmi-Heinemann et al. 2016). Granulosa cells start expressing follicular stimulating hormone (FSH) receptors. The theca cells are also

differentiated into two cell types: theca interna and externa cells harbouring luteinising hormone (LH) receptors on their surfaces (Macklon and Fauser 1998).

At this point, when somatic cells have differentiated and the antrum has formed, the follicle is known as an antral follicle. It switches from relying on bidirectional signalling between the oocyte and granulosa cells through cell-cell junction to relying on the gonadotrophins FSH and LH for its development and maturation (Li and Albertini 2013, Rimon-Dahari, Yerushalmi-Heinemann et al. 2016). The production of FSH and LH from the pituitary glands is triggered by the pulsatile release of the gonadotrophin-release hormone (GnRH) by the hypothalamus (Holesh, Bass et al. 2020).

FSH levels rise until they reach a threshold level, triggering the growth of a group of small antral follicles. FSH stimulates granulosa cell proliferation, as well as LH receptor expression by theca cells and the formation of more FSH receptors (Rimon-Dahari, Yerushalmi-Heinemann et al. 2016). The granulosa cells, activated by FSH, and the theca cells, activated by LH, work hand in hand to produce oestradiol. Indeed, LH signalling causes theca cells to convert cholesterol into androstenedione and testosterone, which is, in turn converted to oestradiol by granulosa cells, in response to FSH (Macklon and Fauser 1998). Once the FSH threshold is reached, FSH levels will drop, causing most of the developing follicles to become atretic. The follicles themselves will cause this fall in FSH levels by secreting the FSH inhibitors inhibin A and oestradiol. Only dominant follicles will survive in this FSH-poor environment as they are more sensitive to FSH: they have more granulosa cells, that express higher levels of FSH receptors, allowing them to survive. As FSH levels continues to fall, the dominant follicles are believed to become FSH-independent through processes that are not completely understood, relying on LH for their development (Mihm and Evans 2008).

Oestradiol release by the granulosa cells causes the production of even more oestradiol, via a positive feedback mechanism. This signals back to the hypothalamus, increasing the frequency of GnRH pulses, leading to a LH surge (Holesh, Bass et al. 2020). There is constant cross-talk between the hypothalamus, which secretes GnRH, the pituitary glands, which secrete LH and FSH and the ovaries themselves which produce oestrogen, along the hypothalamic-pituitary-gonadal axis (Rimon-Dahari, Yerushalmi-Heinemann et al. 2016).

LH signalling triggers changes in the follicle, including the dissolution of its basement membrane, its vascularisation and cumulus cell expansion (the production of extra-cellular matrix proteins). These events will cause the ovarian follicle to rupture, releasing the cumulus-oocyte complex (COC) from the ovary: this process is known as ovulation (Robker, Hennebold et al. 2018).

Additionally, the surge in LH inhibits cGMP production within the granulosa cells, leading to a decrease in cAMP and CDK1 to be activated so the oocyte matures. This leads to the resumption of meiosis I in the oocyte, it will then start meiosis II and arrest at the metaphase stage. At this point, the oocyte is fully matured and awaits sperm entry to resume meiosis II and be fertilised. Following ovulation, LH signalling will cause remodelling of the granulosa and theca cells into small and large luteal cells. These luteal cells will form the corpus luteum (CL), a temporary endocrine system whose function is to secrete progesterone, causing changes in the endometrium to prepare it for pregnancy (Bagnjuk and Mayerhofer 2019).

2.2. The formation of sperm cells

Similar to oocytes, sperm cells originate from immature germ cells. However, unlike oocytes, sperm cells are constantly produced throughout a man's lifetime. The formation of a spermatozoon from a germ cell happens in two main stages: the foetal and neonatal stage and spermatogenesis (Culty 2009).

The first stage starts in the foetus as primordial germ cells migrating to the genital ridge and undergo genome-wide methylation, differentiating into gonocytes. These gonocytes undergo rounds of mitoses in the foetal and postnatal stages, separated by a period of quiescence. It is during the neonatal period that the gonocytes move to the seminiferous tubules of the testes, where they can fully differentiate into spermatogonial stem cells (SSCs), which can self-renew, allowing sperm to be produced throughout a man's lifetime. The second phase; spermatozoa maturation, known as spermatogenesis, can then start converting SSCs into mature spermatozoa: a process which lasts 74 days in humans (Gunes, Al-Sadaan et al. 2015). The SSCs start by undergoing rounds of cell divisions, forming differentiating spermatogonia. This is followed by two rounds of meiotic divisions, forming haploid spermatids containing 23 chromosomes with one chromatid. Paracrine signalling from Sertoli cells in the seminiferous tubules controls the proliferation and differentiation of SSCs as well as meiosis (Chen and Liu 2015). The final step of spermatogenesis is characterised by important changes in morphology, from round spermatids to fully formed spermatozoa, with a sperm head and a tail (Gunes, Al-Sadaan et al. 2015).

3. Cell Divisions

To understand how the embryo develops, following the fertilisation of the oocyte by the spermatozoon, it is crucial to look at mitotic cell divisions, which allow one cell to divide into the ~50 million cells that constitute us human beings.

The cell cycle in human somatic cells is ~24 hours long and can be separated into interphase (23 hrs) and an M phase (1 hrs) during which time one cell divides into two. Interphase is composed of the G1, S and G2 phases. During G1, the cell either

become quiescent (G0) or enters S phase, during which time the DNA and centrosomes are replicated and duplicated (Hochegger, Takeda et al. 2008).

This transition from G1 to S relies on the inhibition of the anaphase promoting complex/cyclosome (APC/C) in association with the co-activator Cdh1 by the inhibitor Emi1. Without inhibition by Emi1, the APC/C-Cdh1 will degrade cyclins and proteins involved in DNA replication, preventing the cell from moving on to S phase. After S phase, the cell will enter G2: the growth phase where organelles, such as mitochondria and ribosomes, are synthesised. After G2, mitotic entry relies on the maturation-promoting factor (MPF), which is defined as a molecular complex which triggers M-phase in eukaryotic cells. MPF consists of the cyclin B/Cdk1 complex and the Greatwall kinase (Hara, Abe et al. 2012, Kishimoto 2015). Cdk1 activity remains relatively constant throughout the cell cycle whilst cyclin B accumulates during G2 and associates with Cdk1. The Cyclin B/Cdk1 complex is kept in an inactivation through the inhibitory phosphorylation of T15 and Y14 until the end of G2 when these phosphorylations are removed by Cdc25, triggering mitotic entry (Perry and Kornbluth 2007).

Mitosis is defined as nuclear division and can be subdivided into prophase, prometaphase, metaphase, anaphase and telophase (see below for details). Cytokinesis, the process that divides the cell into two new daughters and starts during anaphase as the chromosomal passenger complex is recruited to the spindle midzone (Landino, Norris et al. 2017). The final separation of daughter cells requires abscission, during which the cell membrane is cut, giving rise to two daughter cells. In human somatic cells, mitosis takes around 37 mins and cytokinesis takes 4 mins (Spira, Cuylen-Haering et al. 2017).

3.1. Mitosis

3.1.1. Prophase

During prophase several key events occur to prepare cells for mitosis. Firstly, chromosomes start to condense through the recruitment of condensin complexes to assume the morphology of mitotic chromosomes (Skibbens 2019). These chromosomes are composed of two sister chromatids which have a long (q) and short (p) chromosome arm, which join at the centromere. Before mitosis begins, the two sister chromatids are held together by cohesin complexes composed of two structural maintenance of chromosomes (SMC) subunits, one stromalin subunit and one kleisin subunit. They assemble along the chromosome arms and the centromere during S phase. During prophase, these cohesin complexes are removed from chromosome arms but are retained at the centromeres to ensure the sister chromatids remain associated until anaphase onset. This happens via the phosphorylation of the stromalin subunit STAG2 by Polo-like kinase (PLK) and Aurora B, causing the dissociation of cohesin complexes along the chromosome arms (Brooker and Berkowitz 2014).

Centromeres at this stage are bound by the constitutive centromere associated network (CCAN) which remains on centromeres throughout the cell cycle. The microtubule binding components of the kinetochore, a large multiprotein structure which assembles on the centromere and is essential for mitosis, begin to assemble forming, microtubule attachment sites (Nagpal and Fukagawa 2016). During this time the nuclear envelope remains intact separating chromosomes and kinetochores from the two microtubule asters. Each aster is formed of microtubules nucleated from the two centrosomes. The centrosomes move apart across the nuclear envelope during prophase until they are positioned opposite each other. This is important for efficient formation of the bipolar mitotic spindle in prometaphase (Gadde and Heald 2004).

3.1.2. Prometaphase

Nuclear envelope breakdown (NEB) marks the start of prometaphase and the mitotic spindle can start to assemble. It is a dynamic structure made of microtubules, which are themselves composed of α and β tubulin polymers. NEB allows microtubules, nucleated from the centrosomes, to bind kinetochores, linking the centromere of the chromosomes to the two centrosomes, which are the spindle poles. Microtubules polymerise and depolymerise from their plus end before disassembling. By growing and shrinking, they can explore the space around them until they come into contact with a kinetochore to which they bind, resulting in microtubule stabilisation (O'Connell and Khodjakov 2007). This is known as the search-and-capture model (Kirschner and Mitchison 1986). The microtubule nucleation process can also be driven by chromatin. The guanine-nucleotide-exchange factor (GEF) regulator of chromosome condensation 1 (RCC1), which is associated with chromatin, converts Ran-GDP into Ran-GTP, forming a Ran-GTP gradient around the chromosomes. The elevated Ran-GTP concentration around the chromosomes encourages microtubule polymerisation and microtubules stabilising factors around the chromosomes supports microtubule capture (O'Connell and Khodjakov 2007). During prometaphase, kinetochores are attached to microtubules in different orientations: some chromosomes have both sister chromatids attached to opposite spindle poles (amphitelic attachment), some are unattached, some have only one sister chromatid attached, some have one kinetochore attached to both spindle poles (merotelic attachment) and some have both sister chromatids attached to the same pole (syntelic attachment) (Fig. 2). Aurora B destabilises both merotelic and syntelic attachments (Auckland and McAinsh 2015), through a process known as error correction, see section 3.3.1. When sister kinetochores are attached to opposite poles (amphitelic), chromatid cohesion at the centrosome counterbalances the forces produced by microtubules, generating tension. This

tension pulls Aurora B away from the kinetochore proteins it phosphorylates, preventing the destabilisation of kinetochore-microtubules attachments, see section 3.3.1, (Nagpal and Fukagawa 2016).

3.1.3. Metaphase

During metaphase, the chromosomes migrate to the spindle equator, known as the metaphase plate. The movement of chromosomes towards the spindle equator is known as congression and is driven by changes in microtubule polymerisation. Indeed, microtubules can depolymerise at the + end, causing the attached kinetochore to move towards the pole and polymerise at the - end, causing a movement away from the spindle pole (Auckland and McAinsh 2015). Error correction, which started in prometaphase is still correcting erroneous attachments and the spindle assembly checkpoint (SAC) ensures that all the chromosomes are attached, through interactions which will be detailed in section 3.3. Prometaphase and metaphase together have been shown to last about 24 mins in human somatic cells (Silio, McAinsh et al. 2015, Alper and Fauser 2017).

3.1.4. Anaphase

Once all the chromosomes are bi-orientated and aligned on the metaphase plate, the two sister chromatids are pulled towards opposite spindle poles: an event known as anaphase onset. Sister chromatid separation requires cohesion to be lost at the centromere. This process is driven by the anaphase promoting complex/cyclosome (APC/C) which destroys the inhibitor securing, causing separase to be active and cleave RAD21 (the kleisin subunit of the cohesin complex) (Brooker and Berkowitz 2014). This causes the cohesin ring to open and the sister chromatids to separate. Anaphase happens in two stages, known as anaphase A and B. During anaphase A, the microtubules are depolymerised,

pulling on the kinetochores to move the chromosomes towards the spindle poles. This is followed by anaphase B during which the spindle poles further separate, thanks to motor proteins (Gadde and Heald 2004).

3.1.5. Telophase

During Telophase the chromosomes reach the two spindle poles and a nuclear envelope forms around each of the DNA masses. Once a nucleus is formed in each cell, the chromosomes can start to decondense (Gadde and Heald 2004).

3.2. Cytokinesis

Cytokinesis is the process during which the cytoplasm of the dividing cell is partitioned and the plasma membrane is cleaved, giving rise to two distinct daughter cells. This process starts early in anaphase. Indeed, as the chromosomes move towards the spindle poles, microtubules form antiparallel arrays at the spindle midzone, forming the central spindle. The central spindle serves as a signalling platform which will direct cytokinesis. An important factor in cytokinesis is the chromosomal passenger complex (CPC): a heterotetramer of Aurora B, INCENP, borealin and survivin that relocates from the centromeres to the central spindle. At this point, the small GTPase Rho A is activated around the spindle midzones, driving the process of cytokinesis furrow ingression, where the cytoplasm is partitioned into two (Mierzwa and Gerlich 2014). Rho A phosphorylates myosin II, triggering actin filament nucleation by formins, leading to the formation and contraction of an actomyosin ring at the spindle equator (Pollard and O'Shaughnessy 2019). As the actomyosin ring constricts, two cells form, linked by an intracellular bridge made of antiparallel microtubules, with a structure known as the midbody at its centre. The midbody is essential for the completion of cytokinesis as it serves as a platform for the assembly of abscission

machinery, which will eventually cleave the plasma membrane (Hu, Coughlin et al. 2012, Mierzwa and Gerlich 2014). As the intercellular bridge matures, the cortex adjacent to the midbody constricts, causing the plasma membrane to split. This process is known as abscission as it is mediated by the endosomal sorting complex required for transport-III (ESCRT-III). Abscission relies on APC/C activation as Plk1 suppresses ESCRT-III until it is degraded by the APC/C at mitotic exit (Mierzwa and Gerlich 2014). Whilst the contraction of actomyosin ring and the formation of two cells connected by an intracellular bridge takes about 10 minutes in human somatic cells, abscission can take hours to be completed and happens once the cell has already exited mitosis and is in G1 (Nahse, Christ et al. 2017). The duration from cytokinesis furrow ingression to the completion of abscission varies in different human somatic cell lines, from about 60 minutes in HeLa cells (Steigemann, Wurzenberger et al. 2009) to about 125 mins in RPE1 cells (Gershony, Pe'er et al. 2014).

3.3. Checkpoints (spindle assembly checkpoint, abscission checkpoint).

During the cell cycle, checkpoints exist to make sure the cell progresses through the cell cycle without accumulating errors. The spindle assembly checkpoint (SAC) makes sure that all the chromosomes are attached to microtubules before initiating anaphase. The abscission checkpoint controls the final stage of cytokinesis where the plasma membrane is cleaved, creating 2 distinct daughter cells.

3.3.1. Error-correction mechanisms

For anaphase to happen without lagging chromosomes, all the chromosomes must form amphitelic attachments, where the two sister chromatids are attached to

opposite spindle poles. The cell therefore has mechanisms in place to correct the three types of erroneous microtubule attachments: monotelic (only one kinetochore is attached), syntelic (both kinetochores are connected to the same pole) and merotelic (one kinetochore is connected to both poles) (Fig. 2). This is known as error-correction, a process dependent on Aurora B, which happens in prometaphase and metaphase. Aurora B is the kinase subunit of the CPC, which is also composed of the proteins Survivin, Borealin and INCENP (Carmena, Wheelock et al. 2012). The CPC is recruited to the kinetochore early in mitosis by histone H3 phosphorylation by the Haspin kinase or by Histone H2A phosphorylation by Bub1 (Carmena, Wheelock et al. 2012, Kataria and Yamano 2019). Aurora B phosphorylates the kinetochore proteins Ndc80 and Ska, destabilising the incorrect kinetochore-microtubule attachment. The cell is able to make the distinction between correct and incorrect attachments as, when an amphitelic attachment forms, the two centrosomes are pulled towards opposite spindle poles, generating tension. This increases the distance between the kinetochore and Aurora B, which is on the inner centromere. Aurora B is therefore unable to phosphorylate the kinetochore proteins of correctly attached chromosomes (Kataria and Yamano 2019) (Fig. 3).

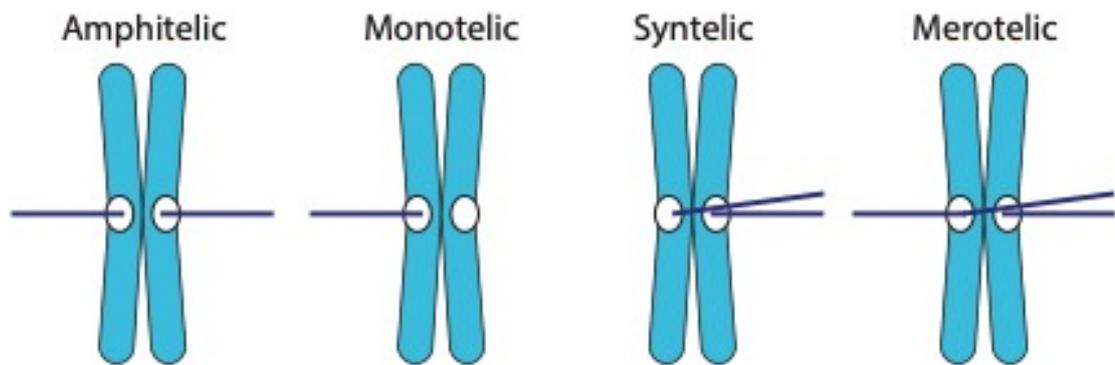


Figure 2: Kinetochores microtubule attachment types. The chromosomes are correctly attached when each kinetochore is attached to opposite spindle poles (merotelic attachment). Incorrect attachments include: monotelic attachments (only one kinetochore is attached), syntelic attachments (both kinetochores are attached to the same spindle pole), Merotelic attachments (one kinetochore is attached to both spindle poles).

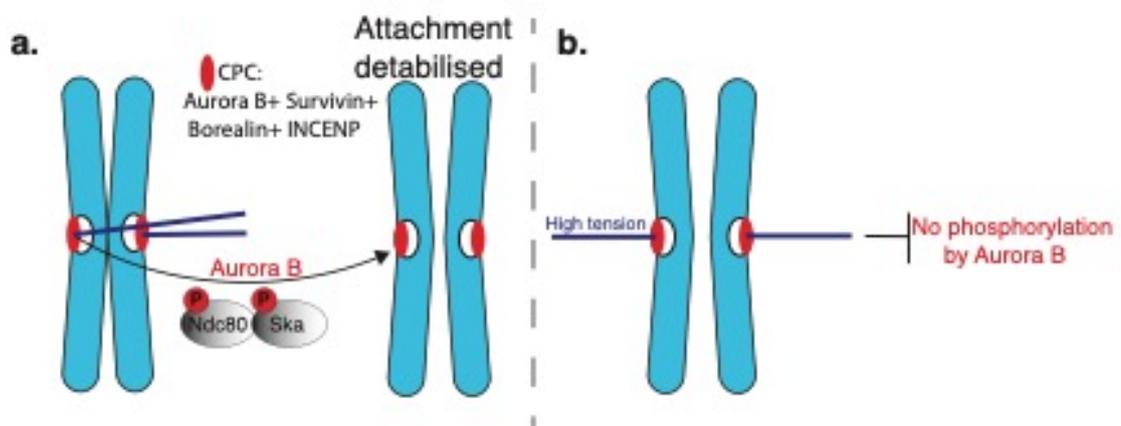


Figure 3: Overview of error correction. (a) Error correction of an erroneous kinetochore microtubule attachment. Aurora B, the kinase subunit of the chromosome passenger complex (CPC) phosphorylates Ska and Ndc80, causing the erroneous attachment to be destabilised. (b) When the sister kinetochores are correctly attached, this creates tension, pulling Aurora B away from Ndc80 and Ska away from Aurora B, preventing their phosphorylation. The attachment is therefore stable.

3.3.2. The Spindle Assembly checkpoint

Unattached kinetochores, as a result of a destabilised attachment by Aurora B or simply because the kinetochore has not made any microtubule attachments, will activate the spindle assembly checkpoint (SAC) which makes sure the cell remains in metaphase until all the chromosomes are properly attached (De Antoni, Pearson et al. 2005).

Indeed, unattached kinetochores will recruit the kinase monopolar spindle 1 (Mps1), which binds the kinetochore proteins Ndc80, initiating a phosphorylation cascade. Knl1 becomes phosphorylated as a result, recruiting Bub1-Bub3, which in turn recruits BubR1. Bub1 becomes phosphorylated by Mps1 and Cdk1, allowing Mad1: Mad2 complexes to bind the kinetochore. Mad2 can assume an “opened” and a “closed” conformation. When Mad2 is bound to Mad1, it is in its “closed” conformation and serves a receptor for “opened” cytoplasmic Mad2, triggering a conformational change to “closed” Mad2 (De Antoni, Pearson et al. 2005). “Closed” Mad2 binds Cdc20, which allows BubR1 and Bub3 to bind, forming the mitotic checkpoint complex (MCC) heterotetramer (Stukenberg and Burke 2015, Kataria and Yamano 2019). Cdc20 is the coactivator of the APC/C, a 15 subunit E3 ubiquitin ligase whose role it is to target cell cycle proteins for degradation to allow the cell to irreversibly move on to the next stages of the cell cycle. Because Cdc20 is sequestered by the MCC, the APC/C remains inactive, causing a mitotic delay (Fig. 4). Mad1 and Mad2 can also be recruited to unattached kinetochores by the RZZ complex, via an alternative pathway (Silio, McAinsh et al. 2015).

When all the chromosomes are bioriented, the MCC is disassembled, causing Cdc20 to be released and to associate with the APC/C. APC/C-Cdc20, which triggers anaphase onset by degrading many of the proteins required to keep the cell in metaphase including Cyclin B1 and separase. Cyclin B1 proteolysis causes

Cdk1 to be inactivated, leading the cell to exit mitosis and enter G1 (Stukenberg and Burke 2015).

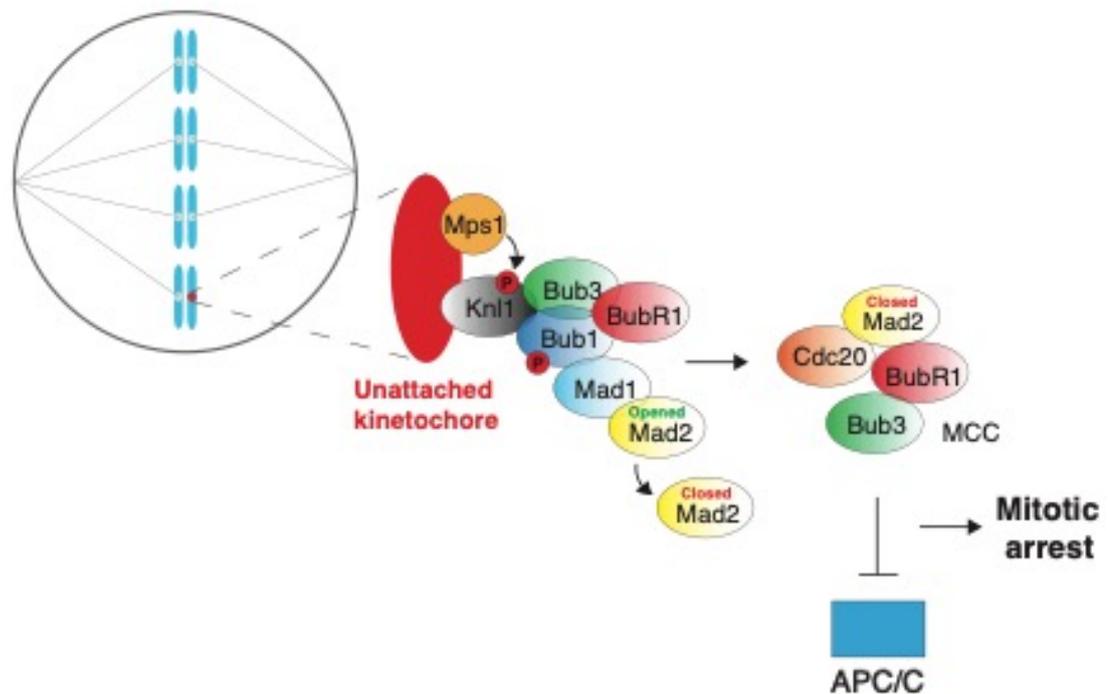


Figure 4: Spindle assembly checkpoint signalling. Unattached kinetochores trigger spindle assembly checkpoint (SAC) signalling by recruiting Mps1 which will phosphorylate Knl1 which will lead to the recruitment of Bub1 and Bub3, which will in turn recruit BubR1. Mps1 phosphorylates Bub1, causing Mad1 to bind. Mad2 in its open conformation binds Mad1 and undergoes a conformational change to assume its closed conformation. This causes cytoplasmic Mad2 to have a closed conformation. "Closed" Mad2 binds to Cdc20, BubR1 and Bub3 then bind, forming the mitotic checkpoint complex (MCC). This causes the coactivator Cdc20 to be sequestered away from the anaphase promoting complex /cyclosome (APC/C). The APC/C is inactive, causing a mitotic arrest.

3.3.3. Spindle assembly checkpoint silencing

Once chromosomes are bi-oriented and the SAC is satisfied, it needs to be silenced to allow the cell to exit mitosis. This is dependent on the removal of SAC proteins at the kinetochore, on inhibition of further SAC signalling and on pools of cytoplasmic MCC to be disassembled.

One way by which SAC proteins are removed is through a mechanism known as “stripping”, during which Mad1 and Mad2 are physically pulled away from the kinetochore by dynein (Stukenberg and Burke 2015). Moreover, many dephosphorylation events are needed to counteract the phosphorylation of kinetochore and SAC proteins which activated the SAC in the first place. A key player in this dephosphorylation cascade is the phosphatase PP2A-56 directly, which opposes Aurora B by dephosphorylating its targets. Notably, PP2A-B56 reverses Aurora B phosphorylation of the PP1 binding site of KNL1, allowing PP1 to be recruited to the inner kinetochore. PP1 dephosphorylates KNL1 on its MELT motif (previously phosphorylated by MPS1), causing Bub1, BubR1 and Bub3 to be removed. These phosphorylation events therefore cause the SAC proteins to be taken off the kinetochore (Nijenhuis, Vallardi et al. 2014).

PP1 also works to silence the SAC through direct interactions with other kinetochore proteins such as CENP-E, which stabilises mature attachments once dephosphorylated, and Ska, which works to silence the SAC (Conti, Gul et al. 2019, Kataria and Yamano 2019).

Many events associated with SAC silencing are promoted by PP1, such as the stabilisation of correct kinetochore-microtubule attachments and nuclear envelope assembly. Indeed, PP1 is delivered by the stabilising protein Astrin next to Ndc80's C terminus, promoting the recruitment of more Astrin, strengthening kinetochore-microtubule interactions (Conti, Gul et al. 2019). PP1 becomes associated to the Repo-Man complex, which is activated late in mitosis, as a result of Aurora B and Cdk1 inhibition. Repo-Man-PP1 localises to the chromatin and dephosphorylate the histone H3 at different positions, an important step in establishing heterochromatin once again. Repo-Man/PP1's action also supports nuclear envelope assembly by interacting with Importin β (Vagnarelli and Earnshaw 2012).

To fully silence SAC signalling, cytoplasmic MCC pools must be disassembled. This happens via the action of the protein phosphatases P31^{comet} and TRIP13, which oppose Mad2 phosphorylation by MPS1, causing Mad2 to switch back to its opened conformation and to be released from the MCC. (Stukenberg and Burke 2015).

In summary, the SAC is silenced by a big wave of dephosphorylation, leading to the activation of the APC/C, triggering a cascade of events which will lead to mitotic exit.

3.4. Abscission checkpoint

Another key checkpoint regulating cell division is the abscission checkpoint which delays abscission in the presence of errors such as chromatin trapped in the cleavage plane, DNA replicative stress, high membrane tension and defects in the nuclear pore complex. If abscission goes ahead despite these errors, it can result in aneuploidies, the formation of micronuclei, chromothripsis (chromosomes broken down into many pieces and aberrantly repaired) and chromosomal instability (Nahse, Christ et al. 2017).

This process is dependent on Aurora B which is present in the midbody and acts as an abscission inhibitor. It is still unclear how Aurora B senses these errors but proteins such as the checkpoint kinase 1 (CHK1) and CLK1,2,4 have been shown to activate Aurora B. As a result, Aurora B phosphorylates several proteins involved in ESCRT-III activation, inhibiting abscission until these errors are corrected (Mierzwa and Gerlich 2014, Nahse, Christ et al. 2017).

4. Meiosis: a specialised cell division

I have described mitosis, which is preceded by a round of DNA replication and gives rise to two identical diploid daughter cells. However, this is not the only type of cell division; meiosis is the specialised cell division that gives rise to male and female gametes. Two meiotic cell divisions take place to form the sperm and oocyte from a diploid cell. Whilst some features are conserved between meiosis and mitosis, they follow the stages of prophase, prometaphase, metaphase, anaphase and telophase and a bipolar spindle forms and moves the chromosomes to opposite sides of the cells; key differences make these divisions completely unique (Ohkura 2015). During the first meiosis, the two sister chromatids remain joined together during the entire division, due to cohesin being conserved at the centromere and to recombination (detailed below). This division gives rise to haploid two cells with 23 chromosomes. Importantly, this division is not followed by DNA replication. During the second meiotic division, the sister chromatids are separated, resulting in daughter cells with 23 chromosomes composed of one chromatid.

In this chapter, I will focus on the key differences which set meiosis apart from other divisions.

4.1. Extended prophase I: a key feature of meiosis

The prophase of the first meiotic division is unique as, unlike in mitosis and meiosis II, homologous chromosomes form crossovers to ensure that they are segregated together. This step is comprised of the following phases: leptotene, zygotene, pachytene, diplotene and diakinesis. During leptotene, the chromosomes start to condense and proteins of the cohesin complex, such as Rec8 and structural maintenance of chromosomes 1B (SMC1B), and of the synaptonemal complex

(SC), such as SYCP 2 and 3, are recruited (Nasmyth and Haering 2009). Double strand breaks (DSB) are induced in the DNA sequence by SPO11, a step which catalyses recombination, a process during which genetic material is exchanged between chromosomes. Indeed, DSBs lead to the invasion of a chromatid strand into the chromatid strand of its homologues. This triggers the process of homologous recombination repair, via proteins such as DMC1 and RAD51, during which the damaged part of the DNA sequence is repaired (Handel and Schimenti 2010, Baudat, Imai et al. 2013).

During zygotene, homologous chromosomes are finally paired and fuse together at the sites of the DSBs. Importantly, synapsis happens during this stage. It is a process during which the SC proteins present on each chromosome interact with each other, causing homologous chromosomes to “zip” and the SC to form (Handel and Schimenti 2010). By pachytene, the chromosomes are fully synapsed, resulting either in crossovers, in which the two chromosomes swap the sequence after the chiasmata, or non-crossovers where only a small fragment of the homologue sequence is acquired (MacLennan, Crichton et al. 2015). The purpose of recombination is to create genetic diversity and to physically link homologous chromosomes. In diplotene, the SC disassembles, the chromosomes condense and move apart slightly, only connected by the chiasmata (Handel and Schimenti 2010). The final stage of diakinesis is characterised by the spindle starting to assemble and the nuclear envelope breaking down (Rosenberg and Rosenberg 2012).

4.2. Meiosis in the oocyte

Meiosis in the oocyte is complex as it undergoes two meiotic arrests: one during this prophase I that I just described, which can last decades, and one during metaphase of the second division until sperm entry. The meiotic progression is coordinated with ovulation and fertilisation.

As mentioned in section 2.1.1, the oocyte starts undergoing the first meiotic division during embryonic development, before the primordial follicles are formed. It is then arrested during the diplotene stage of prophase I, the homologous remain linked together for years, until after sexual maturation, or until the oocyte's follicles become atretic. The diplotene arrested oocyte is known as the germinal vesicle (GV), characterised by the presence of a nucleus, known as GV, around the chromosomes. To ensure that the oocyte stays arrested in meiotic prophase I, CDK1 levels are kept low to prevent transition to metaphase (Jaffe and Egbert 2017). This is thanks to signals within the oocyte, via the production of cAMP which inhibits CDK1 by phosphorylating it via signalling pathways involving protein kinase A (PKA). The arrest also requires signalling from the rest of the follicle. Indeed, surrounding granulosa cells inhibit cAMP hydrolysis, keeping its levels high, by producing cGMP which enters the oocyte via gap junctions (Pan and Li 2019).

4.3. Overview of meiosis after puberty

The first meiotic division is resumed many years later, after puberty, during ovulation. This happens in response to a surge in LH which inhibits cGMP production within the granulosa cells, leading to a decrease in cAMP. This will cause the inactivation of the protein kinase A (PKA) and the translation of c-mos mRNA (Gebauer and Richter 1997). Mos, the product of the proto-oncogene c-mos, will in turn activate the MAPK proteins MEK and Erk2, causing a positive feedback loop, further amplifying MAPK signalling. This will cause MPF activation and therefore meiosis I resumption, starting with GV nuclear envelope breakdown and chromosomes condensation (Sen and Caiazza 2013). The two homologous chromosomes are still connected by the chiasmata, like they were years before, and align on the metaphase plate. The physical link provided by the chiasmata, together with the mono-orientation of the chromosomes, generates tension

within the first meiotic spindle (MacLennan, Crichton et al. 2015). At this point during metaphase, the spindle migrates to the cell cortex. Studies in mice have shown this process to be actin-dependent. Indeed, an actin cortex borders the oocyte's plasma membrane and actin-nucleator proteins, such as Formin and Spire1/2, create a dynamic cytoplasmic actin network. Myosin 2 is activated at the spindle poles and attaches the spindle to the actin cortex, pulling the spindle towards the cell edge. Later stages of meiosis I spindle migration are supported by the process of cytoplasmic streaming, which happens thanks to the cytoplasmic actin network (Uraji, Scheffler et al. 2018).

The migration of the spindle is essential as it causes the oocyte to become polarised. This occurs due to signalling from the chromatin that induces major changes within the cell cortex. Because chromatin signalling is distance-dependent, the part of the cell where the spindle is located will be very affected by this chromatin signalling, unlike the other side of the cell. This is the root cause of cell polarity in the oocyte (Li and Albertini 2013). The chromatin signals the actin cortex adjacent to it to thicken, creating the actin cap (Uraji, Scheffler et al. 2018). Once the spindle reaches the cortex, homologous chromosomes migrate to opposite spindle poles (anaphase onset) and cytokinesis takes place. Polarity in the oocyte allows this extremely asymmetric division to take place, giving rise to a large oocyte and small polar body (PB), which is there simply to allow the oocyte to eliminate half of its diploid genome and will eventually degenerate.

This cell polarity is maintained as a second meiotic spindle forms (Li and Albertini 2013). During the second meiosis, chromosomes are bi-orientated and aligned on the metaphase plate. At this point, during second meiotic metaphase, meiosis is arrested until fertilisation. This arrest is maintained by the cytostatic factor (CSF) which inhibits the APC/C and therefore stabilises MPF via the c-mos-MAPK pathway (Sen and Caiazza 2013). Indeed, mouse oocytes in which Mos is

suppressed fail to maintain the metaphase arrest and therefore meiosis II is resumed in the absence of sperm (parthenogenesis) (Dupre, Haccard et al. 2011).

During the arrest period, the second meiotic spindle is anchored to the cell cortex, below the actin cap (Uraji, Scheffler et al. 2018). Sperm entry triggers calcium oscillations, triggering a plethora of cell processes including meiosis II resumption. The sister chromatids separate and move to opposite spindle poles and cytokinesis start, creating a large mature oocyte and a second polar body.

The resumption and completion of both meiotic divisions relies on maternal mRNAs, which need to be translated and stored, as well as other processes requiring the reorganisation of organelles in the cytoplasm, such as those in place to respond to the high energy needs of the oocyte and the production of calcium oscillations. Such processes must take place before meiotic resumption to support meiosis but also fertilisation and early embryonic development, through a process known as cytoplasmic maturation (Conti and Franciosi 2018).

4.4. Meiotic spindle nucleation

It is worth diving deeper on how the spindle is nucleating during the two meiosis spindles as, despite what their similar morphology would suggest, the processes underlying meiotic spindle nucleation differ from mitosis.

These differences in spindle nucleation are rooted in the fact that the oocyte does not possess centrosomes. Instead, spindle formation relies on microtubule organising centres (MTOCs) and on a Ran-GTP gradient in mice (Schuh and Ellenberg 2007). A staggering 80 MTOCs were observed before NEBD. They congregate together via MTOC-MTOC interactions and mediate a Ran-dependent increase in microtubule nucleation after NEBD. Chromosomes are then separated into bivalents and move to the surface of this microtubule ball. Multiple spindle

poles are then formed, via kinesin 5 activity. These poles fuse together until 2 dominant poles are formed, giving rise to a bipolar spindle (Schuh and Ellenberg 2007).

In human oocytes, the spindle nucleation progress is also dependent on a Ran-GTP gradient but happens independently of MTOCs, unlike in mice (Holubcova, Blayney et al. 2015). Spindle nucleation starts with the formation of a small microtubule aster. As it grows, the chromosomes become separated into single bivalents, on the surface of the aster. An early bipolar spindle then forms, with the chromosomes spread throughout the spindle. Next, the chromosomes start moving to the centre, still oscillating, with stable attachments only forming just before anaphase onset. As metaphase progresses, the spindle volume increases, forming a barrel-shaped spindle, with no astral microtubules. Meiotic spindle nucleation, in human oocytes, is characterised by high levels of instability. Indeed, many spindles observed reverted back to being apolar or underwent many cycles of multipolarity (Holubcova, Blayney et al. 2015).

Meiotic spindle assembly is a lengthy process, lasting 3-5h in mice (Schuh and Ellenberg 2007) and about 16h in humans (Holubcova, Blayney et al. 2015), compared to just 30 mins for mitotic spindle assembly in somatic cells (Meraldi, Draviam et al. 2004, Silio, McAinsh et al. 2015).

4.5. Meiosis in the sperm

The times at which the meiotic divisions happen as well as the cells that arise from these divisions are very different in the oocyte and spermatozoon. Indeed, the two meiotic divisions are not coupled with ovulation and fertilisation like in the oocyte, they simply happen one after the other without any extended arrest periods. Meiosis in the sperm corresponds to the second stage of spermatogenesis and happens continuously from puberty. I mentioned previously the importance of

establishing polarity in the oocyte to make sure this asymmetric division occurs properly. This is not the case in the sperm, where 4 haploid spermatozoa with 23 chromosomes composed of one chromatid form after the two meiotic divisions (Handel and Schimenti 2010).

5. Both types of cell divisions can be a source of errors

Aneuploidies, as a result of chromosome segregation errors are very common in human embryos. Single cell analyses of human morulae and blastocysts have revealed that a staggering 80% of human embryos are aneuploid (Starostik, Sosina et al. 2020). Aneuploidies in human gametes and embryos can cause developmental disorders such as Down's syndrome (trisomy 21) and trisomies of the chromosomes 13, 18 and the sex chromosomes. These aneuploidies have been shown to be a cause of infertility and pregnancy loss (van den Berg, van Maarle et al. 2012, Hardy and Hardy 2015).

5.1. Errors of meiotic origin

5.1.1. Aneuploidies during the first and second meiotic divisions

The rates of aneuploidies of meiotic origin in the oocytes increase dramatically with maternal age from the age of 35, from about 20% to over 60% (Capalbo, Hoffmann et al. 2017, McCoy 2017). This increase is consistent with the growing proportion of trisomic pregnancies with age (Hassold and Hunt 2001).

Similar error-rates have been reported in the two meiotic divisions (Webster and Schuh 2017). However, the amount of errors in each division has been suggested to be age-dependent: for younger women, the errors arising in meiosis I appear to be predominant, whereas as maternal age advances, errors in meiosis II most commonly occur (Fragouli, Alfarawati et al. 2013)

Different types of errors can happen during meiosis, leading to aneuploidies. These include nondisjunction events where homologous chromosomes during meiosis I or sister chromatids during meiosis II fail to separate, causing them to end up in the same daughter cell. Sister chromatids can fail to separate but they can also split prematurely, causing them to segregate independently, often resulting in errors: this is referred to as premature separation of sister chromatids (PSSC). Studies sequencing the two polar bodies have shown that errors resulting from PSSC can be rescued during the second meiosis. For example, a loss during meiosis I can be compensated by a gain during meiosis II (Hassold and Hunt 2001, Handyside, Montag et al. 2012, Webster and Schuh 2017).

Studies looking at the genetic composition of polar bodies have revealed that the sister chromatids of homologous chromosomes can be segregated during meiosis I similar to what usually happens in meiosis II. This is known as reverse segregation. Most of the time (60 to 80% of cases), this is rectified during meiosis II, giving rise to euploid oocytes (Handyside, Montag et al. 2012, Capalbo, Hoffmann et al. 2017, Webster and Schuh 2017)

5.1.2. Potential origins of meiotic errors

During meiosis I, sister chromosomes are connected to the same spindle pole and segregate together. It is only during anaphase onset of the second meiotic division

that they will finally separate and migrate to opposite spindle poles. In mice and other organisms, the sister kinetochores are fused and segregated as one unit. This is not the case in humans, where sister kinetochores are not physically fused and need to form individual attachments to k fibres (Patel, Tan et al. 2015, Capalbo, Hoffmann et al. 2017). As sister chromatids have to stay joined for up to several decades, it is not very surprising that chromosome splitting in mice has been shown to increase with maternal age (Capalbo, Hoffmann et al. 2017). As mentioned before, sister kinetochore are not fused in humans but an increase in inter-kinetochore distance with age has been reported during meiosis I (Patel, Tan et al. 2015).

Experiments in mice mutants not expressing the cohesin protein $\text{Smc}\beta$, have shown that cohesion loss leads to chromosome splitting (Hodges, Revenkova et al. 2005). The mechanisms causing an increase in univalents in humans are not well understood. Although the total levels of cohesin proteins decrease in women over 40 (Tsutsumi, Fujiwara et al. 2014), the proportion of separated sister kinetochores and the localisation of cohesin proteins to kinetochores remain constant with age (Garcia-Cruz, Brieno et al. 2010, Patel, Tan et al. 2015). This suggests that, unlike in mice, the decrease of overall cohesin protein expression is unlikely to be a major factor in the increase of meiotic errors in the oocyte with maternal age.

As mentioned in part 3, meiotic spindle nucleation happens without the centrosome, making it more unstable. This effect seems to be exacerbated with maternal age as abnormal spindles are more common in older women (Cimadomo, Fabozzi et al. 2018), giving a potential cause for the increasing error-rates with advancing maternal age. The SAC (detailed in part 2.4.1) has also been postulated to be weakened in oocytes of older women, based on the observation that Mad2 and Bub1 expression decreases with advancing maternal age (Steuerwald, Cohen et al. 2001).

Other potential causes of errors during the meiotic divisions include telomere shortening, as telomerase is important for spindle stability in mice, mitochondrial dysfunction, which decreases with age and provides energy to the oocyte for every single step of its development and division, and recombination events, which can lead to the premature separation of chromosomes (Capalbo, Hoffmann et al. 2017, Cimadomo, Fabozzi et al. 2018)

5.2. Errors of mitotic origin

5.2.1. Mosaic embryos as a result of mitotic errors

Errors of meiotic origin only explain part of the aneuploidies observed in embryos, mitotic errors are also very prevalent, leading to mosaic embryos, whose blastomeres have different chromosome compositions. Single cell sequencing analyses have revealed that about 75% of cleavage stage embryos are mosaic, meaning that these aneuploidies occurred as a result of errors during these initial cleavage divisions (Vanneste, Voet et al. 2009, Starostik, Sosina et al. 2020).

Unlike errors of meiotic origin, errors of mitotic origin are independent of maternal age. Preimplantation genetic screening analyses of approximately 28,000 day 3 embryos have shown that for women under 40 years of age, most of the errors observed were of mitotic origin (McCoy, Demko et al. 2015). These errors of mitotic origin seem to predominantly explain pregnancy loss before the blastocyst stage. This is based on the observation shown that mitotic errors were more prevalent in patients who had previous IVF failures, whereas meiotic errors were common in patients who had previous pregnancy losses (McCoy, Demko et al. 2015)

5.2.2. Segmental chromosomal abnormalities.

Microarrays analyses that looked at specific error types have shown that, unlike in meiosis where the errors observed were whole chromosomes gains or losses, errors of mitotic origin also included segmental chromosome abnormalities (Vanneste, Voet et al. 2009, Chavez, Loewke et al. 2012, Fragouli, Alfarawati et al. 2013, Starostik, Sosina et al. 2020).

These segmental chromosomal abnormalities can occur as a result of chromothripsis: a process where chromosomes present in a micronucleus undergo many breaks, giving rise to complex chromosomal rearrangements (Liu, Erez et al. 2011). Whether or not chromothripsis occurs in human embryos is not known but micronuclei have been observed in human and non-human primates (Chavez, Loewke et al. 2012, Daughtry, Rosenkrantz et al. 2019). Studies in humans and non-human primates have shown that some chromosomes can end up in cellular fragments in which higher levels of DNA damage were observed (Chavez, Loewke et al. 2012, Daughtry, Rosenkrantz et al. 2019). This would provide another explanation for these complex abnormalities.

5.2.3. What is the fate of aneuploid cells?

A drop in aneuploidies from 83% to 58% has been reported from the cleavage stage to the blastocyst stage. This was also accompanied with a decrease in the number of chromosomal errors observed within an embryo (Fragouli, Alfarawati et al. 2013). The decreased rates of complex aneuploidies observed suggests that these embryos have a reduced survival rate. However, the fact that more than half of the blastocysts were aneuploid shows that embryos are able to progress and survive despite some mosaicism. This was further proven by a study of 32 women who underwent frozen-thawed mosaic embryos transferred. This resulted in 9

pregnancies, including 4 miscarriages which is consistent with the pregnancy and miscarriage rates for their age group (the median age was 40.6) (Yang, Rito et al. 2021).

Interestingly, in mosaic embryos, a dramatic decrease in the proportion of aneuploid to euploid cells was observed after day 3 (during the transition from cleavage to blastocyst stage) (Fragouli, Alfarawati et al. 2013, Yang, Rito et al. 2021). An aneuploidy rate of 81.82% was detected at day 3, this dropped to 11.1% by day 4 and to 5.56% at day 7. Moreover, chromosomal analyses in an embryo that was miscarried during the first trimester, following implantation of a mosaic embryo, was chromosomally normal (Yang, Rito et al. 2021). Taken together, this data suggests that euploid cells have preferential survival rates and that a mechanism exists to remove some of these aneuploid cells.

Experiments in mice have shown that these aneuploid cells can be eliminated via apoptosis in the blastocyst (Lightfoot, Kouznetsova et al. 2006, Singla, Iwamoto-Stohl et al. 2020). However, less than 1% of mice embryos are aneuploid, which is in stark contrast to humans. Very recent experiments in gastruloids made from human embryonic stem cells stimulated with BMP4. The authors created aneuploidies in these stem cells by treating them with the Mps1 kinase inhibitor Reversine, which they mixed at a 1:1 ratio with euploid stem cells. The embryos had their euploid cell population enriched, following the apoptosis of aneuploid cells. Interestingly they also observed an increased level of the apoptosis marker CASP3 and of the DNA damage marker p53 in the embryonic germ layer of the gastruloids compared to the extra-embryonic layer. This suggests that apoptosis happens preferentially in the inner cell mass and that the trophectoderm, which is composed of more differentiated cells, is more resistant to aneuploidies (Yang, Rito et al. 2021). This also appears to be the case in human embryos as Nocodazole treated embryos displayed higher apoptosis rates (Jacobs, Van de Velde et al. 2017).

6. The first cleavage division in mammals

6.1. Parental genome separation and formation of a dual spindle

The first cleavage division is unique as it is the first time the maternal and paternal chromosomes will be segregated along one another. However, recent work in mice and bovines has challenged what was previously known about this division. It has been shown that two distinct mitotic spindles form independently and then fuse together to form a barrel-shaped spindle containing all of the embryo's genetic material. Growing microtubules start clustering around the pronuclei in prophase. After NEBD, two individual bipolar spindles start to form around each parental genome, the two spindles will then align and fuse together (Reichmann, Nijmeijer et al. 2018) (Schneider, de Ruijter-Villan et al., 2020).

Importantly, the maternal and paternal genomes remained spatially separated, with two distinct spindles forming. This was shown by live-cell imaging of mice embryos, resulting from a cross between two strains, allowing the maternal and paternal genomes to be differentially labelled (Reichmann, Nijmeijer et al. 2018). The dual spindle was also shown to be the cause of the parental genome separation observed in zygotes. Indeed, when the authors depolymerised the spindle using Monastrol and Nocodazole, then washed it out, one large bipolar spindle formed and the parental chromosome mixed.

Moreover, the observation that congression was not necessarily simultaneous until anaphase onset and that metaphase plates formed at different angles provides further evidence for the formation of two independently regulated spindles (Reichmann, Nijmeijer et al. 2018). In bovine zygotes, the two pronuclei

were broken down asynchronously, once again suggesting that during the early stages of mitosis, paternal genomes are governed by two distinct machineries (Schneider, de Ruijter-Villan et al., 2020). An image of a fixed human zygote with a dual spindle suggests that this is conserved in humans (Xu, Li et al. 2019). However, more research needs to be done to prove this.

This unique spindle formation during the first cleavage division is a potential cause of aneuploidy. It has been shown in mice that a failure to align the two spindles resulted in anaphase in different directions, leading to binucleated blastomeres. This experiment was done by increasing the distance between the 2PNs, causing the spindles to be too far from each other to align (Reichmann, Nijmeijer et al. 2018). Moreover, it has been reported in bovine zygotes that 38% of dual spindles failed to merge, causing parental genomes to be segregated independently (Brooks, Daughtry et al., 2020). I can hypothesise that failure to fuse both parental spindles could lead to daughter cells inheriting the genetic material of one parent only. Indeed, embryos with blastomeres containing the genomes of one parent known as uniparental genome segregation have been observed in cattle (Destouni, Zamani Esteki et al. 2016).

6.2. Spindle nucleation during the first cleavage division

Unlike in humans and bovines where the sperm brings two centrosomes, the first mitotic division happens without centrosomes in mice. In mice, during the first cleavage division, microtubules were shown to be nucleated at the chromosomes, with MTOCs only becoming associated with the microtubules later on (Reichmann, Nijmeijer et al. 2018). This raises the following question: how does spindle nucleation happen during the first cleavage division in species that have centrosomes? Furthermore, because the zygote only has two sperm-derived centrosomes but also has two distinct spindles, the idea that spindle nucleation happens in the same manner as in somatic cells has to be ruled out.

Live cell imaging of bovine zygotes revealed that centrosomes were not located at opposite sides of the pronuclei. Two spherical or monopolar spindles form around parental genomes, and it is only later during mitotic progression that the centrosomes became incorporated into the spindles. In the majority of zygotes, the two aligned and fused spindles will eventually have a centrosome at each pole. However, errors in centrosome localisation, including localisation to the same pole or to the midzone, were not corrected. In some zygotes, spindles remained far apart and failed to align, with each spindle having a centrosome associated with one of their poles in most cases. Anaphase onset still took place in these distant spindles and the spindle morphology was not too dissimilar to fused spindles. This suggests that centrosomes are not essential in establishing a spindle or triggering mitotic exit in the first cleavage division (Schneider, de Ruijter-Villan et al., 2020).

6.3. Elongated mitosis I timings

The elongated timing of the first cleavage division sets it apart from other embryonic divisions. In mice, it lasts about 120 mins, which is almost twice as long as the second cleavage division which lasts about 70 mins (Sikora-Polaczek, Hupalowska et al. 2006). This also appears to be the case in human embryos. In ICSI embryos, where the oocyte was fertilised by injecting a spermatozoon, NEBD took place about 24h after ICSI (Minasi, Colasante et al. 2016) and 2 cells formed about 27h after ICSI (Kirkegaard, Ahlstrom et al. 2015). This indicates that the timing from NEBD to the 2 cell stage is around 3h in human zygotes, with NEBD to cytokinesis lasting 2.7 hours and cytokinesis 20 mins (Vera-Rodriguez, Chavez et al. 2015). This is over 3 times as long as mitosis in human somatic cells, where it takes under 1h (Spira, Cuylen-Haering et al. 2017).

The mechanisms behind this have not been studied in humans but experiments in mice zygotes have shown that it is only partly due to the SAC. It could also be due to the atypical spindle nucleation and fusion that happens during the first mitotic division. The APC/C regulates mitotic progression by degrading cell cycle proteins (see part 2.2.6). A delay in cyclin A2 and cyclin B1 degradation was observed in mice zygotes. Indeed, cyclin A2 was only degraded over 30 mins after NEBD compared to somatic cells and in embryos at the 2 cells cell stage or after in which it happens at the time of NEBD, with a similar delay in cyclin B1 also observed. This could be due to a delay in APC/C activation. As the SAC inhibits cyclin B1 degradation in somatic cells and slows down cyclin A2 degradation, the authors inhibited the SAC using the Mps1 inhibitor reversine. They observed an acceleration in the degradation of both cyclins by 10 to 20 mins, which was not enough to account for the 30+ min delay observed (Ajduk, Strauss et al. 2017). The SAC provides part of the answer of the mechanism behind the elongated mitosis I timings but there are also other factors contributing to this. Moreover, the spindle assembly checkpoint proteins Mad2 and RSN (CLIP-170 in humans) disappeared from the kinetochores even before the metaphase plate formed. This suggests that the SAC cannot be responsible for the increase in metaphase timings observed in the first cleavage division in mice (Sikora-Polaczek, Hupalowska et al. 2006). The protein Plk1 could also be a potential player in this delayed degradation of cyclin A2 and B1 as overexpression in mice zygotes accelerated the degradation of the two cyclins (Ajduk, Strauss et al. 2017). The role of the SAC zygotes remains very enigmatic in mice and, to our knowledge, has not been looked into in human embryos.

After this initial division, data in mice embryos has shown that the SAC exhibits different levels of activity at different stages of embryonic development. Indeed, challenging mitotic spindle polymerisation using low dose nocodazole treatment increased mitotic timings during the second mitotic division and in the blastocysts but not at the 4 to 8 cell stage or in the morulae (Vazquez-Diez, Paim et al. 2019).

At the morula stage, most embryos had fully aligned chromosomes, but anaphase onset happened regardless in embryos which did not. It appears that the SAC is not as efficient as in somatic cells. However, it is still active, as shown when inhibiting the SAC protein Mad2 and MPS1, which resulted in higher rates of aneuploidies and micronuclei (Bolton, Graham et al. 2016, Vazquez-Diez, Paim et al. 2019). The authors have also showed that severe spindle damage will lead to mitotic arrest, by treating embryos with high doses of Nocodazole. This arrest was accompanied by Mad2 localisation at the kinetochores, suggesting that this was driven by the SAC (Vazquez-Diez, Paim et al. 2019).

In conclusion, the SAC's activity remains elusive during the first mitotic division; it is active during the second division but it appears to be weak from the 4 cell stage to the morula stage in mice embryos. Indeed, severe chromosome alignment errors results in a mitotic arrest but a few unaligned chromosomes failed to cause an arrest. It was hypothesised that this could be due to mismatched stoichiometry between the APC/C and the SAC (Vazquez-Diez, Paim et al. 2019).

However, this could be different in human embryos as, unlike mice, they display extremely high error rates and therefore the SAC might therefore be working differently. To our knowledge, this has not been looked at in zygotes and cleavage stage embryos. Some experiments have been done, treating preimplantation embryos (day 3 to 7) with Nocodazole, then fixing them, in an attempt to elucidate the SAC's role in these embryonic divisions. The authors have observed a higher proportion of cells at the metaphase stage in treated embryos compared to the control group, suggesting that the SAC does could cause a delay in metaphase. Moreover, the Nocodazole-treated embryos showed high levels of apoptosis compared to the control, but only from day 5 onwards, suggesting that any eventual aneuploid cells formed as a result of Nocodazole treatment could carry on surviving until then (Jacobs, Van de Velde et al. 2017). Chromosome

movements and localisation of SAC proteins need to be looked at in human embryos in order to gain insight into the SAC's activity.

7. In vitro fertilisation (assisted reproductive therapy): provides access to eggs and embryos for research.

In order to study the first cleavage divisions of human embryos, I have been using oocytes and embryos that were donated to research following *In vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) treatment (see material and methods section).

In vitro fertilisation (IVF) is an assisted reproductive procedure used by individuals experiencing infertility.

After years of research in animals (Edwards 1965), then in humans (Edwards, Bavister et al. 1969), honing in on the culture media compositions, maturation conditions and studying the morphology and timings of both oocyte and spermatozoa; the first IVF baby was born in 1978 (Steptoe and Edwards 1978)! IVF is life-changing for many people who would not otherwise be able to conceive. 54,000 patients underwent IVF in the UK in 2018, resulting in live births for 23% of the under 43-year-old population, following embryo transfer (HFEA, 2020).

At the start of IVF treatment, the growth and maturation of several follicles concurrently is induced by administering FSH to the patient. This maintains FSH and LH levels above a certain threshold, stimulating follicle development. This can be accompanied by a GnRH agonist or antagonist to prevent a premature surge in LH. The final oocyte maturation steps and ovulation are triggered by an injection of a GnRH agonist and/or human chorionic gonadotrophin (hCG) (Alper and Fauser 2017). HCG mimics the effects of the LH surge in the follicle. Just before ovulation,

about 36 hours after the hCG dose triggers oocyte maturation, the oocytes are retrieved from the patient via surgical aspiration of the follicles. The sperm sample is also prepared, removing the seminal fluid. During IVF, the oocyte within its cumulus cells, and spermatozoa are cultured together in the same dish, allowing sperm entry to happen. If the sperm quality is judged as insufficient, due for example to low sperm numbers or motility, Intracytoplasmic sperm injection (ICSI) treatment can be used instead. During ICSI treatment, a sperm is directly injected into a mature oocyte (at metaphase II stage) using a micromanipulator. (Fig. 5). As mentioned previously, sperm entry catalyses meiosis resumption and the extrusion of a second polar body. After sperm entry, its DNA is decondensed and 2 pronuclei (PNs) form, one with maternal DNA and one with paternal DNA. These 2 PNs are what embryologists look for to classify the oocyte as fertilised.

The embryos are cultured in the embryology lab, until they reach the blastocyst stage around day 5 after oocyte collection. Their development is monitored throughout. A blastocyst is transferred back into the patient where it can implant and give rise to pregnancy. If there is more than one healthy blastocyst, those not transferred can be cryopreserved and used by the patient for a later treatment.

When more than one blastocyst is formed, the embryology team has the difficult task of assessing which blastocyst has the best chances of forming a healthy baby and should therefore be transferred back to the patient. Morphological characteristics such as blastomere nucleation, cellular fragmentation and cell number are traditionally used to assess blastocyst quality (Cruz, Garrido et al. 2012). The timings of the first cleavage divisions can be used to assess whether or not a successful blastocyst will form. Indeed, the timings of the first cytokinesis, timings from 2 cell to 3 cells and the synchronicity of the second division have been shown to predict blastocyst quality (Wong, Loewke et al. 2010, Chavez, Loewke et al. 2012, Cruz, Garrido et al. 2012).

Increasing our knowledge of aneuploidies occurring in the sperm, oocyte and embryo thanks to research could help predict embryo quality, increasing the chances of success of IVF and ICSI treatment.

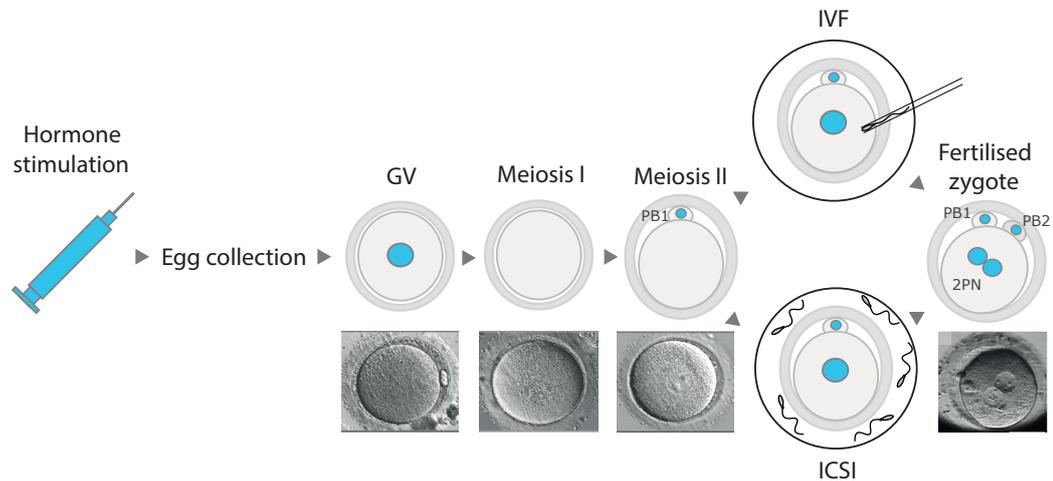


Figure 5: Overview of IVF and ICSI treatment. The patient undergoes hormone stimulation to produce plenty of oocytes, which are then collected. The oocytes mature in the embryology lab from germinal vesicle (GV), characterised by a nucleus and no polar bodies, to meiosis I, at the end of which a first polar body, the oocyte will then undergo meiosis II. The oocyte is then fertilised by sperm injection (ICSI) or by an oocyte and sperm being placed in the same dish (IVF). Sperm entry will result in the resumption of meiosis, leading to the extrusion of a second polar body and the formation of two pronuclei (PN). The images of the oocytes (Schnauffer 2019) and zygotes (Araki et al., 2018) were imaged with an embryoscope

8. Aims of this thesis

Seventy five percent of cleavage stage embryos were reported to be mosaic, meaning that they contain karyotypically different cell lineages (Vanneste, Voet et al. 2009, Starostik, Sosina et al. 2020). This indicates that these embryos segregate their chromosomes incorrectly during one or several of their cleavage divisions, leading to aneuploidies. Although aneuploid embryos can be viable, complex aneuploidies have been associated with poor embryonic development, infertility

and pregnancy loss (Fragouli, Alfarawati et al. 2013, McCoy 2017, Kahraman, Cetinkaya et al. 2020). It is therefore crucial to understand the mechanisms causing aneuploidies in human embryos.

In this project, I aim to set up, for the first time, live cell imaging in human embryos to follow chromosome movements during these early mitotic divisions. Using these live cell imaging methods, I aim to uncover the nature of these errors as well as during which divisions they occur. I also aim to investigate whether or not there are mechanisms in place in human zygotes to prevent aneuploidies from occurring. In somatic cells, the SAC causes a delay in metaphase until all the chromosomes are aligned on the metaphase plate in human somatic cells (Stukenberg and Burke 2015, Kataria and Yamano 2019, Kahraman, Cetinkaya et al. 2020). I aim to investigate whether or not the SAC is active in the early cleavage divisions of human embryos.

Chapter 2: Materials and Methods

1. Sources of Human Embryos

1.1. Human embryos donated to research

This research project (04/Q2802/26) was approved by the NHS Research Ethics Committee under the Human Fertilisation and Embryology Authority (HFEA) research licence RO155 (see Appendix C). Patients undergoing in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) treatment gave informed consent at the Centre for Reproductive Medicine (CRM), University Hospitals Coventry and Warwickshire (UHCW) NHS trust (for consent forms and information, see Appendix C). Oocytes and embryos which could not be used for the consenting patients' ICSI or IVF treatments and would otherwise be discarded were donated to research. These included immature oocytes which would not be injected with sperm for ICSI treatment. These oocytes would be at the germinal vesicle (GV) or meiosis I (MI) stage and are recognisable as they do not have a polar body on their surface. GV oocytes are identified by their large central nucleus containing usually a single or occasionally two prominent nucleoli. MI oocytes lack any visible nucleus and are assumed to be at MI because of the lack of a polar body. These are donated to research on the day of egg collection: day 0 (D0), after the embryologists check which oocytes are mature and can be used for patient's ICSI treatment (MII) and which oocytes are immature and can be donated to research (GV and MI). I also received oocytes on day 1 (D1), which had been exposed to sperm, as part of ICSI or IVF treatment, but which were not fertilised ~17 hours after insemination. Unfertilised oocytes from ICSI cycles are at the MII stage, recognised by the absence of pronuclei (PN), and in the presence of one polar body on their surface. These can potentially become fertilised at a later time (delayed

fertilisation), giving rise to an embryo. Unfertilised oocytes from IVF cycles may also include oocytes arrested at GV and MI stages, because oocytes are not assessed for maturity before insemination by IVF. On day 1, some embryos have PN numbers that differ from the expected 2PNs. These typically display 1PN or >2PNs. 1PN embryos may be parthenogenetic (containing oocyte-derived chromosomes only), normally fertilised 2PN where one pronucleus has either entered syngamy already or where one pronucleus has merged into the other, or displaying an anomaly. Where more than two pronuclei are visible, the oocyte is abnormally fertilised due to the presence of an extra set of sperm or egg chromosomes.

The clinical embryologists at the CRM are responsible for assessing which oocytes and embryos should be donated to research and checking the consent forms, with another member of the embryology team witnessing their removal from the clinical pathway. To acquire material, a member of the research team attended CRM, checked the consenting documents again and signed, dated and recorded on the patient's notes that which was taken for research. They then assigned an anonymised code to each patient (recorded in a separate folder which stays at the CRM). This anonymised code is then used in the research notes and data, maintaining patient confidentiality. A full record of all the oocytes and embryos used for this study can be found in tables 1-6.

1.2. Egg sharer program

The egg sharing to research project (19/WM/003) was approved by the NHS Research Ethics Committee under HFEA research licence RO155. Patients wishing to undergo IVF or ICSI treatment, who are no more than 35 year of age and who have a good chance of producing at least 10 eggs with no history of poor fertilisation or embryo quality can voluntarily choose to be part of the egg sharing program. In this program, their treatment is provided for a reduced price and they

donate half of their freshly collected eggs to research while the other half are used for their treatment. In the event of an odd number of eggs produced, the extra egg is used for the patient's treatment (Patient information sheet for Egg sharing, see Appendix C). This program provided a source of freshly collected, mature oocytes from young women, which were likely to represent normal oocytes as a control group for comparison with oocytes that were deselected from clinical treatment. Some MII eggs donated to research were injected with donor sperm by ICSI during the course of the research to create embryos for research use. The sperm donor had previously given informed consent (for donor sperm consent form and information, see Appendix C) and his sperm sample was stored in liquid nitrogen for up to 10 years.

During clinical treatment, female patients had received ovarian stimulation to promote the production of multiple follicles through exposure to elevated levels of follicle stimulating hormone (FSH). Oocyte maturation was triggered 36 hours before egg collection by an injection of human chorionic gonadotrophin (hCG). Oocytes were then collected surgically via needle aspiration under transvaginal ultrasound guidance. Within the clinical laboratory, oocytes and embryos were cultured at 37°C in individual drops of commercially available culture media (Fert then Cleav media, Origio) under mineral oil and under an atmosphere of 6% CO₂, 5% O₂ and 89% N₂ for up to 6 days. Embryos were selected for transfer to the patient and/or cryopreservation during this period by the clinical embryologists, with deselected embryos becoming available for research use in accordance with patient consent.

1.3. Access to embryoscope data

Patients having ICSI or IVF treatment can opt to have their embryos imaged using an Embryoscope™ incubator (ES-D model, Vitrolife) which provides time lapse images collected at 7 planes through each embryo. The embryos were imaged

using Hoffman modulation contrast optics every 10 mins for up to 6 days, allowing the embryos' development to be closely monitored (this is used for clinical decision making). In this study, I analysed Embryoscope™ images collected as part of ICSI treatment from 330 embryos. The selection and anonymisation of the embryoscope movies was carried out by Geraldine Hartshorne at the CRM. The image sets collected were those where the individual embryo's fate was known. These embryos comprised four different categories: transferred pregnant (fetal heart beat recorded), transferred not pregnant, not transferred normal cytokinesis (first two cell divisions giving rise to 2 cells and then 4 cells) and not transferred abnormal cytokinesis (the zygote divided into 3 or more cells instead of the expected 2).

The anonymised Embryoscope™ movies were analysed, recording the time of NEB, start of cytokinesis formation and finally the formation of 2 or more distinct cells. From these events, the timings from NEB to cytokinesis and the cytokinesis timings were calculated.

2. Visualising chromosomes: staining with SiR-DNA

Oocytes and embryos donated to research were collected by the research team after the clinical embryology team had selected the oocytes/embryos to be used for the patient and those which could be donated to research. Research embryos were placed in a drop of Cleav™ media (Origio), which is a medium designed to support early embryo development and which is also used for clinical embryos. During initial experiments to set up live embryo imaging with SiR-DNA, the zona pellucida (glycoprotein layer surrounding the oocyte/embryo) was perforated using a Saturn 5 Laser (CooperSurgical, Denmark), to allow the SiR-DNA to enter the oocyte/embryo. For all of the embryos presented in this thesis, the zona

pellucida (glycoprotein layer surrounding the oocyte/embryo) was removed by placing the embryo in a drop of acid Tyrode's solution (Sigma) and pipetted up and down until the zona pellucida loosened around the oocyte/embryo and finally come off. They were immediately placed back into CleavTM media drop to remove any residual acid Tyrode's solution. The handling of oocytes and embryos was performed manually using an RI EZ-Tip handling pipette (135 micron diameter) (CooperSurgical, Denmark). Embryos were then transferred to a Fluorodish (WPI) containing a 5 μ l drop of 2 μ M SiR-DNA (Spirochrome), diluted in CleavTM media covered with mineral oil. The dish was previously pre-warmed at 37°C and the embryos transported in a portable incubator (K Systems) at 37°C, about 14 km from the UHCW CRM to Warwick Medical School (WMS).

3. Small molecule inhibitors: reversine treatment

To test if the SAC was active during the first cleavage divisions of human embryos, the small molecule inhibitor reversine, which inhibits the SAC kinase Mps1, was used. Mps1 was originally established as an Aurora B inhibitor and later found to inhibit Mps1 10-20 folds more efficiently (Hiruma, Koch et al. 2016). Reversine inhibits the Mps1 kinase by binding to its ATP binding pocket, preventing ATP binding. Mps1 is therefore inactive, unable to catalyse the transfer of a phosphate group from ATP to a target protein, inhibited SAC signalling (Hiruma, Koch et al. 2016).

The zona pellucida was removed as described above (see section 2.1.). The embryos were randomly split into two groups: one control group treated with DMSO and one group treated with reversine. Embryos assigned to the reversine group were placed in a CleavTM media drop in a Fluorodish containing 2 μ M SiR-DNA diluted (as in section 2.1.) with 1 or 10 μ M reversine (Sigma-Aldrich) diluted

in DMSO. Embryos assigned to the control group were placed in a drop with an equivalent concentration of DMSO in the place of reversine, in the same volume.

4. Live cell imaging

4.1. Long term imaging with a widefield microscope

The oocytes and embryos were imaged immediately upon arrival at the Centre for Mechanochemical Cell Biology (CMCB, Warwick Medical School) with a DeltaVision Elite microscope (Applied Precision, LLC) equipped with a CoolSNAP HQ camera (Roper Scientific) using a 40x oil-immersion 1.3 NA objective (Olympus). The following image stacks: 60 x 1.5 μm optical sections, 1 x 1 binning were acquired every 10 or 15 mins for 24 to 48 hours. Brightfield images were acquired with a laser attenuated to 10% with an exposure time of 0.1s. Fluorescent images were acquired using an InsightSSI solid state illuminator (Applied Precision, LLC) attenuated to 32% and a Cy5 filter set with an exposure time of 0.05s. The temperature was maintained at 37°C and 5% CO₂ using a stage-top incubator (INU; Tokai Hit) stabilised with a microscope enclosure (Weather station, Precision Control) maintained at 37°C. A calibrated probe (Fluke 52) was used to check the temperature. The imaging data was analysed by hand using OMERO (Open Microscopy Environment).

4.2. Long term imaging with a spinning disk confocal microscope

The oocytes and embryos were imaged immediately upon arrival using the Marianas spinning disk confocal from 3i (Intelligent Imaging Innovations) equipped with 2x Photometrics 95B Prime sCMOS cameras, using a 40x oil-immersion 1.46, alphaPlanApo (Zeiss) objective. I also took the following image

stacks: 60 x 1.5 μm optical sections, 1 x 1 binning every 15 mins for about 48 hours. Brightfield images were acquired attenuated to 10% with an exposure time of 40ms. Fluorescent images were acquired with a laser attenuated to 5% with an exposure time of 50ms. The temperature was maintained at 37°C and the CO₂ at 5% using a stage top incubator (Okolab). A calibrated probe (Fluke 52) was used to check the temperature. The imaging data was analysed by hand using the Slidebook software.

4.3. Data plotting and statistics.

Calculation of the timings of the different mitotic stages was conducted by looking at the time of nuclear envelope breakdown (NEBD), the formation of a metaphase plate, anaphase onset, cytokinesis furrow ingression and the formation of 2 or more daughter cells. Histograms were plotted using Microsoft Excel (version 16.46) and box and whisker plots using R studios (R 4.1.0 GUI 1.76 High Sierra build). All figures were created using Adobe Illustrator 2020. The medians were compared using Mann-Whitney U tests (Fig. 14, 16), which is used to compare two independent groups which are not normally distributed. To check if the data was normally distributed, Anderson-Darling tests were performed. When comparing error rates in different populations, the p values were calculated with Fisher's exact tests, which are used to find out if there are non-random associations between two nominal variables (Fig. 13, 15, 17). All statistical tests were performed using MATLAB R2020A (Mathworks) inbuilt functions.

Chapter 3: The first cleavage divisions of human embryos are error-prone

1. Reasoning

The first mitoses of human embryos remain enigmatic because chromosome movements have never previously been imaged in live human embryos. However, preimplantation genetic screening and single cell analyses of human embryos have revealed that 75% of preimplantation embryos are mosaic, meaning that they had chromosome segregation error at some point during their mitotic divisions (see Chapter 1.4) (Vanneste, Voet et al. 2009, Chavez, Loewke et al. 2012, Fragouli, Alfarawati et al. 2013, Vera-Rodriguez, Chavez et al. 2015, Starostik, Sosina et al. 2020). Studies on fixed embryos do not tell the complete story as they represent a snippet in time from which I can only infer how different observed chromosome segregation errors lead to different aneuploidy types. I therefore aimed to establish live imaging in human embryos to gain insight into the origins of chromosome segregation errors in the early cleavage divisions.

2. Challenges associated with working with live human zygotes

Whilst fresh human zygotes are invaluable as they provide us with a window into the human early embryogenesis, working with such precious material has its own challenges. The main limitation encountered is the scarcity of the material. This makes optimising experiments very tricky as every oocyte or embryo is very precious and cannot be wasted on an experiment that is not going to work. Moreover, this makes collecting enough data a complex and slow process. Indeed, the number of oocytes or embryos available for research is very unpredictable.

The material used for this project include mature (MII) oocytes which have been in contact with sperm and mis-fertilised zygotes (1PN or 3 or more PNs), see material and methods. The number of oocyte or embryos available for research therefore depends on the number of patients that had egg collection the previous day and then out of these patients the number of them which gave consent to research, the number of follicles produced and how well the patient's oocytes fertilised. This means that some weeks, I could have 0, 1 or over 10 embryos available for research. Establishing a clear pipeline of experiments and knowing what to do with oocytes and embryos at different stages allowed me to make the most out of every embryo available. Moreover, good communication with the embryology team, as well as always being ready to travel to the hospital to collect even just a single embryo was crucial to the success of this project.

Another major challenge comes from transporting the oocytes or embryos back from the centre for reproductive medicine (CRM) at UHCW, which is a 25 min drive away from the lab at Warwick university. This required a portable incubator set at 37°C. The temperature of the portable incubator, the temperature and CO₂ concentration of the incubator at the CRM and of the microscopes at Warwick university were carefully checked to make sure the embryos were always in optimal conditions and that I was not wasting any material. Comparing mitotic timings of our research embryos to what was already published allowed me to ensure that the transport and imaging conditions used were not negatively impacting the embryos.

3. Establishing live cell imaging in human oocytes and embryos

To stain the chromosomes of live human oocytes or embryos, I used the cell permeable dye Silicon Rhodamine (SiR)-DNA (Lukinavicius, Blaukopf et al. 2015).

In an initial series of experiments, I simply placed an oocyte or embryo in a media drop containing SiR-DNA, similar to the established method for somatic cells. This was unsuccessful. Our hypothesis was that the dye could not diffuse through the zona pellucida, the proteinaceous membrane surrounding the oocyte/embryo. I therefore tried different techniques to breach the zona to allow the dye to enter the oocytes/embryos. Perforating the zona pellucida using a laser was only successful when the hole was close to the area where the chromosomes happened to be. The oocyte/zygote is 110-120 μm in diameter, excluding the zona pellucida, and the chromosomes in MII are peripheral. This method did not therefore result in reliable DNA staining of every oocyte/embryo. Indeed SiR-DNA entering from the hole in the zona would have to diffuse through up to 110-120 μm to reach the small spindle (Nazari, Khalili et al. 2011). To our knowledge, the mitotic spindle has not been measured in human zygotes. In mice zygotes, the spindle is 24 μm long in a ~ 70 μm diameter zygote (Courtois, Schuh et al. 2012), highlighting the challenge of staining the spindle as SiR-DNA will have to travel through the large cytoplasmic volume before reaching the chromosomes. I therefore switched to removing the zona pellucida entirely, using acid Tyrode's. The zona-free oocytes and embryos were then placed in a media drop containing SiR-DNA. This successfully allowed visualisation of chromosomes within an oocyte or embryo (Fig. 6a). Moreover, I was able to acquire long term (24-48 hrs) time lapse image stacks (60 x 1.5 μm z-section every 15 mins) using a widefield microscope. This allowed visualisation of chromosome movements during the first and sometimes second cleavage divisions of the zygote. I term these divisions Mitosis I and Mitosis II respectively. (Fig. 6a).

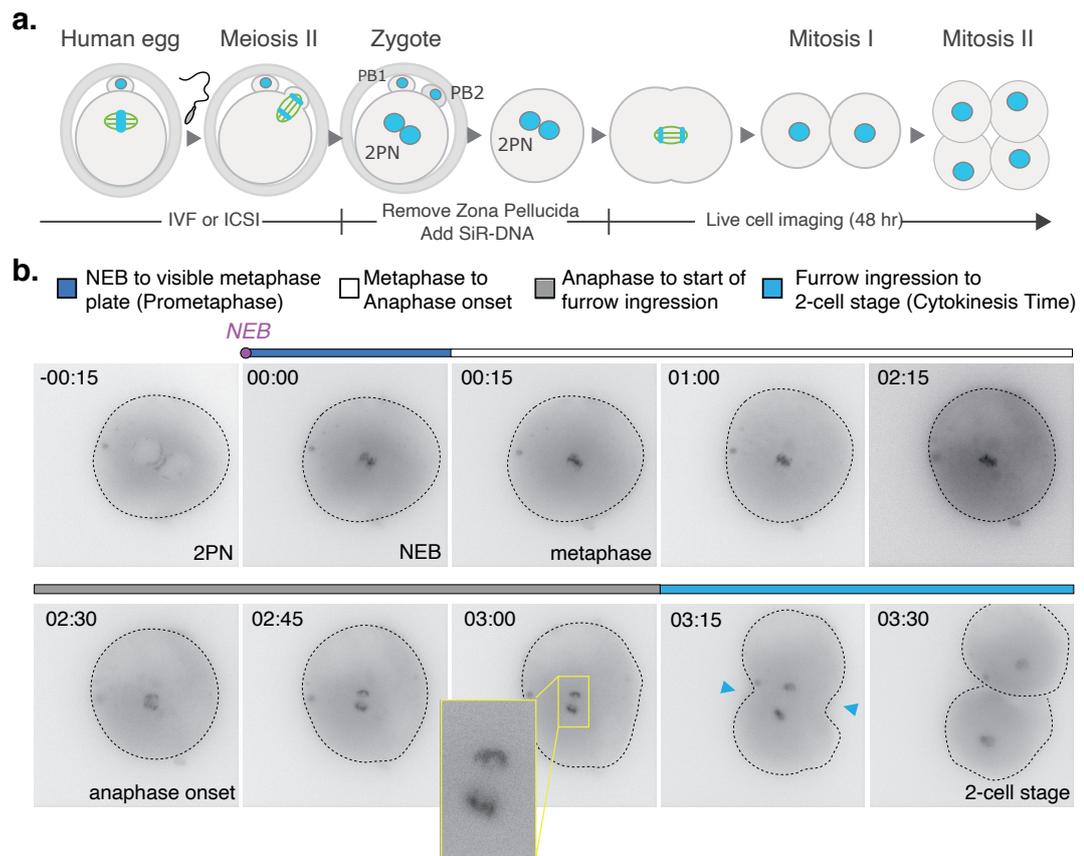


Figure 6: Live cell imaging of the first mitotic division in live human deselected embryos. (a) schematic outlining important steps in embryo preparation for live cell imaging of human zygotes. (b) Time lapse imaging of the first mitotic division, the chromosomes were stained using SiR-DNA (embryo 3083vii, see table 1). Time in hours:mins. Blue arrows indicate the onset of cleavage furrow ingression. The top bar shows the different mitotic phases measured in our dataset: prometaphase, metaphase, anaphase onset to start of furrow ingression and cytokinesis.

4. The first mitotic division of human zygotes takes several hours

These live cell imaging experiments allowed quantification of the durations of the different mitotic stages: The time of nuclear envelope breakdown (NEB) was denoted as T=0 mins. From this I could measure the time to formation of a

metaphase plate (this phase involves formation of the bipolar mitotic spindle, capture of chromosomes and their congression to the spindle equator – I term this “prometaphase time”). The time from this point to anaphase onset was considered “metaphase time” with the combination of prometaphase and metaphase time giving the NEB to Anaphase onset time. The beginning of cytokinesis was marked when the cleavage furrow first started to ingress and I also scored the time at which two daughter cells formed (Fig. 6b). The duration between these two events was termed “cytokinesis time”. I imaged a total of 38 deselected embryos undergoing mitosis I on a widefield microscope (Appendix B, table 1).

The prometaphase time in human embryos during mitosis I was 75 ± 75 mins (n=21) and the metaphase time was 60 ± 70 mins (n=27; Fig. 7, 8). When measuring median timings of each stage, I only included embryos in which the entirety of the stage was imaged to ensure an accurate measurement of the duration of each stage. This resulted in the removal of a number of data points because some had progressed during collection and transport from the clinical facility. I imaged 38 deselected human embryos (Appendix B, table 1), NEB was not visible in 17 embryos (the event had already happened when the image acquisition started), so a complete prometaphase was imaged in 21 embryos (38-17). The timings of all 38 embryos are shown in Figure 7, with the pink and black dots indicating whether NEB was imaged. A complete metaphase was imaged in 27 embryos. The median time of metaphase + prometaphase was 135 ± 30 min (2 hours 15 minutes), n=21, which is significantly longer than the ~24 mins measured in immortalised human somatic cells (Silio, McAinsh et al. 2015) or ~35 mins in cancer cells (Meraldi, Draviam et al. 2004). These elongated prometaphase and metaphase timings during the first mitotic division could potentially be explained by inefficient spindle assembly and chromosome capture and biorientation. Moreover, the SAC could cause a mitotic delay in response to unaligned chromosomes, which would cause elongated metaphase timings. However, when plotting prometaphase timings compared to metaphase timings, I observed that

they were inversely proportional (Fig. 9). Therefore, it seems that it is an intrinsic timer which sets the combined duration of these two phases, not the SAC.

The time from anaphase onset to the start of furrow ingression and then to the formation of separate daughter cells was also extended compared to somatic cells. Each of these 2 phases lasted 30 minutes (median time of 30 ± 30 minutes), $n=36$ and $n=38$ respectively. In human somatic cells the combination of both phases lasts ~ 7 min (Spira, Cuylen-Haering et al. 2017). Taken together, the first mitotic division in human embryos lasted 3.5 hours ± 63.8 minutes in embryos which were imaged all the way from NEB to the formation of 2 cells ($n=20$). When assessing the entire set of mitosis I data (including those embryos where NEB was not visualised because it had already happened), the median total time was 3 hours 7.5 ± 128.75 minutes ($n=38$) but this is an underestimation as only part of prometaphase was imaged in some embryos as NEB had already taken place when I started imaging. The mitosis I of human embryos is much longer than the 2 hours observed in mouse embryos (Sikora-Polaczek, Hupalowska et al. 2006) and about 5 times longer than the 37 minutes observed in somatic cells (Spira, Cuylen-Haering et al. 2017). It is important to note that, there is a lot of variability from embryo to embryo when it comes to mitotic timings, hence the high inter quartile ranges (Fig. 7, 8).

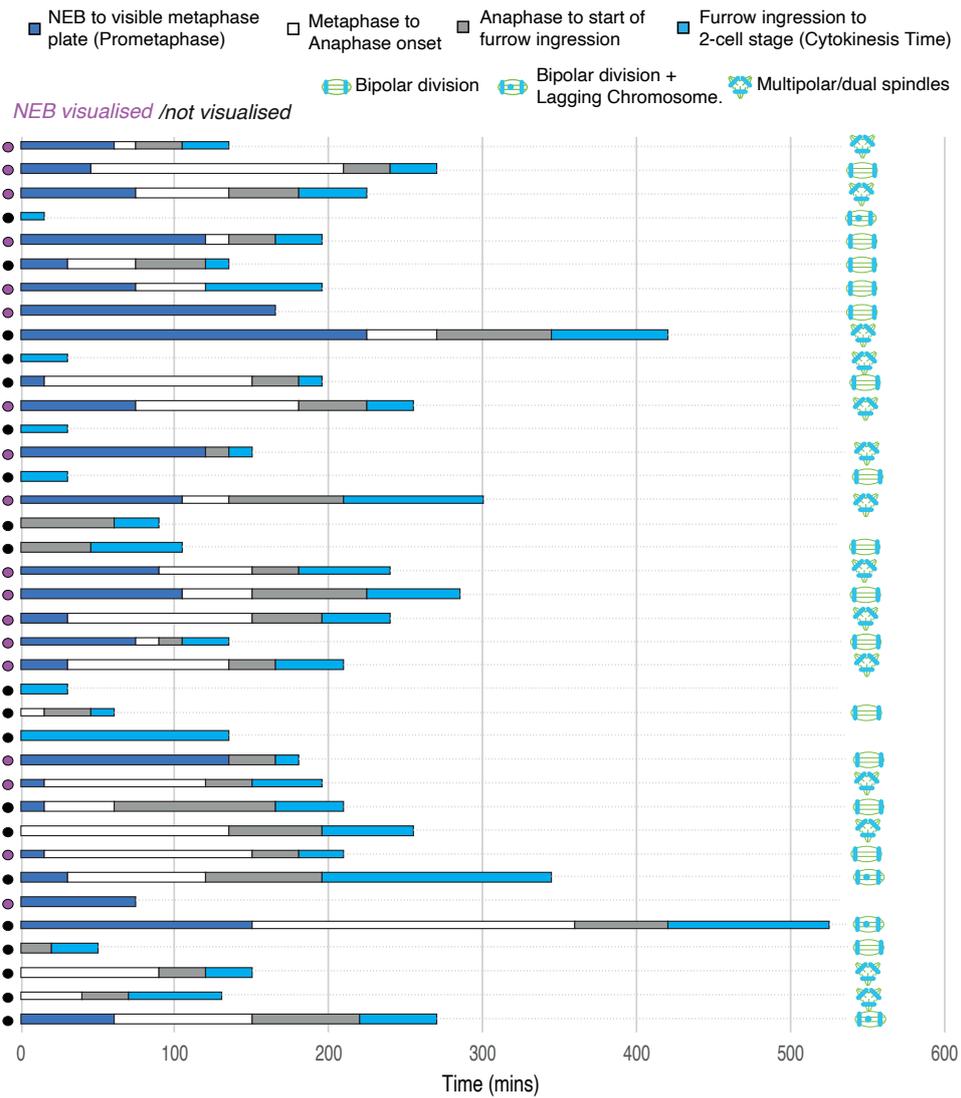


Figure 7: History plot of embryos imaged undergoing mitosis I using a widefield microscope. The chromosomes were stained with SiR-DNA. The different coloured bars of the histogram show the timing of each mitotic stage and cartoons indicate the phenotypes observed. The phenotype is not indicated for some of the bars as the quality of the staining and/or the orientation of the zygote did not allow to accurately tell the phenotype. Pink/black dots indicate whether NEB was imaged.

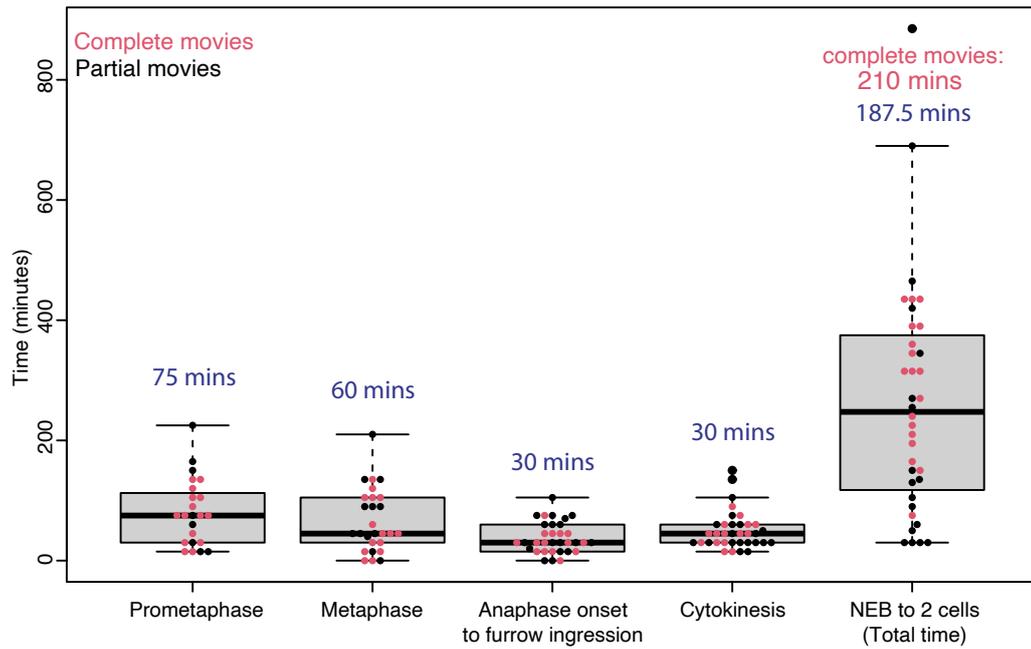


Figure 8: Quantification of each mitotic stage during mitosis I based on live cell imaging of deselected human embryos with a widefield microscope. The median times for each stage are indicated above each bar and the black/red dots indicates movies in which NEB was imaged.

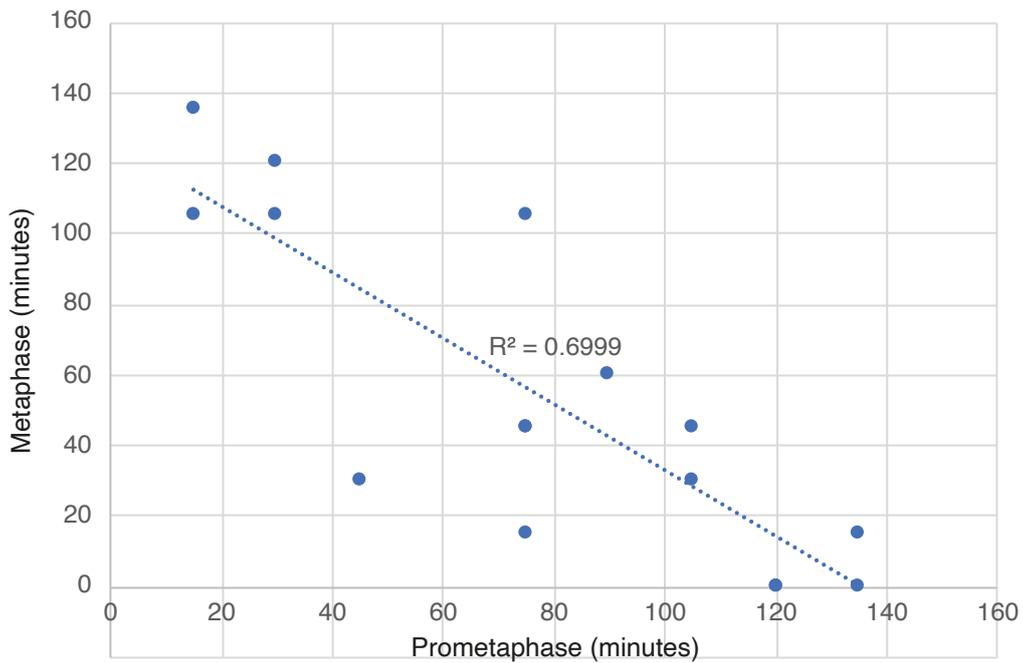


Figure 9: Correlation of prometaphase and metaphase timings in embryos where these stages were filmed in their entirety, $R^2 = 0.699$.

5. Mitosis I is very error-prone

Imaging chromosome segregation in live human embryos allowed us to study chromosome segregation errors during mitosis I and how these can lead to aneuploidies.

5.1. Failure to assemble a bipolar spindle

At fertilisation, maternal and paternal chromosomes segregate alongside each other for the first time. A spindle has to form and capture both sets of parental chromosomes which came from two different pronuclei. Forming this bipolar spindle during the first mitotic division appears to be a very error-prone process. Indeed, 39.5% of human zygotes (n=38) failed to form a bipolar spindle, multipolar metaphase plates were observed instead (Fig. 10). Multipolar spindles are known to occur as a result of fertilisation by two spermatozoa, creating a zygote with 3 pronuclei (Kai, Kawano et al. 2021). Indeed, all the 3PN zygotes which divided with a multipolar spindle were IVF embryos, meaning that they could have been fertilised by two spermatozoa. However, 2 out the 8 embryos which divided with multipolar spindles and in which I imaged NEB (25%) started as normally fertilised 2PN zygotes, showing that multipolar chromosome segregation can arise in normally fertilised zygotes. At anaphase, these multipolar spindles segregated the chromosomes into more than 2 DNA masses (Fig. 10a,b). This process was, however, poorly coupled with cytokinesis, giving rise to embryos with cells with varying numbers of DNA masses, including daughter cells with multiple nuclei (Fig. 10) and sometimes cells with no nuclei, which were effectively large cytoplasmic fragments. 61.5% of embryos with multipolar spindles divided into 2 cell embryos and the remaining 38.5% divided into 3 or more cells (n=13). From my work, it is clear that multipolar spindles create aneuploidies in human embryos by creating cells with varying numbers of DNA masses and multiple nuclei.

Moreover, it appears that mitotic I spindle nucleation happens in a way that is unconventional in mammalian embryos. Indeed, experiments in cattle and mice have shown that, during the first mitotic division, two individual spindles form and then fuse together before anaphase onset (Chapter 1.5.1). Whether or not this is the case in humans is uncertain. In these experiments, the spindle was not stained so the mechanisms of spindle assembly and fusion cannot be directly visualised in our data. The position of chromosomes, however, is enough in some divisions to point towards the formation of two distinct spindles. Some of the multipolar spindles that were imaged looked like two separate spindles. The embryo presented in Fig. 11a is a clear example of this as it displays spindles which remained spatially separated the entire time and which underwent anaphase onset at different times, giving rise to two main DNA masses each and therefore four nuclei. Fig. 11b is another example of what could be two spindles which fail to align and fuse. Indeed, the two metaphase plates appear to be at a 90° angle from each other. Once again, anaphase results in the formation of several DNA

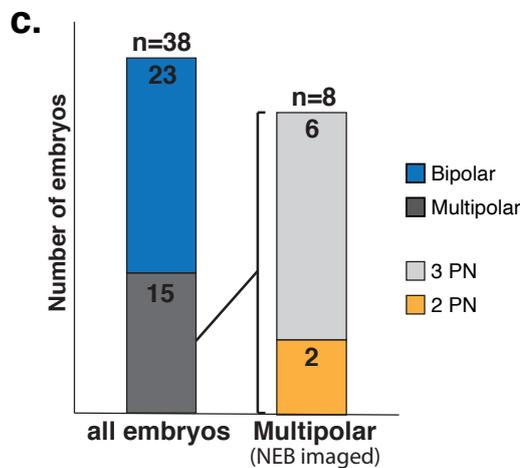
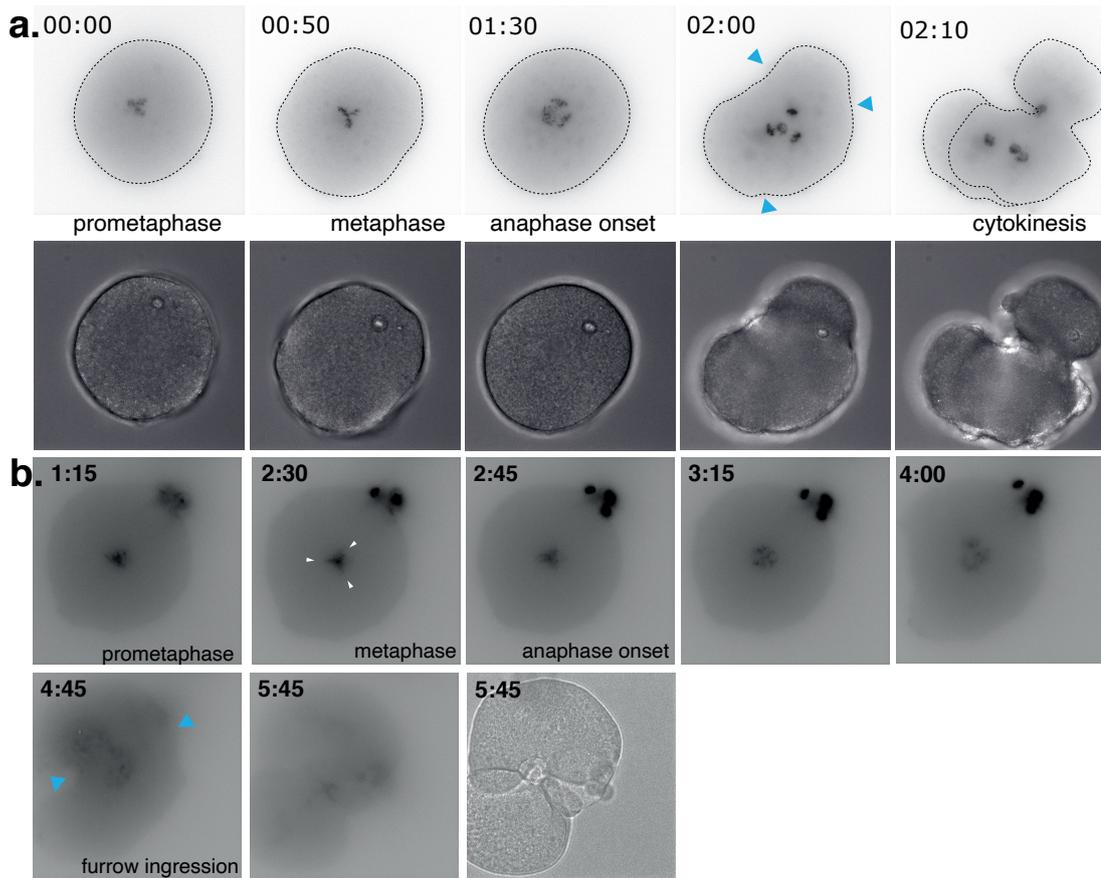


Figure 10: Multipolar spindles observed during mitosis I. Time lapse imaging of embryos undergoing mitosis with a multipolar spindle. The DNA was stained with SiR-DNA, white arrows indicate where the spindle poles appear to be and blue arrows indicate the onset of cytokinesis furrow ingress. Time in hours:mins (a) embryo 3034vii, see table 1 (b) embryo 3233v, see table 1. (c) Quantification of deselected embryos displaying a multipolar or a bipolar spindle (left bar).

Quantification of the number of embryos which divided with a multipolar spindle which had 2PN vs. 3PN (right bar). The data presented in the right bar includes all embryos imaged undergoing mitosis I and the data in the right bar only includes embryos i which NEB was imaged, hence the difference in n.

masses and subsequently multiple nuclei. One hypothesis could be that assembling and merging two distinct spindles is an inefficient and hence variable

process, explaining the heterogeneity in mitotic timings observed from embryo to embryo. Moreover, some of the multipolar divisions observed could be a result of distinct bipolar spindles which fail to align and fuse.

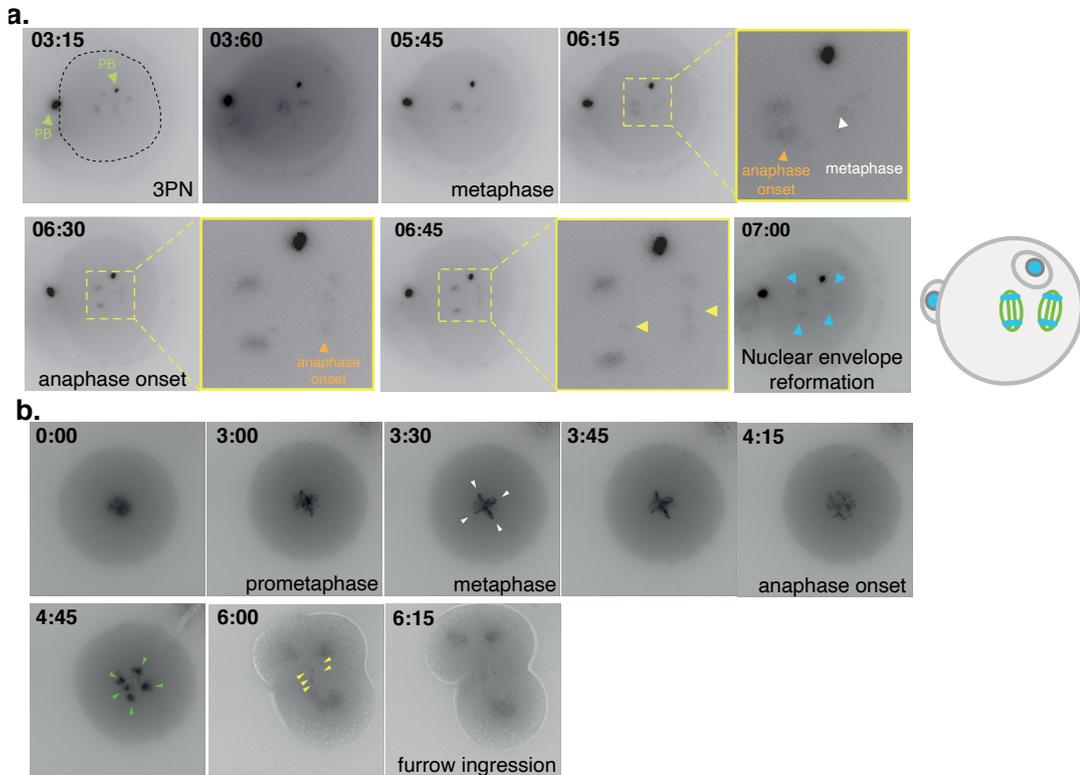


Figure 11: Evidence of dual spindles during mitosis I in human embryos.

Chromosomes stained with SiR-DNA, time in hours:mins (a) Time lapse of a 3PN human zygote undergoing mitosis I with a dual spindle. Anaphase onset happened earlier in the left spindle (6:15) compared to the right spindle (6:30). The yellow arrows show lagging chromosomes and the blue arrows the 4 nuclei formed. The schematic depicts the positions of the 2 spindles observed, as well as the polar bodies (PB) (embryo 3326v, see table 1). (b) Time lapse imaging of a human zygote of unknown PN status undergoing mitosis I with a quadrapolar spindle, or what looks like two spindles at 90 degrees of each other. White arrows indicate the four pole, green arrows indicate the 5 DNA masses formed and the yellow arrows the lagging chromosomes (embryo 3233v, see table 1).

5.2. Lagging chromosomes which can lead to the formation of micronuclei

Lagging chromosomes are chromosomes which are left behind on the spindle equator after anaphase onset. Embryos which divided with a bipolar spindle at first mitosis represent 60.5% of all embryos analysed (n=38). However, 17.4% of these (n=23) displayed at least one lagging chromosome (Fig. 12). This is likely to be a result of merotelic kinetochore-microtubule attachments, where a kinetochore is attached to both spindle poles, as is the case in human somatic cells (Cimini, Howell et al. 2001). Lagging chromosomes do not always result in aneuploidies as they may still end up in the correct daughter cell. However, a lagging chromosome pulled into the wrong daughter cell will cause whole chromosome aneuploidy. Indeed, lagging chromosomes have been associated with aneuploidy in somatic cells (Cimini, Howell et al. 2001).

Moreover, a nuclear envelope can assemble around lagging chromosomes, forming micronuclei in somatic cells (Cimini, Howell et al. 2001). Whether or not this is how micronuclei form in human embryos has not previously been reported. I therefore followed the fate of lagging chromosomes in our image sequences to determine if micronuclei can form. I observed micronuclei forming in 3 out of 23 of embryos that underwent a mitosis I with a bipolar division spindle. In two of these embryos, it was clear that the micronuclei formed as a direct result of the lagging chromosomes (Fig. 12b, c, see yellow arrow heads). This data provides the first evidence of micronuclei formation around lagging chromosomes in human embryos. Since micronuclei are frequently observed clinically in human embryos, this is an important finding of clinical relevance (Kort, Chia et al. 2016).

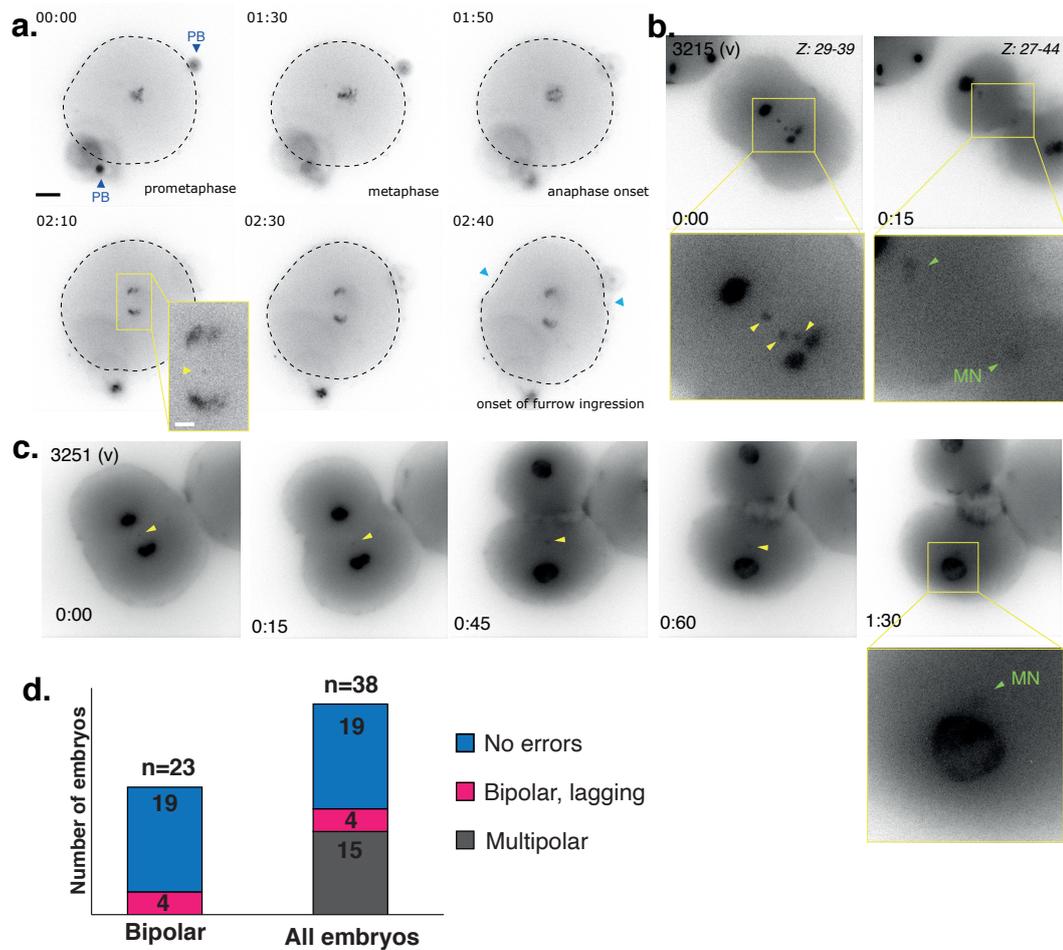


Figure 12: Lagging chromosomes during mitosis I. Time lapse imaging of human embryos undergoing mitosis I with lagging chromosomes, chromosomes were visualising using SiR-DNA, image with a widefield microscope. Time in hours:minutes. Yellow arrows indicate lagging chromosomes and green arrows micronuclei. (a) Embryo dividing with one lagging chromosome (embryo 3004iii, see table 1). (b) Embryo dividing with 3 lagging chromosomes, giving rising rise to 2 micronuclei (embryo 3215v, see table 1). (c) Embryo dividing with one lagging chromosome, leading to the formation of one micronuclei (embryo 3251v, see table 1). (d) Histogram showing the proportion of lagging chromosomes in embryos which divided with bipolar spindles (left bar) and of lagging chromosomes and multipolar spindles in all embryos imaged undergoing mitosis I (right bar).

From sequencing-based analysis of preimplantation embryos, it has been proposed that errors of a mitotic origin are not associated with maternal age, unlike errors of a meiotic origin (McCoy, 2017). I therefore wanted to investigate if errors which happened during the first mitosis specifically were also maternal

age independent. To test this the dataset was divided into two groups: embryos from patients where the female partner was aged 35 and younger and aged 36 and older. The age of 35 is considered to be the age from which aneuploidies of meiotic origin are reported to exponentially increase (McCoy, Demko et al. 2015). For the 35 and younger age group, 46.1% of embryos divided with a bipolar spindle and no lagging chromosomes (n=26), for the 36 and older age group, it was 50% (n=12) (Fig. 13). I performed a Fisher's exact test, the p value was 0.55 indicating no significant difference between the two age groups (Fig. 13). Thus, this data provides additional evidence to support the idea that the frequency of mitotic errors is not sensitive to maternal age. Overall, this set of results shows that mitosis I is highly error prone, with only around half of zygotes dividing with a bipolar spindle and no lagging chromosomes. The chromosome segregation errors encountered included multipolar spindles and lagging chromosomes, which sometimes gave rise to micronuclei. Moreover, maternal age did not appear to have an association with the frequency of chromosome segregation errors during mitosis I in human embryos.

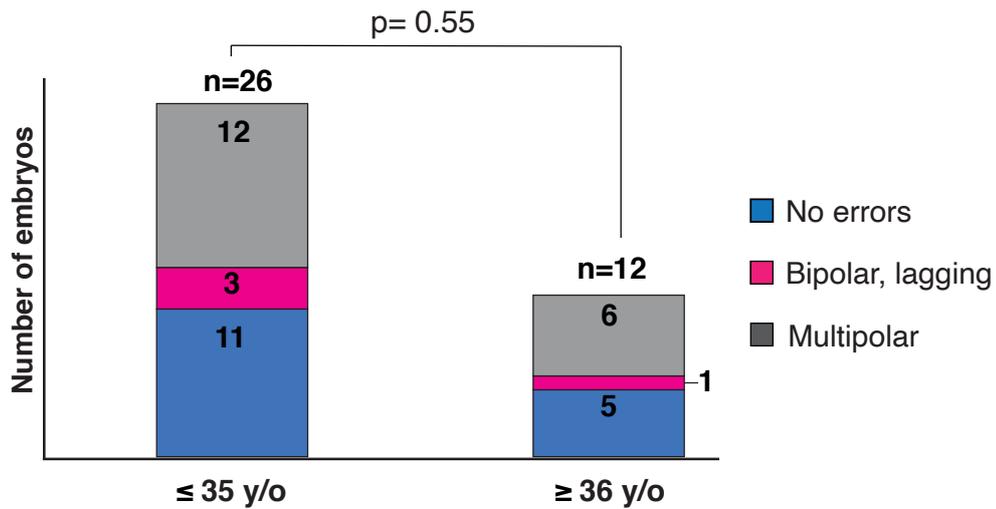


Figure 13: Maternal age impact of chromosome segregation errors. The proportion of multipolar spindles and of bipolar spindles with lagging chromosomes were analysed in two groups of patients aged 35 or under (left bar) or 36 and older (right bar) based on time lapse imaging of human embryos stained with SiR-DNA on a widefield microscope. Significance was calculated using a Fisher's exact test to compare the embryos with chromosome segregation errors vs. no errors (No errors vs. Bipolar lagging + Multipolar).

6. The second mitotic division is shorter and less error prone.

Given that I have shown how the first mitotic division is highly error prone I next sought to establish whether this was true for the second mitotic division. As for mitosis I imaging, the zona pellucida was removed with acid Tyrode's, the chromosomes were stained with SiR-DNA and imaged for about 48h. The second mitotic division was imaged in 21 cells from 15 embryos (Appendix B, table 2).

For two embryos, mitosis II was imaged after mitosis I had already been imaged in the same embryo. In this select group, the median interphase time following the first mitosis and before the second was 15 hours 15 minutes. The other 13 embryos were two cell embryos on the day when first placed on the microscope for imaging and on the day when they were donated for research. This second mitotic division was 25% shorter than mitosis 1 (145 minutes, n=20 vs. 210, n=20,

$p= 0.028$). Each of the mitotic timings measured was about 15 minutes shorter in mitosis II compared to mitosis I. The median prometaphase, metaphase, anaphase onset, furrow ingression and cytokinesis timings were respectively 60, 45, 22.5 and 15 for mitosis II and 75, 60, 30 and 30 minutes for mitosis I, $p= 0.23, 0.17, 0.11, 2.0 \times 10^{-4}$ (Fig.14, 15). The difference in timings observed during these two divisions is less dramatic than observed in mice, in which the first mitosis is almost twice as long as the second (Sikora-Polaczek, Hupalowska et al. 2006).

I also compared the error rates between the two division. Fewer errors were observed in mitosis II compared to mitosis I: 3 (16.6%) of the 18 mitosis II divisions were multipolar ($n=18$), compared to 39.5% during the first mitotic division. 2 out of the 15 bipolar divisions observed in mitosis II displayed lagging chromosomes (13.3%) compared to 17.4% in mitosis I. Taken together, 27.8% of the cells imaged undergoing mitosis II displayed chromosome segregation errors (multipolar spindles and/or lagging chromosomes). The chromosome segregation error rate during mitosis I is almost double that in mitosis II, with about half of embryos displaying lagging chromosomes or multipolar spindles. The p value of 0.15 shows that these differences could be due to chance, which is likely a consequence the relatively small sample sizes, ($n=38$ and $n=18$) (Fig. 15). This data suggests that the first mitotic division is uniquely error prone; more data collection would allow for a more robust conclusion about the error rates of both divisions.

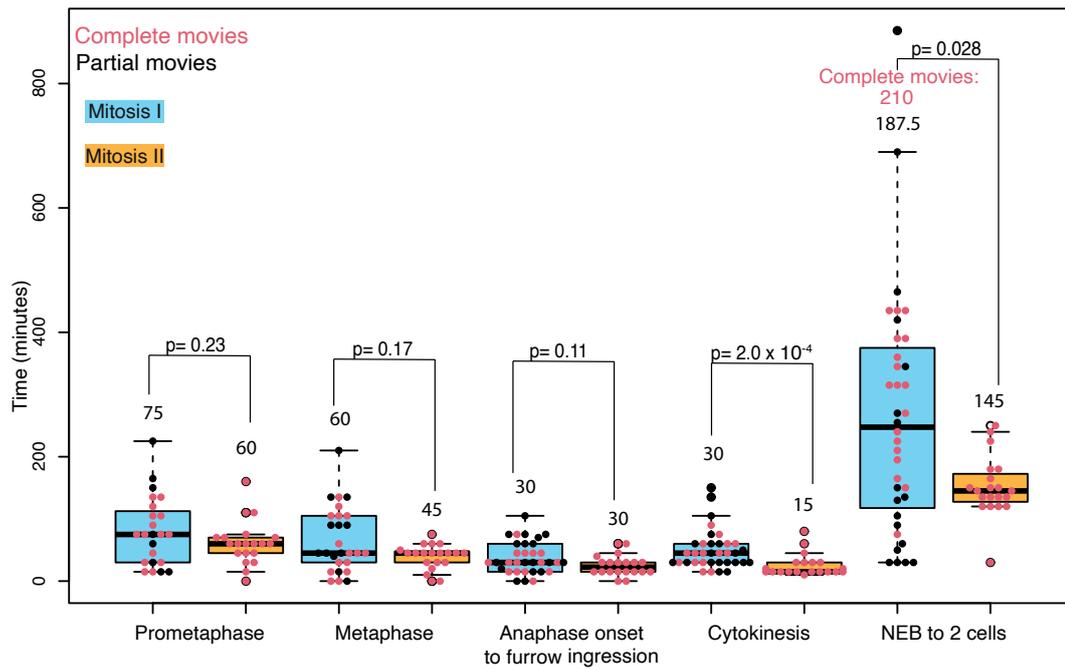


Figure 14: Comparing the timings of the first two mitotic divisions. Quantification of mitotic stages of deselected human embryos imaged on a widefield microscope and stained with SiR-DNA. The median time of each mitotic stage is displayed at the top of each box and whisker bar. Red/Black dots indicate whether or not NEB was imaged. The p values were calculated using a Mann Witney U test.

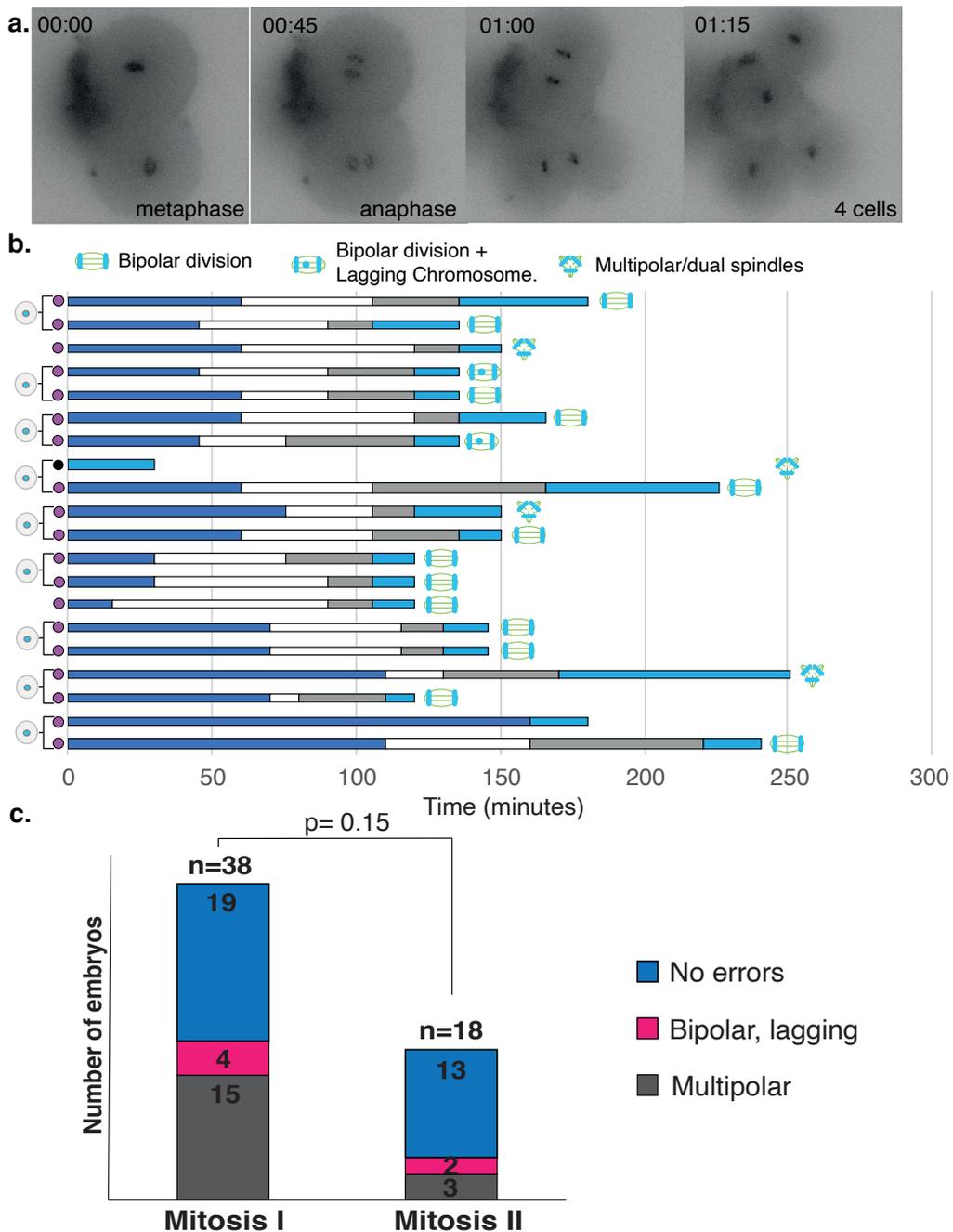


Figure 15: Live cell imaging of mitosis II in live deselected human embryos. Time lapse imaging of human embryos undergoing mitosis II without lagging chromosomes. Imaging was done using a widefield microscope and chromosomes were visualising using SiR-DNA. Time in hours:minutes. (a) Stills of one of the two cells of the embryo undergoing mitosis II with a bipolar spindle and no lagging chromosomes (embryo 3160iv, see table 2) (b) History plot of all the deselected embryos imaged undergoing mitosis II with a widefield microscope. The different coloured bars of the histogram show the timing of each mitotic stage and cartoons indicate the phenotypes observed. The phenotype is not indicated for some of the bars as the quality of the staining and/or the orientation of the zygote did not allow to accurately tell the phenotype. (c) Histogram showing the proportion of lagging chromosomes and multipolar spindles in all embryos imaged undergoing mitosis II. The p values were calculated using a Fisher's exact test.

Chapter 4: Integration of clinical and research data through the use of oocytes and embryos used for patient treatment and human oocytes shared to research

1. Reasoning

The data presented so far is from deselected human embryos following ICSI or IVF treatment. The zygotes that I imaged dividing came from mis-fertilised zygotes, i.e. zygotes with a number of PNs varying from the usual 2 (1PN or 3PNs), or zygotes which appeared unfertilised (MII stage) at the time when the clinical decision was made and then went on to become fertilised and divide. This starting material might potentially attract criticism for a lack of relevance to the normal situation. I therefore wanted to check that the timings observed in these embryos were consistent with 2PN embryos used in the clinic for patient treatment and initiation of human pregnancies. I also wanted to ensure that our experimental imaging was not affecting the mitotic timings observed. This chapter presents the results of comparing deselected embryos used for research with embryos used for patient treatment.

2. Clinical embryos have similar mitotic timings to deselected research embryos

The development of oocytes and embryos can be observed and recorded using an EmbryoScope™, which acquires a stack of seven Hoffman contrast images every 10 minutes for each embryo. Oocytes inseminated by ICSI can be placed in the embryoscope immediately so that all stages of their fertilisation can be observed as well as subsequent embryo development. I analysed 330 embryos imaged with an EmbryoScope™ that provided a dataset of ICSI embryos that had been used in the clinic. This set of embryos included those which had developed to blastocysts, which the embryologists assessed as being the healthiest of the cohort available for that patient and which were subsequently transferred to the patient as part of their treatment in a single embryo transfer of a fresh (not cryopreserved) embryo (n=180). A fraction of these embryos gave rise to pregnancies (n=100) whilst others did not (n=80). Following transfer, the success of embryo implantation depends both on embryo quality and on the endometrium as both must mature synchronously and communicate with each other (Salamonsen, Evans et al. 2016). I also analysed the timings of embryos produced by the same cohort of patients who became pregnant as a result of their ICSI treatment, but this time looking at the embryos which were not transferred to the patient or cryopreserved because the clinical embryologists assessed that their development was suboptimal (n=150). These included embryos which successfully divided into 2 and then 4 cells (n=80) and embryos which had erroneous cytokinesis and divided into more than 2 cells at mitosis 1 (n=70). With these 4 categories I can therefore compare the durations of different stages of mitosis I and II, in light of knowledge about the embryos' normal fertilisation with 2PN as well as their developmental potential expressed through subsequent preimplantation development and in some cases, pregnancy outcome.

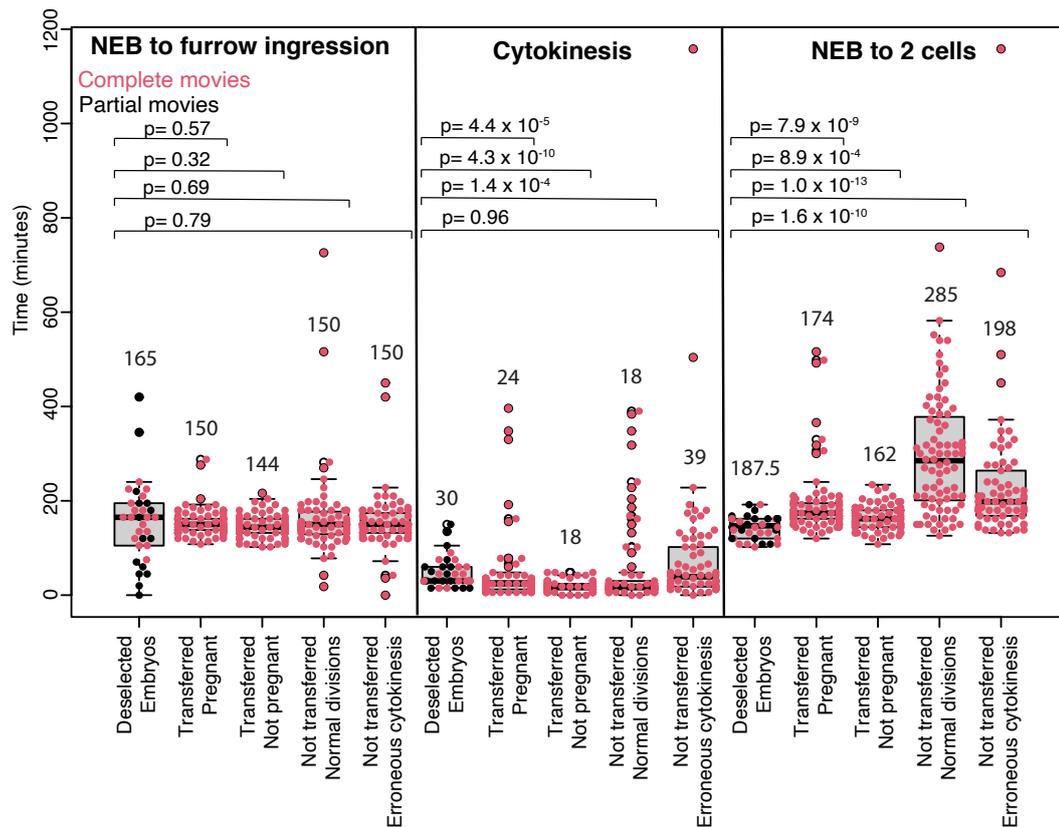


Figure 16: Mitotic timings of clinical embryos used for fertility treatment compared to research embryos. Quantification of mitosis I, of embryos monitored using an EmbryoScope™ of different indicated groups and of research embryos stained using SiR-DNA and imaged on a widefield microscope. Red/black dots indicate whether or not NEB was imaged. The median time of each mitotic stage is displayed at the top of each box and whisker bar. The p values were calculated using a Mann Whitney U test.

Because these clinical embryos were imaged using the embryoscope, the chromosomes were not visualised. However, the following key cell biological events could be visualised: NEBD, furrow ingression, and the formation of 2 (or more) cells. The median timings from NEBD to cytokinesis were very consistent across the four groups: 150 minutes for both transferred groups and the non-transferred abnormal cytokinesis groups and 144 minutes for the non-transferred normal cytokinesis group. Median cytokinesis timings ranged from 18 to 24 minutes for the groups with normal cytokinesis, while the group of embryos which divided into more than 2 cells at mitosis I displayed a longer cytokinesis time (39 minutes), probably due to the erroneous nature of cytokinesis in this group (Fig.

16). With a median time from NEB to furrow ingression of 165 minutes and median cytokinesis time of 30 minutes, our mitotic timing analyses of deselected research embryos appear to be representative of what happens in clinical embryos, which had been normally fertilised (2PN) and which had not been stained with SiR-DNA or imaged on our live cell imaging microscope. The total mitosis I timings (NEBD to cytokinesis completion) of about 3 hours that were measured in clinical embryos are consistent with what I reported in our deselected research embryos in which mitosis I was imaged in its entirety (3.5 hours) and with published findings from Embryoscope™ movies of human clinical embryos from other groups (Vera Rodriguez 2015).

Moreover, I observed that good quality blastocysts which were transferred to the patient displayed a shorter median mitosis I time than the poorer quality embryos which were not transferred. Indeed, the transferred embryos which gave rise to pregnancy underwent mitosis I in a median time of 174 minutes and the transferred embryos which did not give rise to pregnancy in 162 minutes. The embryos which were not transferred on the other hand, had a median mitosis I time of 285 and of 195 minutes for the embryos which had normal early cytokineses and the embryos which had erroneous cytokineses respectively (Fig. 16). This is consistent with the mitotic timings of clinical human embryos previously reported (Jacobs, Nicolielo et al. 2020). There is a correlation between the time of sperm injection to NEB and to 2 cells and the developmental potential of the blastocyst. Blastocyst quality was assessed in the clinic using morphological criteria when deciding which embryo to transfer to the patient. Blastocysts of good quality tended to have faster timings to NEB and to 2 cells compared to poor quality blastocysts (Jacobs, Nicolielo et al. 2020).

Interestingly, the transferred group of embryos displayed a tighter distribution in timings compared to the non-transferred groups. The interquartile ranges observed were 31.5 and 36 compared to 60 and 87, suggesting that most embryos

which developed into healthy blastocysts had timings closer to the median. However, it is important to point out that some embryos displaying very long mitosis I timings (from over 200 to almost 500 minutes) successfully gave rise to pregnancies (Fig. 16). The fact that embryos with elongated mitotic timings can lead to pregnancies shows that despite a correlation between median mitotic timings and outcome, mitosis I timings alone cannot be used as a predictor of treatment outcome.

3. Micronuclei and multiple nuclei are a sign that chromosome segregation errors occur in embryos giving rise to pregnancies

I have reported multiple nuclei as well as micronuclei as a result of lagging chromosomes in our research embryos. Once again EmbryoScope™ movies of clinical embryos provided a very important control showing that what I observed in our research embryos is representative of what happens in the clinical setting. I compared our research data on nuclear numbers and micronuclei to data collected by Lucy Benham Whyte, a trainee clinical embryologist at the CRM, who was analysing nuclear phenotypes in human 2-cell and 4-cell embryos. Nuclei were analysed for every cell during the first two mitotic divisions in 68 human embryos which gave rise to clinical pregnancies when transferred to the patient. After the first mitotic division, 26.5% of these embryos had more than one nucleus, whether micronuclei and/or multiple nuclei in at least one of the two cells, proving that these nuclear abnormalities can arise in a zygote which was correctly fertilised (2PN) and importantly that they are compatible with the formation of healthy blastocysts and pregnancies (Fig. 17a, b). Multiple nuclei are a sign that the chromosomes were segregated into different masses at anaphase onset, with a nuclear membrane forming around the different masses. This phenotype was commonly observed in our research embryos, usually due to a multipolar spindle.

Micronuclei, on the other hand, usually form around lagging chromosomes. Micronuclei were present in 13.2% of the clinical embryos resulting in pregnancy, which shows that micronuclei, and potentially lagging chromosomes, do happen in normally fertilised embryos and are compatible with successful pregnancies (Fig. 17a, b). I have shown the error-prone nature of mitosis I in deselected research embryos. This clinical data shows not only that such errors occur frequently in normally fertilised 2PN embryos, but also that chromosome segregation errors occurring during the first mitotic division are compatible with human life.

Because micronuclei have been shown to be either unilaterally inherited or reabsorbed into the main nucleus at interphase in mice embryos (Chavez, Loewke et al. 2012), I wanted to investigate what happened to the micronuclei during subsequent divisions. As previously mentioned, 26% of the embryos which gave rise to pregnancies had micronuclei and/or multiple nuclei at the 2 cell stage. At the 4 cell stage, that number reduced to 2%, indicating that most of the aberrant nuclei formed during mitosis I in humans ended up being corrected in these good quality embryos which gave rise to pregnancies. The p value is 2.5×10^{-5} meaning that the difference in error rates between the two divisions is statistically significant (Fig. 17c, d). This suggests that, if the lagging chromosomes can be reabsorbed into the main nucleus, a euploid embryo can still form even if micronuclei were present following mitosis I. Importantly, the much lower proportion of multiple and micronuclei following mitosis I compared to mitosis II is consistent with our finding that mitosis I is uniquely error-prone in human embryos.

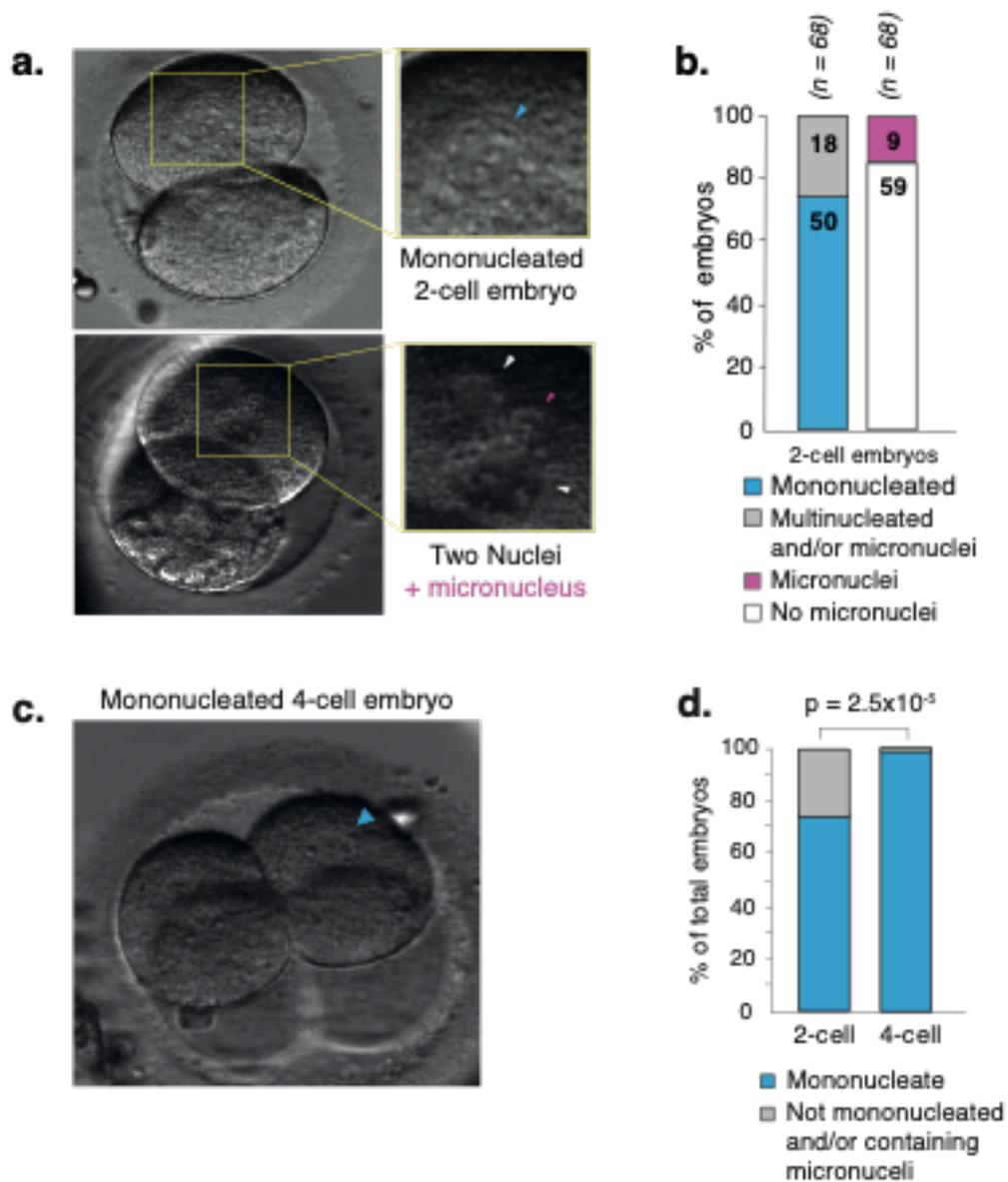


Figure 17: Multiple nuclei and micronuclei in clinical embryos which gave rise to pregnancies. (a) Stills of 2 cell human embryos from an EmbryoScope™ movie, showing a mononucleated embryo (blue arrow) (top) and an embryo with two nuclei (white arrows) and a micronucleus (pink arrow) (bottom). (b) Quantification of abnormally nucleated embryos (multiple and/or micronuclei) (left bar) and of micronuclei (right bar) in a set of 68 2-cell embryos which resulted in a positive pregnancy test. (c) Stills of a mononucleated 4 cell embryo (blue arrow). (d) Comparison of the proportion of mononucleate and non-mononucleate and/or micronuclei containing 2 cell (n=68) and 4 cell (n=65) embryos resulting in positive pregnancy tests. P-value from a Fishers exact test. Data analysis by Lucy Benham Whyte.

4. Establishing an egg sharer program

As previously mentioned, a limitation regarding the deselected embryos that I used for our main dataset is that they deviate from the normally and timely fertilised 2PN embryos used for clinical treatment. In order to access normally fertilised embryos which could be analysed in the same way as our main dataset of deselected embryos (using chromosome staining and live cell imaging), an egg sharer program was established. Patients aged 32 years old or younger who were anticipated to produce plentiful eggs and with a good prospect of success could volunteer to be a part of the egg sharer program and donate half of their mature oocytes to research (see methods for more detail). This was a challenging program to set up, with extensive ethical and administrative issues engaged, but CRM had prior experience in egg sharing from one woman to another for the purposes of producing a baby, so many of the concepts had already been established with precedents. So far, the program has proven very successful; I was able to image embryos from 4 egg sharer patients undergoing the first mitotic division. Three of the four patients joined the program in order to have IVF or ICSI treatment and one of them joined to have their eggs frozen for potential use in the future. Two of the patients attempting pregnancy became pregnant as a result of the treatment and the third patient had healthy blastocysts frozen for an embryo transfer at a later date. This egg sharer program is still ongoing with more patients scheduled to have their fertility treatment as part of the program.

As well as the research embryos that were created using donated sperm and live-imaged, I also had access to the EmbryoScope™ movies for the embryos from the same patient that were used in their treatment. This was a further useful internal control to check whether the experimental imaging conditions were affecting mitotic timings or morphology.

The embryos of the initial 3 egg sharer patients were imaged on our widefield microscope, in the same conditions as our main dataset (see Chapter 3.1 and methods). These initial 3 egg sharer patients will be detailed in this chapter (Appendix B, table 3 and 4). Embryos from the final egg sharer patient were imaged using our spinning disk confocal microscope under different conditions and will be detailed in chapter 5.

The first egg sharer patient was aged 29 and went through ICSI treatment due to blocked fallopian tubes and male factor infertility. The patient had already had a baby as a result of a previous ICSI treatment. 10 oocytes were recovered, 5 of which were donated to research and injected with donor sperm: they fertilised to produce 5 2PN embryos. 2 of the clinical embryos were transferred and the patient became pregnant with dizygotic twins. 4 of the 5 zygotes created for research underwent mitosis I. All these divisions were bipolar, one of them was already in anaphase when the imaging started so I could only quantify the timing of cytokinesis for this one (Fig. 18). Interestingly, one of these embryos divided with a lagging chromosome (Fig. 18b), showing that such errors also occur in normally fertilised 2PN embryos without a delay in fertilisation. Another embryo displayed a lagging chromosome at the start of anaphase, but it then joined the main chromosome mass and therefore was not counted as a lagging chromosome in our analysis (Fig. 18c). The other two embryos divided without lagging chromosomes (Fig. 18a, d). When comparing the research data to the patient's clinical embryos, the mitotic timings were within the same range, confirming again that our timing data in research embryos is similar to the timings of clinical embryos which resulted in pregnancy and that staining with SiR-DNA and live-cell imaging for up to 2 days did not impact mitotic timings (Fig. 18d). Moreover, one of the two embryos implanted had an extended cytokinesis timing, confirming what I saw in our previous analysis of clinical embryos: extended mitosis I timings are compatible with healthy embryo development and pregnancy.

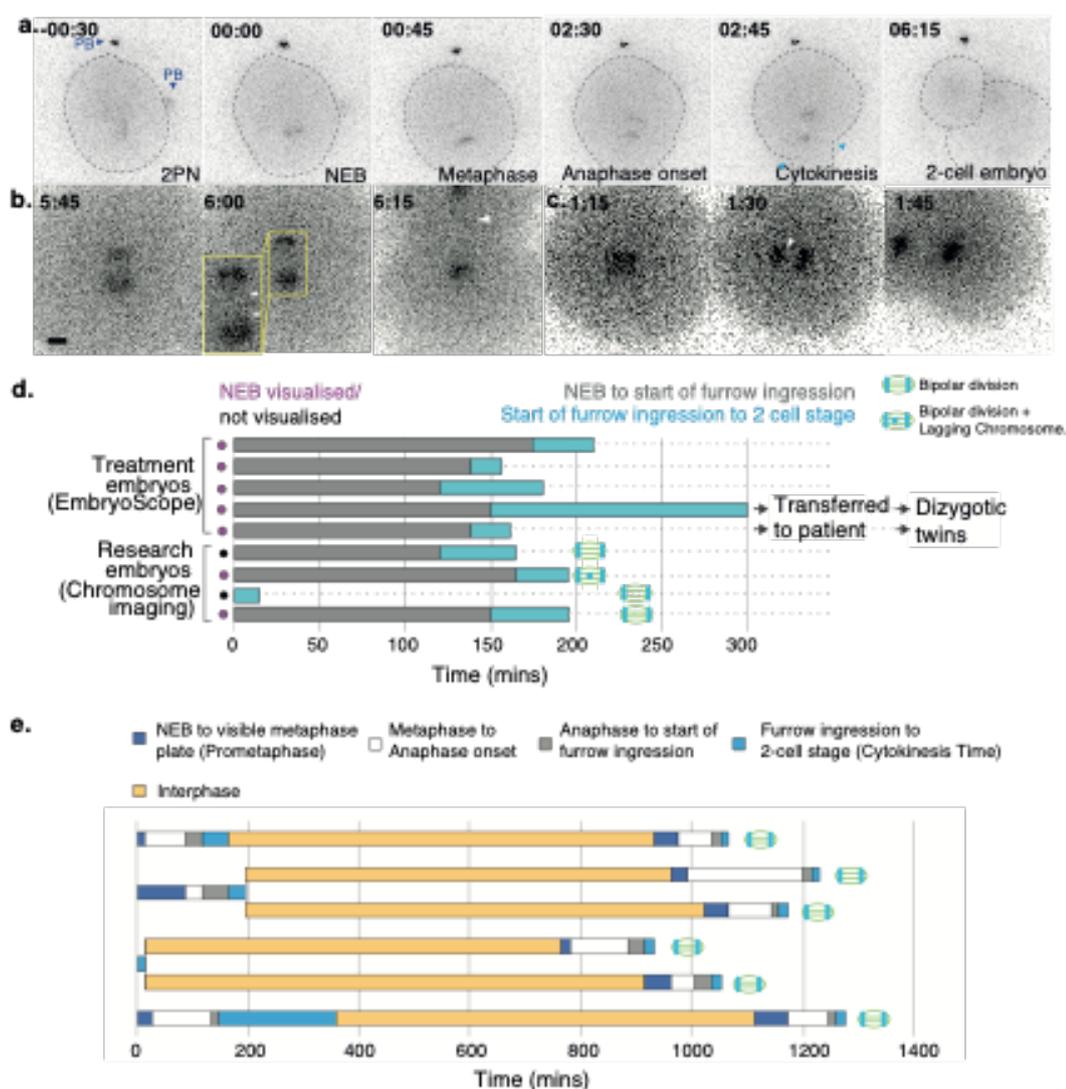


Figure 18: The first mitotic divisions of research and clinical embryos from the first egg sharer patient. (a) Stills from an eggsharer embryo donated to research undergoing mitosis with a bipolar spindle and no lagging chromosomes (embryo 3247ii, see table 3). The chromosomes were stained with SiR-DNA and the embryos were imaged on a widefield microscope. Time in hours:minutes. (b) Same as (a) but with lagging chromosomes (white arrows) (embryo 3247v, see table 3). (c) Same as (b) but the errors is corrected with the lagging chromosome joining the DNA mass, it was not counted as a lagging chromosome in our analysis (embryo 3247iv, see table 3). (d) History plots of research embryos stained using SiR-DNA and imaged on a widefield microscope and clinical embryos imaged using an EmbryoScope™, all from the same patient (see table 4). Cartoons indicate the phenotypes observed. Pink/black dots indicate whether NEB was imaged. (e) Quantification of the timings of mitosis I, mitosis II and interphase for four egg sharer embryos donated to research. After their mitosis I was imaged, two of them had one cell undergoing mitosis II, and the other two had both cells undergoing mitosis II.

The four embryos that were imaged undergoing the first mitosis also went on to the second mitotic division. In two of these embryos, both cells underwent mitosis II and in the two others, one of their two cells underwent mitosis II (Fig. 18e). These 4 embryos remained in interphase (time between the end of mitosis I and start of mitosis II) for 12.75 hours (765 minutes). The median time from NEB to 2 cells for the mitoses II of embryos from the first egg sharer patient was 157.5 minutes which is within the same range as our deselected embryos (median time of 145 minutes).

The second egg sharer was a 19-year-old patient wishing to have their eggs frozen for future use. 7 eggs were donated to research and injected with donor sperm, 5 of these fertilised, with 2PN forming. The remaining two did not fertilise, remaining at the metaphase II stage. 3 embryos underwent the first mitotic division and lagging chromosomes were visible in one of those divisions (Fig. 19), showing once again that chromosome segregation errors also happen in normally fertilised 2PN embryos from young women. The 7 oocytes which were allocated for use by the patient were cryopreserved at the MII stage without fertilisation. There were therefore no clinical embryos to compare with research embryos for this patient.

The third egg sharer patient was aged 31 with subfertility caused by polycystic ovarian syndrome. 24 eggs were produced, 12 were used for the patient's IVF treatment and 12 for research. The oocytes donated to research were injected with donor sperm and 3 of them fertilised with 2PN and underwent mitosis I with a bipolar spindle. Although all 3 embryos divided with a bipolar spindle, one of them had a lagging chromosome. By comparing the mitotic timings of these 3 research embryos to the 8 embryos from the same patient which were used for the patient's fertility treatment and imaged on the Embryoscope™, I was able to show once again that our treatment and long-term imaging of these research embryos did not have an impact on mitotic timings (Fig. 20). The patient became

pregnant as a result of the egg sharer program, following the transfer of one embryo.

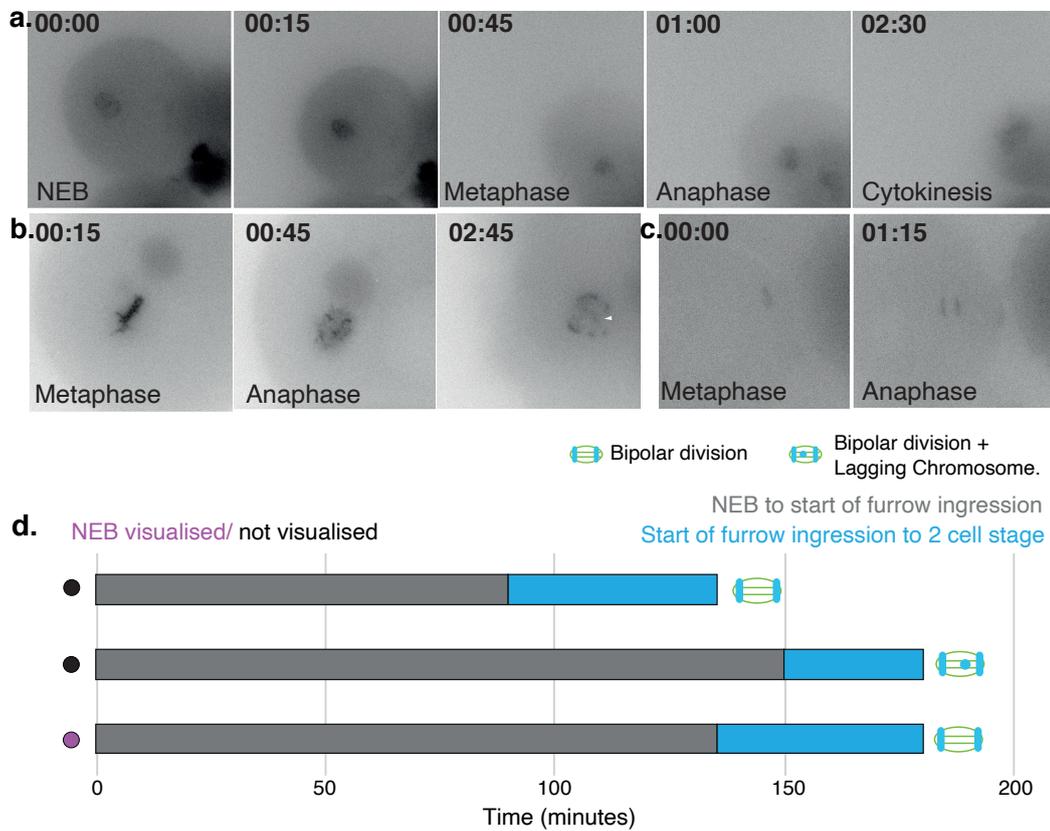


Figure 19: Mitosis I of research embryos from the second egg sharer patient.

(a) Stills from an egg sharer embryo donated to research undergoing mitosis with a bipolar spindle and no lagging chromosomes (embryo 3258ii, see table 3). The chromosomes were stained with SiR-DNA and the embryos were imaged on a widefield microscope. Time in hours:minutes. (b) Same as (a) but with lagging chromosomes (white arrow) (embryo 3258iii, see table 3). (c) Same as (a) (embryo 3258vii, see table 3). (d) History plots of research embryos stained using SiR-DNA and imaged on a widefield microscope and clinical embryos imaged using an EmbryoScope™, all from the same patient. Cartoons indicate the phenotypes observed. Pink/black dots indicate whether NEB was imaged. This egg sharer patient did not undergo fertility treatment, mature eggs were frozen for future use.

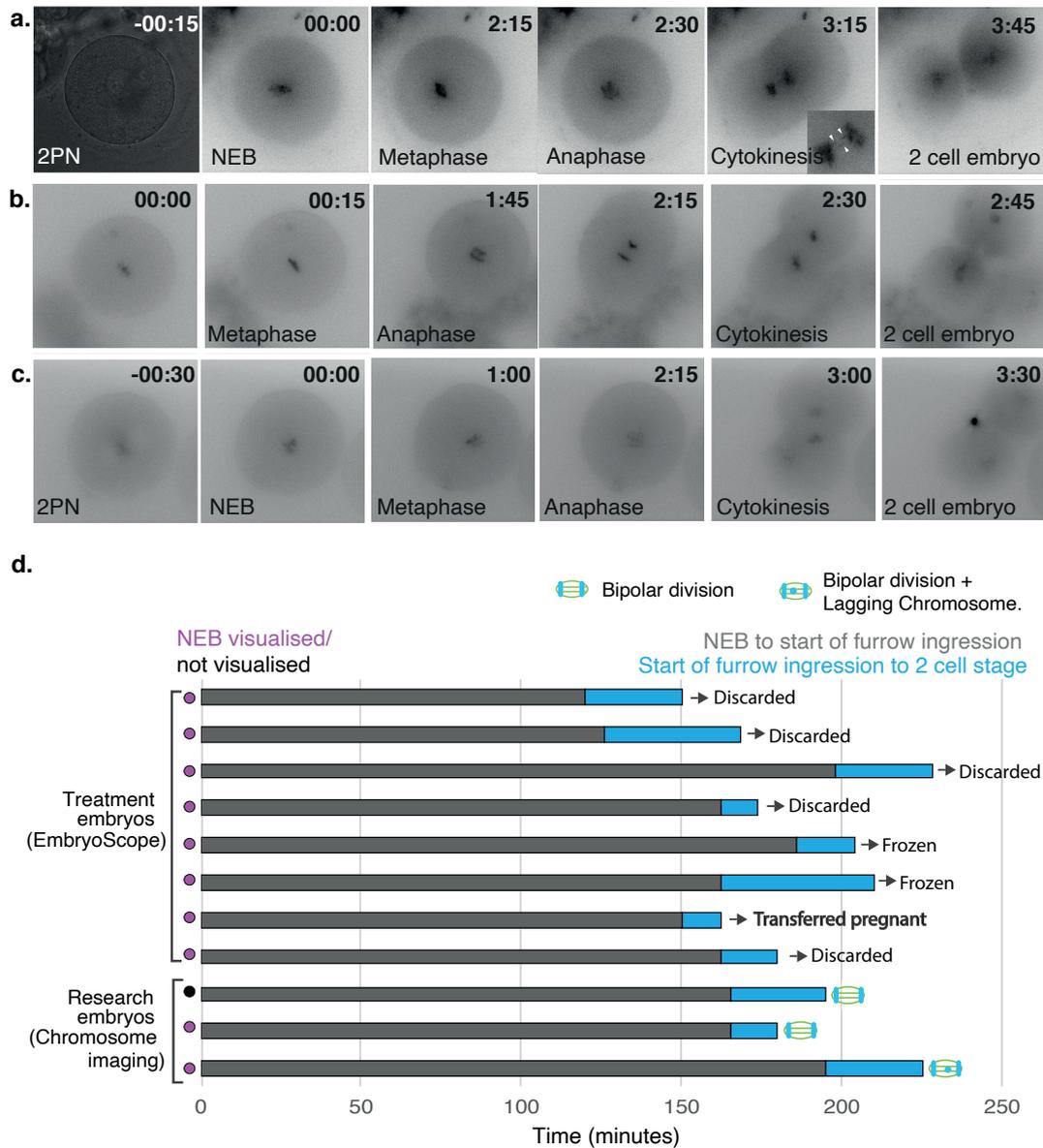


Figure 20: Mitosis I of research and clinical embryos from the third egg sharer patient. (a) Stills from an egg sharer embryo donated to research undergoing mitosis with a bipolar spindle and lagging chromosomes (white arrows) (embryo 3272i, see table 3). The chromosomes were stained with SiR-DNA and the embryos were imaged on a widefield microscope. Time in hours:minutes. (b) Same as (a) but without lagging chromosomes (embryo 3272vi, see table 3). (c) Same as (b) (embryo 3272vi, see table 3). (d) History plots of research embryos stained using SiR-DNA and imaged on a widefield microscope and clinical embryos imaged using an EmbryoScope™, all from the same patient. Cartoons indicate the phenotypes observed. Pink/black dots indicate whether NEB was imaged.

By combining data from all the egg sharer embryos created for research, I have compared deselected embryos to normally fertilised 2PN embryos. Egg sharer embryos had a median time from NEB to cytokinesis furrow ingression of 225 minutes compared to 187.5 minutes in deselected embryos. The prometaphase and metaphase timings were 52.5 and 75 minutes in egg sharer embryos compared to 75 and 60 minutes in our main dataset. The timings from anaphase onset to furrow ingression was 37.5 and 30 minutes in egg sharer embryos and deselected embryos respectively and 30 minutes for both for cytokinesis (Fig. 21). Moreover, the total mitosis I timing was 225 minutes in egg sharer embryos and 210 minutes in deselected embryos.

Overall, egg sharer embryos and deselected embryos display similar mitosis I timings. The heterogeneity in mitosis I timings from embryo to embryo can account for the small differences in median timings observed. Moreover, I observed lagging chromosomes in 33% of egg sharer embryos undergoing mitosis I (Fig. 21), showing that mitosis I is very error-prone even in normally fertilised 2PN embryos. I did not, however, observe any of these embryos dividing with a multipolar spindle. I have shown in this study that multipolar spindles are more likely to arise in 3PN embryos, with 75% of the multipolar spindles observed originated from 3PN embryos in our deselected dataset. In our small series of egg sharers, all of the embryos were fertilised with 2PN and divided with a bipolar spindle, which could explain why I did not observe any multipolar spindles in this dataset. However, due to the low numbers of embryos observed overall, I cannot rule out that multipolar spindles may occur.

In summary, the fact that egg sharer embryos had prolonged mitosis I timings, heterogeneity of timings from embryo to embryo and often had lagging chromosomes, shows that what I observed in our deselected embryo dataset is representative of correctly fertilised normal embryos from young women. The egg sharer program provides the unique opportunity to study live human oocytes and

2PN zygotes, which fertilised on time and are identical to zygotes used for fertility treatment. Moreover, comparing embryos which were stained and imaged for research to embryos which were used for treatment and gave rise to pregnancies, from the same patients in the same treatment cycle provides an ideal control to show that staining with SiR-DNA and long-term imaging did not impact mitotic timings.

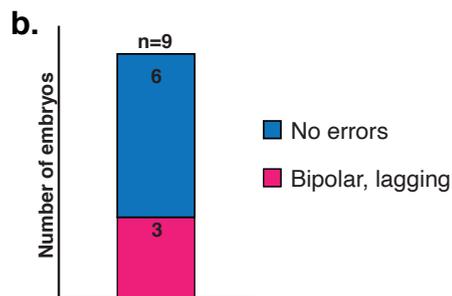
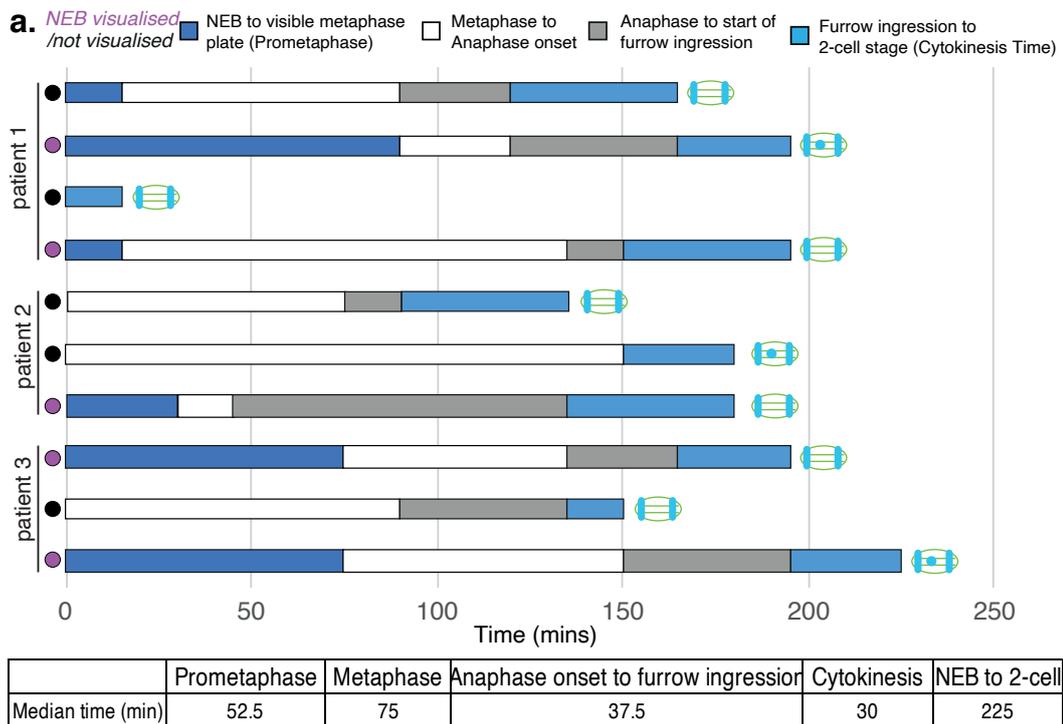


Figure 21: History plot of egg sharer embryos imaged undergoing mitosis I using a widefield microscope. (a) The chromosomes were stained with SiR-DNA. The different coloured bars of the histogram show the timing of each mitotic stage and cartoons indicate the phenotypes observed. Pink/black dots indicate whether NEB was imaged. The median timings for each mitotic stage are displayed in the table. (b) Quantification of embryos displaying lagging chromosome vs. no chromosome segregation errors during mitosis.

Chapter 5: The role of the SAC in error correction during the first cleavage division

1. Reasoning

Our data shows that mitosis I is very error-prone, with only about 50% of research embryos segregating their chromosomes correctly (Chapter 3). Moreover, the finding that 26% of clinical embryos that gave rise to pregnancy had multiple nuclei or micronuclei at the 2-cell stage, indicative of chromosome segregation errors during mitosis I, revealed that such errors, even at mitosis 1, are compatible with healthy embryonic development (Chapter 4). High chromosome segregation error rates would suggest that kinetochore-microtubule attachments form erroneously. Cells normally have mechanisms in place to prevent this from happening, such as error-correction which destabilises incorrect attachments and the SAC that causes an arrest in metaphase until all the chromosomes are aligned on the metaphase plate and attached to the spindle poles (See Chapter 1.2.4). Moreover, the finding that prometaphase and metaphase timings were inversely proportional suggest that it is an intrinsic timer, and not the SAC, that is responsible for setting the time to anaphase onset. I therefore hypothesised that the SAC is weak during mitosis I.

To test this hypothesis, the SAC kinase Mps1 was inhibited using reversine. Reversine binds the ATP binding site of the Mps1 kinase, preventing Mps1 from catalysing the transfer of a phosphate group from ATP to the proteins it targets (Hiruma, Koch et al. 2016). As Mps1 triggers SAC signalling by initiating a phosphorylation cascade (De Antoni, Pearson et al. 2005), Mps1 inhibition silences the SAC. In addition to its essential role in SAC signalling, Mps1 also has a role in

error-correction in human somatic cells (Hayward, Bancroft et al. 2019). In human somatic cells, reversine treatment causes premature mitotic exit as well as high error rates (Santaguida, Tighe et al. 2010).

2. The SAC is not responsible for setting the time from NEB to anaphase onset

2.1. Mps1 inhibition with 10 μ M reversine

To strongly inhibit Mps1, I started by treating human zygotes with a high dose of reversine (10 μ M) in initial experiments. Half the zygotes were treated with 10 μ M and the other half provided a control, treated with DMSO, to which I could compare our treated group, to check if or to what extent reversine caused chromosome segregation errors and an acceleration in mitotic timings. For this initial experiment, 5 embryos were imaged undergoing mitosis I: 3 were treated with reversine and 2 served as a control (Fig. 22, Appendix B, table 5). All embryos were stained with SiR-DNA and imaged for up to 2 days on a widefield microscope. The 10 μ M reversine-treated embryos exhibited a very clear phenotype: they displayed no chromosome alignment on the metaphase plate and anaphase happened chaotically with many small DNA masses moving outwards in many directions (Fig. 22a). This phenotype was characteristic of the 3 embryos tested in our treatment group. The two control embryos both divided with a multipolar spindle, but unlike the reversine-treated embryos, chromosome alignment on the metaphase plate was observed.

Importantly, by quantifying the timings from NEB to anaphase onset, I identified that reversine treatment did not cause a premature mitotic exit, unlike in somatic cells where the SAC is active. The timings from NEB to anaphase onset for the three reversine treated embryos were 75, 45 and 120 minutes, compared to 75 and 120

minutes for the two control embryos (Fig. 22b). This suggests that it is not the SAC is not setting the duration from NEB to anaphase onset during mitosis I. If that were the case, I would expect reversine-treated embryos to have shorter timings to anaphase onset than control embryos, as in somatic cells (Santaguida, Tighe et al. 2010). The fact that SAC inhibition did not result in shorter timings suggests that the SAC is not responsible for setting elongated mitotic timings by causing a metaphase delay until all the chromosomes are aligned in control embryos. More data is needed to confirm this finding.

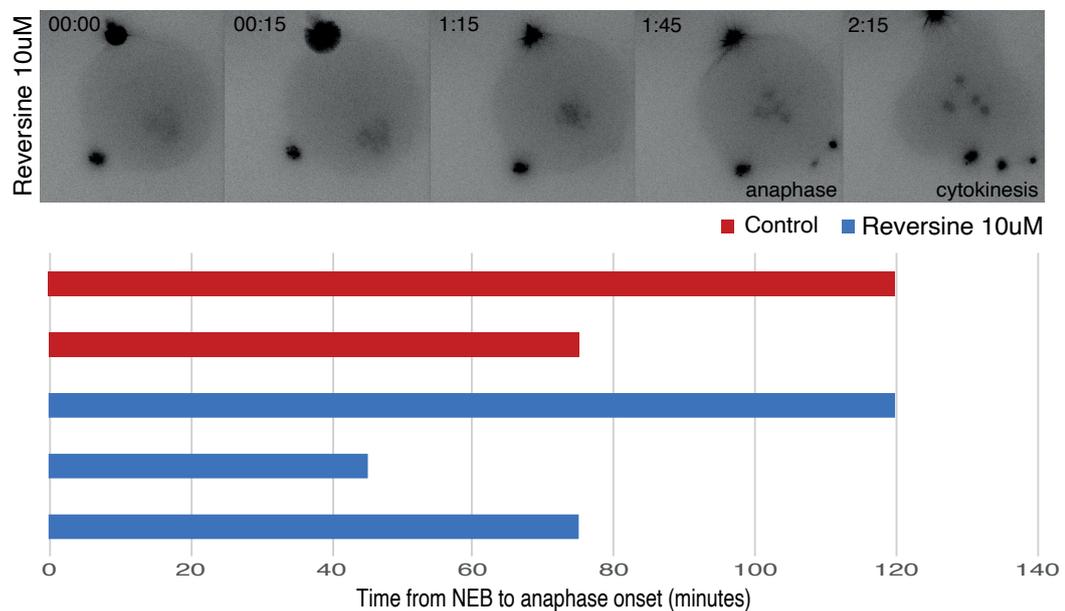


Figure 22: Mps1 inhibition in live human embryos with high dose reversine (10uM). Time lapse imaging of human embryos treated with 10uM or control embryos undergoing mitosis I using a widefield or spinning disk confocal microscope, chromosomes were visualising using SiR-DNA. (a) Stills of a deselected embryos treated with 10uM reversine undergoing mitosis I (embryo 3325iii, see table 5). (b) Timings from NEB to anaphase onset of treated embryos (blue) and control (red) embryos. Cartoons indicate the phenotypes observed for the control embryos, no clear spindle bipolar or multipolar spindles were observed in treated embryos.

2.2. Mps1 inhibition with 1 μ M reversine

The initial experiments show that a 10 μ M reversine treatment created severe biorientation issues, with chromosomes failing to align on the metaphase plate. At high dose, Reversine also inhibits Aurora B as well as other mitotic kinases at lower

levels (Santaguida, Tighe et al. 2010). I therefore decided to use a lower reversine concentration for our future experiments to inhibit Mps1 more specifically. Reversine has been used in early mouse embryos at a concentration of 500nM (Ajduk, Strauss et al. 2017, Singla, Iwamoto-Stohl et al. 2020). However, with a diameter of around 70 μ m mice zygotes are more than half the diameter of human zygotes (110-120 μ m diameter) but less than a quarter of the volume (Quinn and Horstman 1998, Nazari, Khalili et al. 2011). I decided to treat our embryos with 1 μ M reversine to account for human embryos' larger size which means that reversine has to diffuse through a large cytoplasmic volume to get to a proportionally small spindle. Moreover, 1 μ M reversine treatment in somatic cells was shown to cause a potent premature mitotic exit, without significantly inhibiting kinases which control mitosis in vertebrates. At this concentration, Aurora B was still partially inhibited but at a level which was low enough for cytokinesis to be unaffected (Santaguida, Tighe et al. 2010).

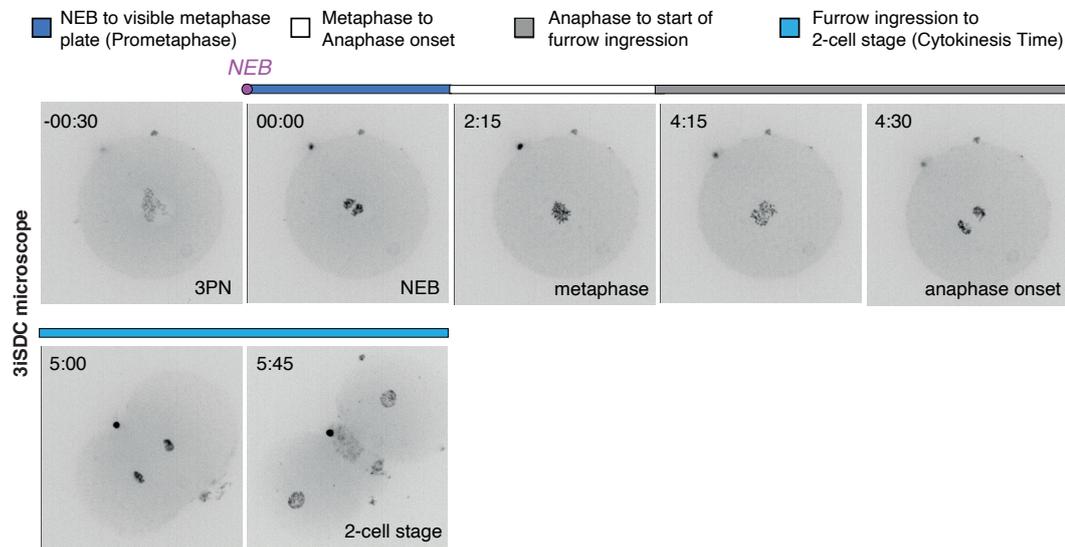


Figure 23: Imaging mitosis I in live human embryos using a spinning disk confocal microscope. Time lapse imaging of the first mitotic division (embryo 3326iii, see table 7), the chromosomes were stained using SiR-DNA. Time in hours:mins. The top bar shows the different mitotic phases measured in our dataset: prometaphase, metaphase, anaphaset onset to start of furrow ingression and cytokinesis.

Half the zygotes donated to research were treated with 1 μ M reversine and the other half were treated with DMSO which served as a control. All embryos were stained with SiR-DNA and imaged for up to two days. Initial experiments were performed using a widefield microscope but, for this experiment, the spinning disk confocal microscope was used to gain improved imaging resolution, allowing individual chromosomes to be seen more clearly. Figure 23 shows an example of a mitosis I in human embryos imaged on the spinning disk confocal microscope. Five reversine-treated embryos and five control embryos were imaged undergoing mitosis I (Fig. 24, 25, Appendix B, table 6). Of the five control embryos, two had bipolar spindles and aligned their chromosomes on a clear metaphase plate with no unaligned chromosomes (Fig. 25). They then segregated their chromosomes into two DNA masses. One of them had a lagging chromosome, which led to the formation of a micronucleus (Fig. 24). The remaining three underwent mitosis I with a multipolar spindle, with poor alignment on the metaphase plate and chromosome segregation into more than two masses during anaphase. Multipolar spindles were identified as a cause of aneuploidy in our mitosis I dataset (Chapter 3.3) as they segregate their chromosomes erroneously, into more than 2 masses. This control data is representative of what I observed in our main mitosis I dataset, in which I observed both multipolar chromosome segregation into more than two masses and bipolar chromosome segregation into two masses, with and without lagging chromosomes.

In the 1 μ M reversine group (n=5), high levels of unaligned chromosomes were observed in all of the embryos (Fig. 24, 25). Two embryos had bipolar spindles, two had multipolar spindles and one displayed no alignment on the metaphase plate making it impossible to identify whether the spindle was bipolar or multipolar. Despite having many unaligned chromosomes, the bipolar spindles still successfully segregated their chromosomes into two masses. The phenotype observed in reversine-treated embryos with a bipolar spindle was consistent with observations in human somatic cells where a metaphase plate is surrounded by

many unaligned chromosomes that remain unaligned for the duration of metaphase (Santaguida, Tighe et al. 2010). Therefore, 1 μ M reversine successfully created errors in biorientation.

The timings from NEB to anaphase onset were quantified for both the control and reversine groups to see whether reversine caused a premature mitotic exit as observed in human somatic cells with an active SAC. NEB was imaged in 4 of the 5 control embryos and 3 of the 5 reversine-treated embryos. The timing from NEB to anaphase onset for the control embryos were 210, 165, 195 and 210 minutes (median of 135 minutes) compared to 180, 165 and 225 minutes (median of 150 minutes) for the reversine-treated embryos (Fig. 25). Inhibiting the SAC did not result in shorter NEB to anaphase onset timings, showing that the SAC is not responsible for setting the time of anaphase onset during the first mitosis of human embryos. This is consistent with our observations of the 10 μ M reversine treatment dataset. Because the unaligned chromosomes persisted through metaphase and these embryos did not enter anaphase prematurely, the biorientation errors observed are likely to arise as a result of the inhibition of error-correction which happens following reversine treatment.

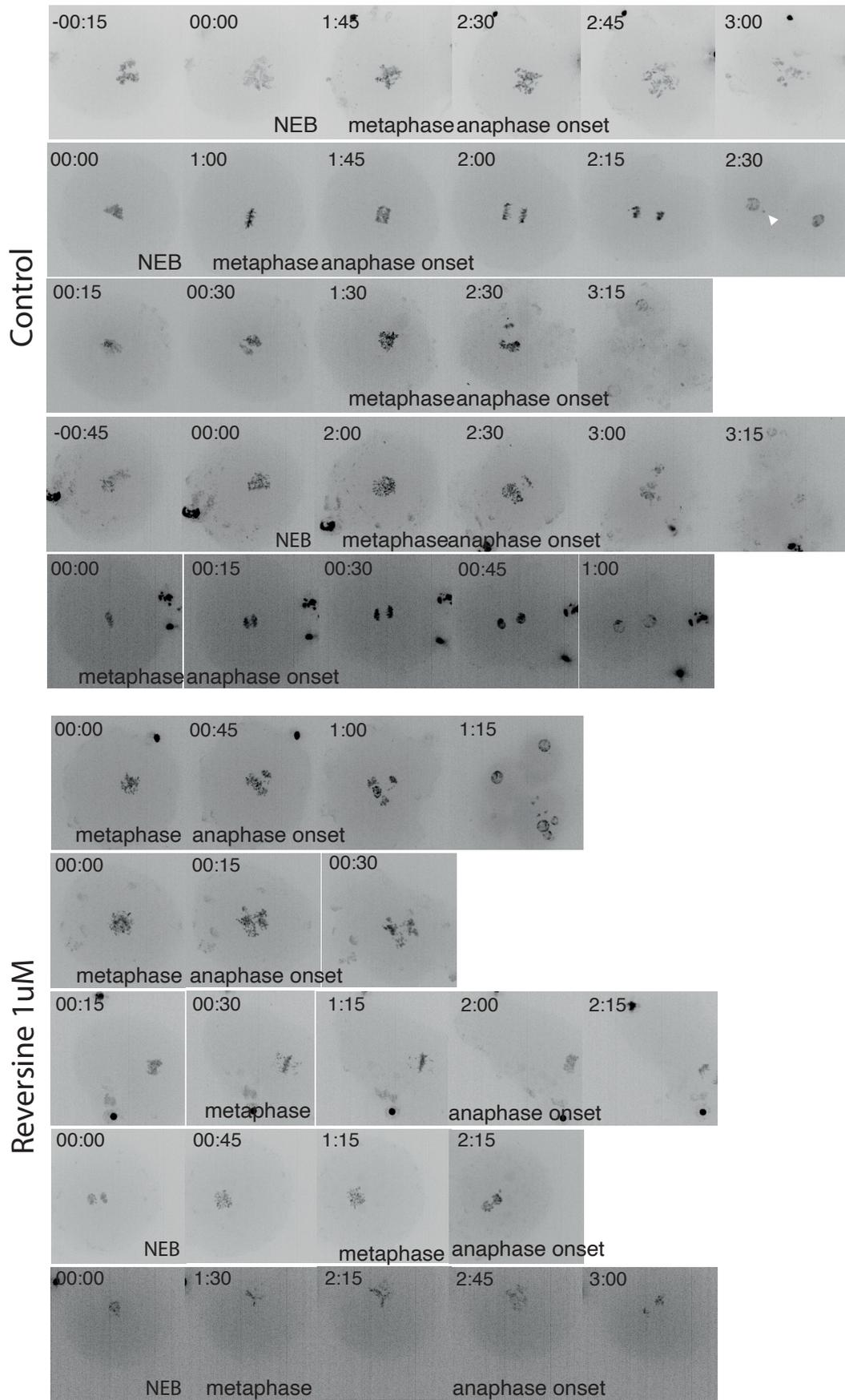


Figure 24: Mps1 inhibition in deselected human embryos with 1 μ M reversine.

Time lapse imaging of human embryos treated with 1 μ M or control embryos undergoing mitosis I using a spinning disk confocal microscope, chromosomes were stained using SiR-DNA. Time in hours: minutes. control: embryo 3346ii, 3348i, 3349i, 3349iii, 3350ii, reversine: 3346iv, 3346v, 3349v, 3349vi, 3350iv (top to bottom, see table 6). The embryos from patient 3346 and 3350 were imaged by Cerys Currie. The white arrow points to a micronucleus.

The total timings for the entire mitosis I division was 3 hours 22.5 minutes (202.5 minutes) and 3 hours (180 minutes) for the control and treatment group respectively. This is consistent with the median times of around 3 hours measured in clinical embryos which developed into healthy blastocysts and were transferred to the patient (Chapter 4.1) and reported in the literature (Vera-Rodriguez, Chavez et al. 2015). This shows that SiR-DNA staining and imaging using the spinning disk confocal microscope did not impact mitotic timings.

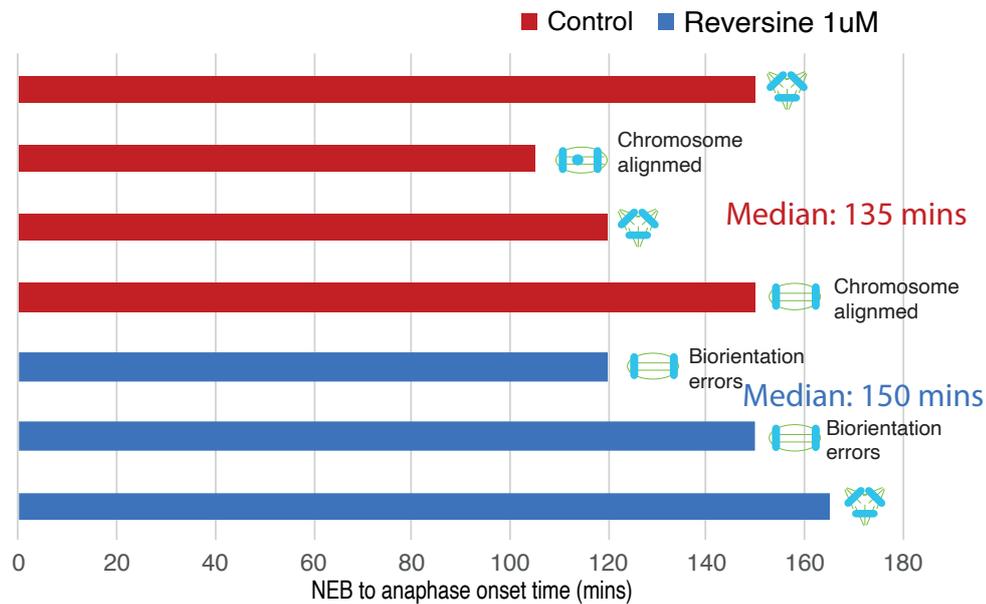


Figure 25: 1 μ M reversine treatment creates biorientation errors but no premature mitotic exit. Quantification of timings from NEB to anaphase onset from all 1 μ M reversine treated and control embryos in which we imaged NEB (stills of these embryos dividing were shown in Fig. 19, table 6). The chromosomes were stained with SiR-DNA and imaged on a spinning disk confocal microscope. The cartoons indicate the phenotypes observed. For the zygotes dividing with bipolar chromosome segregation, the alignment status just before anaphase onset is indicated (chromosome aligned or biorientation errors). The median timings for each mitotic stage are displayed in the table.

I also inhibited Mps1 in embryos from an egg sharer patient. The egg sharer patient was 32 years old and was undergoing ICSI treatment due to subfertility resulting from polycystic ovarian syndrome. This patient donated 8 mature (MII) oocytes to research which were injected with donor sperm. 4 of these embryos were treated with reversine and 4 served as a control. 3 of the reversine-treated embryos and the 4 control embryos underwent mitosis I (Fig. 26, Appendix B, table 7). In the control group, 3 out of 4 embryos underwent mitosis I with a bipolar spindle and no unaligned or lagging chromosomes. The final control embryo had a multipolar spindle and its chromosomes were segregated into 3 masses. In the 1 μ M reversine treated group, 2 embryos divided with a multipolar spindle and 1 divided with a bipolar spindle. All of the reversine-treated embryos had unaligned chromosomes during metaphase and their chromosomes were segregated into more than two masses with lagging chromosomes (Fig. 26a). Importantly, comparing the timings of anaphase onset between treated and control embryos showed that reversine treatment did not cause a premature mitotic exit (Fig. 26b). This data in egg sharer embryos is particularly important because it shows that the phenotype observed following reversine treatment is consistent between deselected and egg sharer embryos. However, due to technical problems with the concentration of CO₂ in the stage top incubator during this experiment, the mitotic timings are extended compared to embryoscope movies of embryos from the same patient. For this egg sharer patient, clinical embryos underwent mitosis in a median time of 174 minutes compared to 300 minutes for research embryos, emphasising the importance of maintaining optimal environmental conditions in the culture system to support optimal development of human embryos.

Overall, the fact that reversine treatment created biorientation errors in all of the treated embryos but failed to cause a premature mitotic exit compared to the control embryos shows that, during mitosis I, the SAC does not set the time to anaphase onset. SAC silencing once all the chromosomes are biorientated is not the trigger for anaphase. Moreover, the fact that the mitotic timings of deselected

embryos imaged with 5% CO₂ on the spinning disk confocal microscope are consistent with the timings of embryos imaged on the widefield microscope and with clinical embryos provides evidence that this new microscope did not impact mitotic timings and progression.

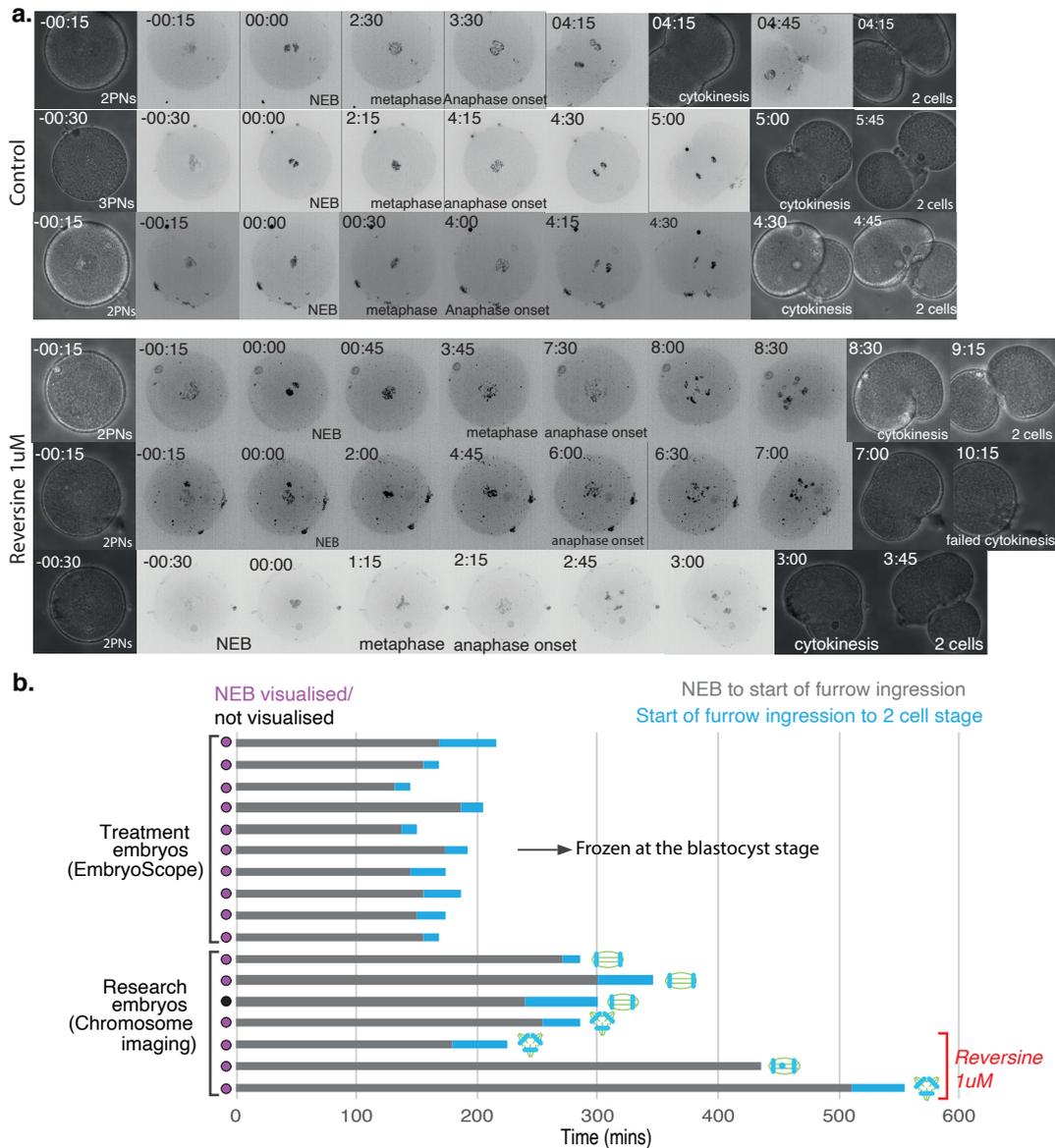


Figure 26: Mps1 inhibition in egg sharer embryos. Time lapse imaging of egg sharer embryos fertilised with donor sperm treated with 1 μ M reversine or DMSO controls imaged with a spinning disk confocal microscope. (a) Stills of control and treated egg sharer embryos from the same patient undergoing mitosis I. The chromosomes were stained using SiR-DNA. Time in hours:minutes. (b) History plots of research embryos stained using SiR-DNA and imaged on a spinning disk confocal microscope and clinical embryos imaged using an EmbryoScope™, all from the same patient (see table 7). The difference in timing between the research and clinical embryos are due to issues with the concentration of CO₂ of the stage top incubator during this particular experiment. Cartoons indicate the phenotypes observed. Pink/black dots indicate whether NEB was imaged.

Chapter 6: Discussion

In this thesis, I aimed to study chromosome movements during the first mitotic divisions to better understand what causes the high incidence of aneuploidy reported in preimplantation embryos (Vanneste, Voet et al. 2009, Chavez, Loewke et al. 2012, Fragouli, Alfarawati et al. 2013, McCoy, Demko et al. 2015, Vera-Rodriguez, Chavez et al. 2015, Starostik, Sosina et al. 2020). I found that the first mitotic division is uniquely error-prone: our embryos displayed lagging chromosomes, micronuclei and multipolar spindles. The second mitotic division was shorter and displayed much lower error rates. Moreover, I show that the spindle assembly checkpoint is weakened during mitosis I and does not determine the duration of metaphase, instead it seems that an intrinsic timer sets the time from NEB to anaphase onset. Indeed, inhibiting the SAC kinase Mps1 in human embryos did not lead to a premature mitotic exit, unlike what is seen in somatic cells, where the SAC is active (Santaguida, Tighe et al. 2010). This, combined with the observation that prometaphase and metaphase timings are inversely proportional suggests that the SAC is not holding the embryos in metaphase, ready to trigger anaphase onset when all of the chromosomes are aligned on the metaphase plate. A weak SAC could be at the origin of the high aneuploidy rates reported in preimplantation human embryos. One potential limitation of this study is that most of the embryos which I stained and imaged are deselected embryos, meaning that they were donated to research following IVF/ICSI treatment because they were mis-fertilised (1 or 3PN) or that they became fertilised after the clinical decision on whether or not the zygote is correctly fertilised and can be used for IVF or ICSI treatment was made (delayed fertilisation). Comparing this dataset of deselected eggs and embryos with egg sharer embryos (normally fertilised embryos donated to research), as well as Embryoscope™ movies of clinical embryos used for patient treatment, shows that

deselected embryos are a robust model for studying the causes of aneuploidies during mitosis I.

1. Use of human embryos in research

Very early human development remains enigmatic, not only due to ethical limitations, but also the sparsity of available material. In this study, I used embryos from different sources to give a more representative sample. I carried out live cell imaging on 38 human zygotes, which could not be used as part of the patients' treatment and were voluntarily donated to research. Accessing this material involved consenting several hundred patients in advance of their treatment beginning, and then following their treatment cycles to identify material that was no longer of use to them. I also imaged 10 zygotes from our egg sharer programme (see methods and Chapter 3.1.1). These donated oocytes were injected with donor sperm and formed normally fertilised 2PN zygotes. Accessing this material was more straightforward practically and quality was more assured but numbers were inevitably low. Finally, I analysed 330 Embryoscope™ movies of clinical embryos, some of which were transferred to the patient and gave rise to pregnancy and some of which did not.

By recording the time of nuclear envelope breakdown (NEB), alignment on the metaphase plate, anaphase onset and cytokinesis furrow ingression, our data provides the first measurements of prometaphase, metaphase and anaphase onset to cytokinesis timings during the first mitoses of human embryos. Indeed, previous mitotic timing studies in human embryos were based on Embryoscope™ movies of clinical embryos which did not have their chromosomes stained (Vera-Rodriguez, Chavez et al. 2015). However, the timing of cytokinesis (furrow ingression to two or more cells) can be measured without any staining, meaning

that I can also measure it from Embryoscope™ movies of clinical embryos. Indeed, I reported median times between 18 and 24 minutes in clinical embryos which divided into 2 cells (our transferred pregnant, transferred not pregnant and not transferred normal cytokinesis clinical datasets, Chapter 4, Fig. 16). In our research embryos, the median cytokinesis timing in our entire deselected embryo dataset was 30 minutes. However, because the embryos were imaged every 15 minutes, it is possible that the real cytokinesis timing is somewhere between 15 and 30 minutes, which is consistent with the literature and with our analysis of clinical embryos. Moreover, embryos which divided into 2 cells have a shorter cytokinesis than embryos which divided abnormally, into 3 or more cells in both clinical and research embryos. Indeed, clinical embryos which divided into more than two cells had a median cytokinesis of 39 minutes compared to 18 to 24 minutes in embryos which divided into 2 cells. The same was observed in deselected embryos which underwent cytokinesis in a median time of 30 minutes for those that divided into 2 cells compared to 60 minutes for erroneous cytokinesis.

Comparing mitotic timings of research embryos to those of clinical embryos showed that chromosome staining with SiR-DNA and long-term imaging of live human embryos did not impact the mitotic progression of human zygotes. Indeed, the median time from NEB to 2 cells of 3 hours 30 minutes measured in our research embryos which I imaged all the way from NEB to the formation of 2 cells was consistent with the timings that I measured in Embryoscope™ movies of clinical embryos which were transferred to the patient (median time of 3 hours 14 minutes and 3 hours 2 minutes in our transferred pregnant and transferred not pregnant groups respectively). Moreover, embryology papers, also based on Embryoscope™ movies, reported timing from NEBD to 2 cells of about 3 hours (Vera-Rodriguez, Chavez et al. 2015), which is consistent with our analysis of mitosis I timings. Comparing the mitotic timings of deselected embryos donated to research following IVF/ICSI treatment to clinical embryos revealed not only that the extended mitotic timings that I reported in our research are representative of

what happens in the clinic but also that clinical embryos which gave rise to pregnancy also displayed embryo to embryo variability. Indeed, embryos which underwent a very long mitosis I (from 200 to 500 minutes) still gave rise to pregnancies. This suggests that deselected embryos are a suitable model for studying the first cleavage division of human embryos.

Moreover, our egg sharer programme provided access to normally fertilised 2PN embryos which I could stain and image, allowing us to demonstrate that the chromosome segregation errors I reported are not specific to deselected embryos but also occur in presumed normal embryos. Deselected embryos from IVF/ICSI treatment may have different numbers of PN (usually 1PN or 3PN) varying from the correct 2PN, or were considered unfertilised at the time when a clinical decision was made and later showed evidence of fertilisation after they had been donated to research (delayed fertilisation). The fact that normally fertilised embryos from egg sharers frequently displayed lagging chromosomes (33%, n=10) shows that these errors do not happen only in deselected embryos and that both deselected and egg sharer embryos are useful models for studying the causes of aneuploidies in human embryos. Moreover, the fact that I reported micronuclei and multiple nuclei, which are indicative of chromosome segregation errors in 26% clinical embryos which gave rise to pregnancies (n=68) indicates that mitosis I is also erroneous in healthy embryos (Chapter 4, Fig. 17). This is consistent with data showing that 48% of human cleavage embryos displayed multiple nuclei (Kort, Chia et al. 2016), showing that chromosome segregation errors occur during one or several of the cleavage divisions. The fact that chromosome segregation errors are common not only in our deselected embryos but also in egg sharer embryos and clinical embryos shows that deselected embryos are an important tool for studying chromosome segregation in human embryos.

Taken together, the consistency of mitotic timings and chromosome segregation errors observed in deselected embryos and clinical embryos, shows that

deselected embryos are a good model for studying early human mitoses. Most previous published work on live human embryos has been based on time lapse movies of clinical embryos, meaning that they could be not stained or treated in any way (Vera-Rodriguez, Chavez et al. 2015, Jacobs, Nicolielo et al. 2020). These movies are taken of embryos as they were developing to monitor their development in the context of IVF/ICSI treatments, checking the embryo's morphology and the timings of different embryonic stages. One advantage of deselected embryos is that, because they have been donated to research and are not required for further treatment, they can be treated with different active compounds and stained to better understand the mechanisms governing mitosis I. Deselected human embryos can be successfully fixed and stained (van de Werken, van der Heijden et al. 2014, Kai, Kawano et al. 2021) but this study shows that chromosome staining and live cell imaging can be used to measure the duration of different stages of mitosis as well as chromosome segregation errors. Currently, our limited understanding of the first embryonic mitosis comes mainly from studies in mice and cattle, these studies have uncovered the processes of spindle assembly, parental genome unification and SAC activity in early embryos (Ajduk, Strauss et al. 2017, Reichmann, Nijmeijer et al. 2018, Cavazza, Takeda et al. 2021). Whether or not the first mitosis happens in a similar fashion to these model organisms in human zygotes is not known. Deselected embryos provide a much-needed model for studying mitosis I in humans. Moreover, our egg sharer program provided us with normally fertilised 2PN embryos, which I can stain and image live, providing an important control group of presumed normal embryos from young women.

Our study provides the first account of chromosome movements during mitosis I in live human embryos, both in abnormally and normally fertilised embryos.

2. Consequences of an error prone Mitosis I

2.1. What chromosome segregation errors cause aneuploidies in human embryos?

The high frequency of chromosome segregation errors reported in our human deselected embryos can potentially account for much of the high degree of aneuploidy reported in early human embryos (Vanneste, Voet et al. 2009, Chavez, Loewke et al. 2012, Chow, Yeung et al. 2014, Starostik, Sosina et al. 2020). Indeed, lagging chromosomes were present in 17.5% of deselected embryos which divided with a bipolar spindle and in 33% of egg sharer embryos (Chapter 4, Fig. 21). Such lagging chromosomes have been shown to happen as result of merotelic attachments (attachment of one kinetochore to both spindle poles) and to cause aneuploidies in human somatic cells (Cimini, Howell et al. 2001). Lagging chromosomes do not always result in aneuploidies as they can still end up in the correct daughter cell. Moreover, I have observed chromosomes which stayed behind at anaphase onset but was then corrected and joined the main chromosome mass and were therefore not scored as lagging (Chapter 4, Fig. 18). It is possible that these chromosomes which started off as lagging chromosomes were resolved by error correction mechanisms, as is the case in somatic cells (Sen, Harrison et al., 2021, preprint). However, these merotelic attachments can also lead to non-disjunction events, causing both sister chromatids to end up in the same cell, resulting in trisomies and monosomies (Fig. 27a). Lagging chromosomes in early mitotic divisions could therefore be a mechanism contributing to high aneuploidy rates in human embryos.

I also identified multipolar spindles (observed in 39.5% of our deselected human zygotes, Chapter 3, Fig. 10) as a mechanism of aneuploidies as they segregated their chromosomes into three or more masses, instead of the expected two masses, creating cells with different chromosome compositions. For example,

when looking at an embryo which segregated its DNA into 3 masses, if the cell divides into two cells, for example, one cell could end up with 1 DNA mass and the other with 2, resulting in more complex aneuploidy patterns (Fig. 27b). Indeed I observed that multipolar chromosome segregation is poorly coupled with cytokinesis, resulting in multinucleated daughter cells (see Chapter 4.1.) Such multipolar spindles could be at the origin of some of the complex aneuploidies (affecting 3 or more chromosomes) reported in the literature and associated with poor embryo development (Fragouli, Alfarawati et al. 2013, Munne, Blazek et al. 2017). Moreover, multipolar spindles have been associated with an increased number of chromosomes attached to multiple spindle poles in somatic cells (merotelic attachments), which have been identified as a cause of lagging chromosomes in somatic cells (Ganem, Godinho et al. 2009). Multipolar spindles could also give rise to aneuploidies by causing lagging chromosomes. It is important to point out that multipolar spindles tend to form in 3PN embryos which were fertilised by two spermatozoa (Kai, Kawano et al. 2021). Therefore, the incidence of multipolar spindles that I observed in our deselected dataset is likely to be an overrepresentation of what happens in the clinic, in 2PN embryos. However, I did observe multipolar spindles in some 2PN embryos (Chapter 3, Fig. 10c), showing that such phenotype can arise in normally fertilised embryos.

Overall, the chromosome segregation errors reported in our data provides an explanation of how aneuploidies occur in early embryos. Indeed, the chromosome composition of early embryos has already been well studied in cleavage and blastocyst stage embryos and, from the type of aneuploidies observed, the causes of such errors were inferred. Imaging chromosome segregation in live human embryos (observing lagging chromosomes and multipolar spindles), enabled me to uncover the mechanisms of aneuploidies during the first cleavage division. Indeed, a lot of our understanding of human aneuploidies comes from preimplantation genetic screening (PGS), where a biopsy of one cell of a day 3 embryo (cleavage stage) or 5 to 10 cells from the trophectoderm (which will form

the placenta) of a day 5 embryo are collected to undergo genetic testing to check for inherited diseases in the context of IVF/ICSI treatment. Analyses of such PGS samples have shown that about 25% of embryos have aneuploidies of mitotic origin (McCoy, Demko et al. 2015). However, one embryonic cell or a few trophoctoderm cells do not provide a complete picture of the genomic composition of an embryo. Indeed, array comparative genomic hybridisation and RNA sequencing analyses of whole frozen/thawed human embryos have revealed that a staggering 75% of preimplantation embryos are aneuploid (Vanneste, Voet et al. 2009, Chavez, Loewke et al. 2012, Chow, Yeung et al. 2014, Starostik, Sosina et al. 2020). The high rates of chromosome segregation errors that I report are consistent with the high rates of aneuploidies of preimplantation human embryos. Indeed, our observation that mitosis I is very error prone suggests that errors of mitotic origin are an important cause of aneuploidy. Varying rates of mosaicism have been reported in the literature from 4 to 90% (Capalbo, Ubaldi et al. 2017).

Additionally, our finding that chromosome segregation errors during mitosis I is maternal age independent is consistent with what was reported in the literature (Chapter 3, Fig. 13). Indeed, PGS studies have shown that, unlike errors of meiotic origin, errors of mitotic origin remain stable with maternal age and are the main cause of aneuploidies in embryos for women under 40 years of age (McCoy, Demko et al. 2015). In conclusion, our analysis of chromosome segregation errors provides an explanation for the aneuploidies of human embryos and the high error rates observed.

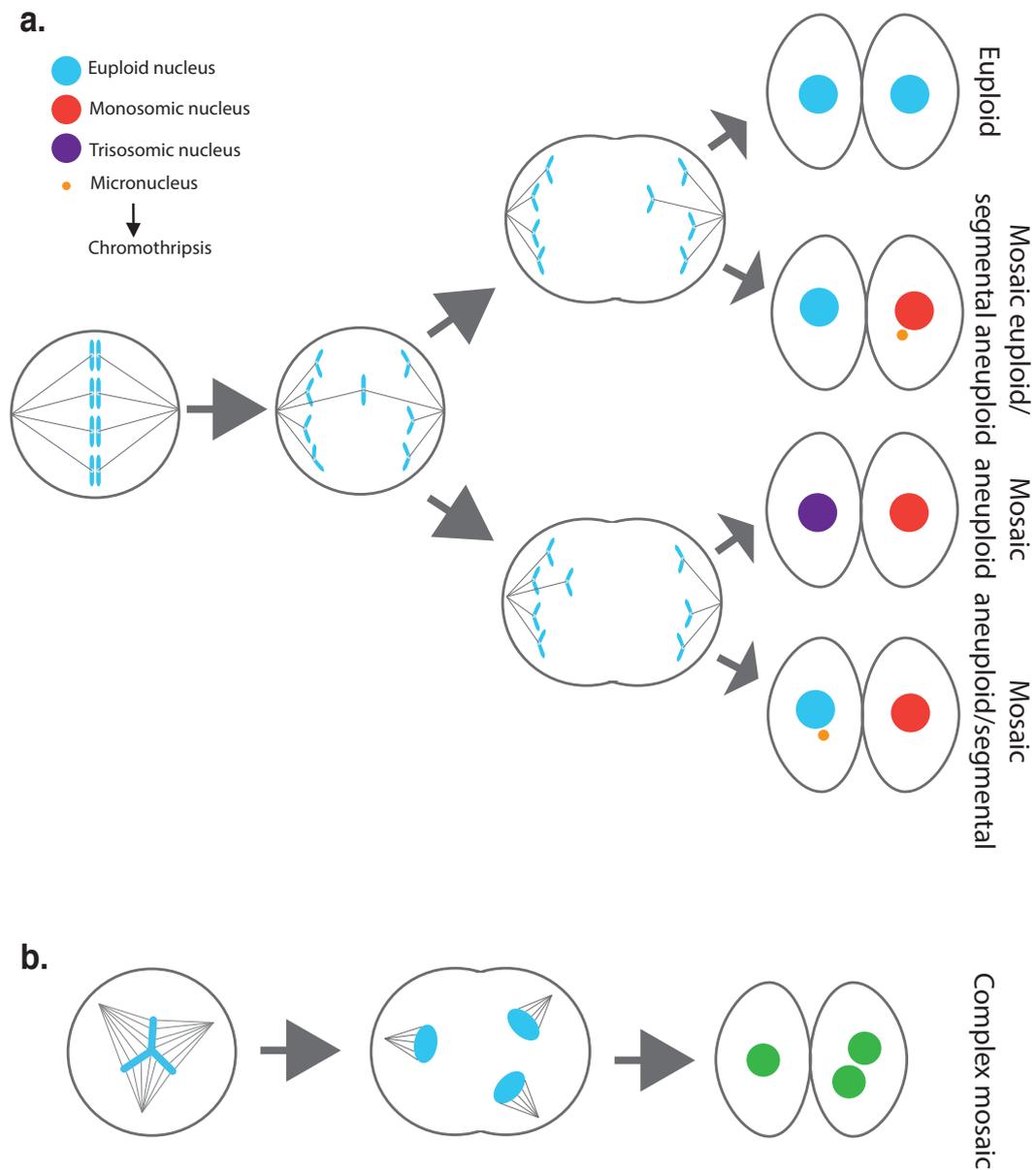


Figure 27: Consequences of chromosome segregation errors. (a) The lagging chromosome can be segregated into the correct cell (top), either giving rise a euploid cell or result in micronuclei formation, causing one of the daughter cells to be aneuploid and the other to have a monosomic nucleus and a micronucleus. Lagging chromosomes can also result in a non-disjunction event, being segregated in the same cell as its sister (bottom), forming a monosomic and a trisomic daughter cell or a monosomic cell and another with an euploid nucleus and a micronucleus. (b) By segregating chromosomes into 3 or more DNA masses, multipolar spindles can give rise to mosaic embryos with complex aneuploidies.

2.2. How does the embryo deal with such aneuploidies?

I have explained how lagging chromosomes and multipolar spindles during mitosis can cause aneuploidies. These mitotic errors will result in mosaic embryos, which contain karyotypically different cell lineages (McCoy, Demko et al. 2015). Mosaic embryos can have both euploid and aneuploid cells or only aneuploid cells, of different kinds. If all the cells harbour the same aneuploidy, this is indicative that the error occurred during meiosis, in the sperm or the oocyte. With this in mind, it is important to point out that not all mosaic embryos are the same, containing different proportions of aneuploid cells, different numbers of karyotypically distinct cell lineages and different types of aneuploidies. These different types/degrees of aneuploidies have been associated with different outcomes when it comes to embryo development, implantation and pregnancy rates. Indeed, FISH on human blastocysts have shown that 26% display a single chromosomal error, 31% have complex aneuploidies, meaning that they display a variety of chromosomally abnormal cells and finally 11% have chaotic aneuploidies, defined as a single embryo containing 4 or more unrelated cell lineages (Coonen, Derhaag et al. 2004). These different types of mitotic aneuploidies are not equivalent. Complex mosaic embryos displayed much lower implantation rates (10%) compared to embryos displaying one or two chromosomal errors (50 and 45% respectively).

To develop properly, human embryos are thought to require a certain number of cells to be euploid. Analyses of biopsies taken for preimplantation genetic screening have shown that there is a link between the proportion of the biopsied cells which were aneuploid and how likely the embryo is to implant. Indeed, embryos which had less than 40% of their biopsied blastomeres which were aneuploid had higher implantation rates (70%) than embryos which had 40-80% of their biopsy which were aneuploid (53%). This was mirrored by the miscarriage rates which were 10% in the less aneuploid group and 25% in the more aneuploid

group (Munne, Blazek et al. 2017). Moreover, aneuploidy rates are higher in cleavage embryos (83%) compared to blastocysts (53%) (Fragouli, Alfarawati et al. 2013), which could indicate that aneuploid cells have lower survival rates or that aneuploid cells are eliminated by apoptosis (Singla, Iwamoto-Stohl et al. 2020), or both. An important takeaway from this is that mosaic embryos can be viable. Moreover, the aneuploidy rates reported are likely to be an under-representation of the actual number of aneuploid embryos as they are based on a blastocyst biopsy of a few cells from PGS, and do not give a full representation of every single cell.

This idea that the proportion of aneuploid cells within an embryo can predict embryo viability is supported by data in mice where chimeric embryos composed of euploid and aneuploid cells were generated. The authors made embryos that were only composed of euploid cells or aneuploid cells, as well as chimeras displaying a 1:1 and 1:3 ratio of euploid to aneuploid cells. None of the embryos containing only aneuploid cells survived; the 1:3 chimeras displayed decreased survival rates and all the 1:1 chimeras survived, along with the euploid embryos (Bolton, Graham et al. 2016). This data shows that mosaic mouse embryos are viable providing that enough euploid cells are present within the embryo. Similar experiments cannot be performed in humans for ethical reasons. Moreover, it is important to note that the idea that aneuploidies negatively correlate with pregnancy is challenged by clinical trials which have not found any significant improvement in pregnancy rates following biopsy and aneuploidy testing in human embryos (Mastenbroek, Twisk et al. 2011).

Later in development, at the blastocyst stage, the embryo has mechanisms in place to deal with aneuploidies. Data in mice showed that aneuploid cells are eliminated via apoptosis and embryo development relies on the proliferation of chromosomally normal cells (or cells which have less severe aneuploidies) (Singla, Iwamoto-Stohl et al. 2020). In human embryos, apoptosis was observed from the

blastocyst stage, but not before, (Hardy 1999, Jacobs, Van de Velde et al. 2017) and there is evidence that human embryos lacking some blastomeres, due to their removal as biopsies or due to damage during cryopreservation, can successfully implant and give rise to pregnancies at similar rates to normal embryos, supporting the idea that human embryos can survive with a smaller number of cells. This is based on retrospective studies of human embryos which were frozen at the 7-8 cell stage: some of them were intact and some of them had one or two blastomeres lysed as a result of the thawing process (Zheng, Liu et al. 2008). This data shows that embryos with some degree of aneuploidy are viable and can lead to pregnancy. Later during development, from the blastocyst stage, some of these very aneuploid blastomeres can also be eliminated via apoptosis. However, embryos displaying very high aneuploidy rates have a poor prognosis. When there are too many highly aneuploid cells, these cannot be corrected with apoptosis as there would not be enough euploid cells left to support embryonic development.

In summary, the viability of embryos with chromosome segregation errors depends on the type of errors occurring and how many cells are affected by them. Our observation that 26% of 2 cell embryos which gave rise to pregnancies had multiple nuclei or micronuclei shows that chromosome segregation errors are both common and compatible with human life (Chapter 4, Fig. 17b). However, embryos with complex aneuploidies or with a high proportion of aneuploid cells have been associated with lower implantation rates. It is therefore crucial to understand how these errors arise, what types of errors occur and how does the embryo deal with such errors in subsequent divisions.

2.3. Micronuclei form around lagging chromosomes during mitosis I and their fate in future divisions

We have shown, for the first time, that micronuclei can form from lagging chromosomes during the mitosis I of human embryos (Chapter 3, Fig. 12). This sheds light on the possible consequences of lagging chromosomes in human zygotes. Bovine and mouse embryos also formed micronuclei as a result of lagging chromosomes (Brooks, Daughtry et al. 2020, Vazquez-Diez, Yamagata et al. 2016). Micronuclei have previously been observed frequently in human preimplantation embryos and in blastocysts (Chavez, Loewke et al. 2012, Kort, Chia et al. 2016) but because, these studies were performed on fixed material or on movies where the DNA was not stained, we can only hypothesise on how these micronuclei were formed. Moreover, our observation of micronuclei in 13.2% of clinical embryos, analysed during the first mitosis, still gave rise to pregnancies, suggests that micronuclei in early mitotic divisions are compatible with successful embryo development and implantation (Chapter 4, Fig. 17b). This raises the question of what happens to these extra nuclei in subsequent divisions.

Our data suggest that multiple or micronuclei occurring during mitosis I can be corrected during subsequent cell divisions. Indeed, in clinical embryos which gave rise to pregnancies, the number of embryos displaying multiple nuclei or micronuclei dropped dramatically from 26.5 % to 2% between the 2-cell and 4-cell stage (Chapter 4, Fig. 17). Data in mouse embryos has shown that micronuclei

remain intact and become inherited by daughter cells (Chavez, Loewke et al. 2012, Vazquez-Diez, Yamagata et al. 2016). Indeed, half of the micronuclei observed in mice fused with the main nucleus and the other half were unilaterally inherited by the daughter cells. The dramatic reduction in the number of micronuclei observed at the 4-cell stage compared to the 2-cell stage in clinical embryos which gave rise to pregnancy, suggest that micronuclei are preferentially reabsorbed into the main nucleus as opposed to unilaterally inherited. This could suggest that persistent micronuclei could hinder embryonic development. This is supported by the literature: micronuclei and multiple nuclei in human cleavage embryos were shown to be associated with aneuploidies, poor blastocyst formation and reduced implantation rates (Kligman, Benadiva et al. 1996, Meriano, Clark et al. 2004, Kort, Chia et al. 2016). Moreover, it appears that multinucleated embryos are associated with more complex aneuploidies. Cytogenetic studies have shown that abnormally nucleated embryos display a high degree of mosaicism compared to embryos with one nucleus which were mostly euploid or had low levels of mosaicism (Kort, Chia et al. 2016). It seems that micronuclei do not form as commonly in later divisions or that many of these cells containing micronuclei are eliminated by apoptosis: a mechanism which was shown to remove very aneuploid cells in the blastocyst (Hardy 1999, Singla, Iwamoto-Stohl et al. 2020). Indeed, studies on fixed cleavage state human embryos reported that 48% of cleavage embryos had multiple nuclei with 16% of their blastomeres having two or more nuclei. When looking at the blastocyst stage, 84% of embryos had at least one blastomere with multiple nuclei. However, the proportion of cells with multiple

nuclei was much lower, with only 5% of blastomeres affected (Kort, Chia et al. 2016). This holds true in mouse models: these abnormally nucleated embryos can give rise to pregnancy, consistent with my findings. Multinucleated mice embryos had blastocyst formation rates of 53% compared to euploid embryos or embryos with minor aneuploidies which had blastocyst formation rates of 93% to 100% (Mashiko, Ikeda et al. 2020). The fact that micronuclei give a developmental disadvantage explains why the clinical embryos which gave rise to pregnancy in our dataset had lower multinucleation rates at the 4-cell stage (2%) than in the previously published data, which was based on cryopreserved embryos and “poor quality fresh embryos” (48%) (Kort, Chia et al. 2016). We expect to see a lower proportion of multinucleated cleavage stage embryos when looking at embryos which gave rise to pregnancies as these nuclear abnormalities provide a developmental disadvantage.

In summary, lagging chromosomes can be sequestered into micronuclei which can merge with the main nucleus in subsequent divisions or become unilaterally inherited (Fig. 27a). Despite being compatible with pregnancies, persistent micronuclei have been associated with poor prognosis and our data showed that micronuclei which were formed during mitosis I were mostly corrected by the end of mitosis in embryos which gave rise to pregnancy.

2.4. Micronuclei are a site of DNA damage.

Micronuclei were associated with segmental aneuploidies, where part of a chromosome is gained or lost in non-human primates (Daughtry, Rosenkrantz et al. 2019). This also seems to be the case in human cleavage embryos: karyotype analyses of 3 cleavage embryos with micronuclei revealed that 2 of them had segmental aneuploidies. This is in contrast with embryos with normal nuclei, which were mostly euploid or had a single whole chromosome aneuploidy and binucleated embryos which had whole chromosome aneuploidies (Kort, Chia et al. 2016). This link between micronuclei and segmental aneuploidies needs to be looked at on a larger scale in human embryos.

Varying rates (15%-70%) of segmental aneuploidy have been reported in human cleavage embryos, based on PGS samples (Vanneste, Voet et al. 2009, Fragouli, Alfarawati et al. 2013). These segmental errors appear to happen following mitotic divisions in embryos and were only rarely seen in the oocyte; meiotic errors tend to affect whole chromosomes, as a result of a loss of cohesion for example (Vanneste, Voet et al. 2009, Fragouli, Alfarawati et al. 2013, Kort, Chia et al. 2016). Segmental errors have been reported to be more than halved from cleavage to blastocyst stage (Fragouli, Alfarawati et al. 2013), indicating that these errors provide a developmental disadvantage.

I have described the association between micronuclei and segmental aneuploidies, as well as their negative impact on embryonic development. However, an interesting question which arises is how can micronuclei cause such segmental errors? Chromosomes sequestered in micronuclei were shown to undergo extensive damage in cancer cells through chromothripsis, which is a mutational process causing many genomic rearrangements to happen as a result of a single event (Liu, Erez et al. 2011, Zhang, Spektor et al. 2015). This results in complex segmental aneuploidies, characterised by multiple DNA breaks and also

duplications in some cases, a phenotype reported in 55% of the cleavage human embryos studied by Vanneste and colleagues (Vanneste, Voet et al. 2009). Chromothripsis in human embryos has not been investigated in detail, however, the observation that embryos with micronuclei have high levels of DNA damage compared to control embryos (87.1% vs. 9.3%) suggests that chromothripsis does in fact take place in human embryos. This is based on experiments staining normally and abnormally fertilised embryos with a marker for DNA damage: phosphorylated histone H2A.X (γ H2AX) (Kort, Chia et al. 2016). This is also the case in non-human primates: single cell sequencing of cleavage embryos has shown that the DNA encapsulated within the micronuclei had double stranded DNA breaks (Daughtry, Rosenkrantz et al. 2019).

This data shows that micronuclei are sites of mutagenesis and DNA damage, resulting in segmental aneuploidies, which are associated with poorer embryo viability and outcome but are not incompatible with pregnancy, as shown by our analysis of clinical embryos. This data sheds light on a potential model explaining the presence of segmental abnormalities in human embryos: lagging chromosomes can be isolated in a micronucleus, inside of which they can undergo DNA damage through the process of chromothripsis. This would suggest that, even if the micronuclei become reabsorbed into the main nucleus, chromothripsis would cause the resulting embryos to have segmental aneuploidies (Fig. 28). DNA damage within micronuclei could cause complex aneuploidies in human embryos.

Furthermore, evidence in mice embryos has shown that chromosomes within these micronuclei fail to assemble a kinetochore. Indeed CREST (anti-kinetochore antibody) failed to localise at the centromere, unlike chromosomes in the main nucleus. They also did not stain for CENP-A, a centromeric protein crucial for kinetochore assembly (Vazquez-Diez, Yamagata et al. 2016). This appears to hold true, to some extent, in human embryos but it is not as clear cut as both aneuploidies with and without centromere were observed in embryos with

multi/micronuclei. Indeed, 16.3% of abnormally nucleated embryos had only aneuploidies lacking centromeres, 48.8% contained a mix of aneuploidies with and without centromeres and 34.9% had only aneuploidies with centromeres (Kort, Chia et al. 2016). This would explain why, in mouse embryos, micronuclei formation during the first mitotic division always led to aneuploidies during the second mitotic division (Mashiko, Ikeda et al. 2020). Chromosomes lacking kinetochores within these micronuclei would be unable to segregate alongside the other chromosomes within the main nucleus, perpetuating aneuploidies. In conclusion, chromothripsis uncovers another mechanism leading to aneuploidies following micronuclei formation during the first mitosis of human embryos.

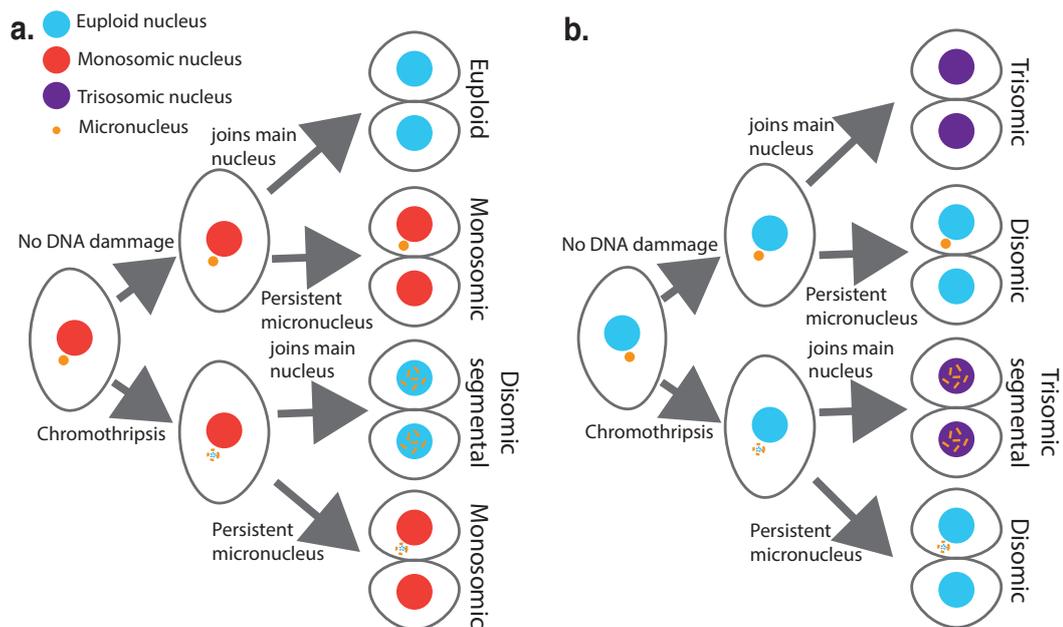


Figure 28: Fate of micronuclei in subsequent divisions. (a) Fate of cell with a monosomic nucleus and a micronucleus (as shown in Fig. 23) in the case of chromothripsis or when no DNA damage occurs. The micronucleus can persist or be inherited unilaterally by one of the daughter cells or join with the main nucleus. This can create euploid, monosomic and disomic with segmental aneuploidies cell lineages. (b) Same as (a) but for a cell with a euploid nucleus and a micronucleus (as shown in Fig. 23). This can create disomic and trisomic cell lineages, with or without segmental aneuploidies.

3. Why are there lagging chromosomes and multipolar spindles?

3.1. Multipolar spindle formation in human zygotes

Our data has shown that multipolar spindles are a common chromosome segregation defect during mitosis I. I wanted to know what are the potential mechanisms by which these multipolar spindles can arise.

Our observation that most multipolar spindles form in embryos which had 3PN suggests that fertilisation with 2 spermatozoa during IVF and the subsequent delivery of two centrosomes is the most common mechanism of multipolar spindle formation (Chapter 3, Fig. 10c). Indeed, unlike mice, where spindle nucleation during the first cleavage divisions happens in the absence of centrosomes (Reichmann, Nijmeijer et al. 2018, Vazquez-Diez and FitzHarris 2018), human embryos inherit a single centrosome and centriole from the sperm upon fertilisation (Sathananthan, Kola et al. 1991). Experiments where pronuclei from human zygotes were transferred into mice zygotes have shown that the centrosome inherited paternally in humans is functional (Van Thuan, Wakayama et al. 2006). In these 3PN embryos, which formed due to fertilisation by two spermatozoa, 4 centrosomes were observed, each forming a microtubule organising centre (MTOC) after NEB, from which the microtubules were nucleated. This resulted in quadrupolar spindles with each centrosome establishing a spindle pole (Kai, Kawano et al. 2021). This shows that, because microtubules are nucleated from the centrosomes, which are brought in by the sperm, incorrect fertilisation by two spermatozoa resulting in 3PN zygotes is a common mechanism for multipolar spindle formation in human zygotes. Importantly however, our live-cell imaging analyses of human embryos have shown that these multipolar

spindles can form in zygotes which have 2PN (correctly fertilised). I observed multipolar chromosome segregation on two embryos which had 2PN (Chapter 4, Fig. 10c). Out of the 2PN deselected embryos undergoing mitosis I (n=9), 22% had multipolar spindles. This shows that there are mechanisms other than dispermic fertilisation that cause multipolar spindles during mitosis I. Moreover, the idea that multipolar spindles can arise in 2PN zygotes is supported by analyses of clinical embryos. Indeed, because multipolar spindles segregate their chromosomes into more than 2 DNA masses (see paragraph 2.1), which results in multinucleated blastomeres (Fig 22.b), the fact that 13% of 2 cell embryos which gave rise to pregnancy had multiple nuclei and no micronuclei suggests that these embryos divided with a multipolar spindle. This shows that, even though multipolar chromosome segregation during mitosis I arises much more commonly in 3PN than in 2PN zygotes, multipolar spindles can form in 2PN zygotes. Moreover, our data on clinical embryos which gave rise to pregnancy suggests that multipolar chromosome segregation during mitosis I, resulting in multinucleated blastomeres is compatible with human life. The question becomes: what causes multipolar spindles to form in normally fertilised embryos?

One hypothesis is that centrosome duplication errors could lead to multipolar spindles, eventually leading to aneuploidies. The centrosome needs to be duplicated before the first mitosis, upon sperm entry. Defective centrosome duplication has previously been associated with aneuploidies in human embryos. Indeed, analyses of preimplantation genetic screening samples from preimplantation human embryos have revealed a correlation between a single nucleotide polymorphism in the centriole duplication replication gene PLK4 and aneuploidies of mitotic origin (McCoy, Demko et al. 2015, Vazquez-Diez and FitzHarris 2018). However, centrosome replication has not been studied during mitosis I specifically. Therefore, I can only speculate as to whether or not defects in centriole replication could be the origin of some of the multipolar spindles observed in our dataset of embryos undergoing mitosis I.

A further potential mechanism for multipolar spindle formation during mitosis I could be explained by the observation that two distinct spindles (one maternal, one paternal) form and then fuse together to form one singular bipolar spindle in mice and cattle (Chapter 1.5.1) (Reichmann, Nijmeijer et al. 2018, Schneider, de Ruijter-Villan et al., 2020). Following these findings in mice, a dual spindle was reported in a single fixed human zygote (Xu, Li et al. 2019). However, $n=1$ and the spindle morphology is not particularly clear. From the literature, it remains uncertain whether dual spindles occur in humans. Our observation of two spatially separate spindles in two live human zygotes suggests that dual spindles can form in humans. This unique spindle type during the first cleavage division could explain some of the chromosome segregation errors observed. Indeed, data in mice showed that a failure to align the two spindles resulted in anaphase in different directions, leading to binucleated blastomeres. Increasing the distance between the 2PN, caused the spindles to be too far from each other to align (Reichmann, Nijmeijer et al. 2018). It has also been reported in bovine zygotes that 38% of dual spindles failed to merge, causing parental genomes to be segregated independently (Schneider, de Ruijter-Villani et al. 2021). Therefore, dual spindles could be at the origin of chromosome errors happening during mitosis I in humans. Failure to align the spindles and merge one or both of their poles could lead to multipolar chromosome segregation and aneuploidies. Indeed, if only two of the poles successfully merge and the other two fail to do so, the spindle would become a tripolar spindle and the DNA would be segregated into 3 masses: one with both parental genomes (where the two poles successfully merged), one with paternal DNA and one with maternal DNA (Fig. 29). I have shown, in our live embryos, that multipolar divisions are not closely coupled with cytokinesis, with 61.5% of them still dividing into 2 cells despite multipolar chromosome segregation. Because cytokinesis normally happens along the spindle midbody but, in such an instance there would be two spindle midbodies, we could end up with one cell containing 2 DNA masses (the one with both parental genomes and

one with only one parental genome) and the other with one DNA mass from one parent. Indeed, this describes uniparental genome segregation, a chromosome segregation error that is observed in bovines and non-human primates where some embryos have blastomeres containing the genome of only one parent (Destouni, Zamani Esteki et al. 2016, Daughtry, Rosenkrantz et al. 2019). Uniparental genome segregation was shown to happen in normally fertilised bovine zygotes, not just in the mis-fertilised ones (Destouni, Zamani Esteki et al. 2016). In summary, I propose the failure of dual spindles to correctly fuse during mitosis I as a potential cause of aneuploidies, leading in some cases to the formation of blastomeres containing the genome of one parent only. Genetic analyses have reported such uniparental blastomeres in bovine embryos. Importantly, this phenotype was observed in normally fertilised 2PN embryos (Destouni, Zamani Esteki et al. 2016). This unique spindle assembly during the first mitotic division could explain why this division is uniquely error prone.

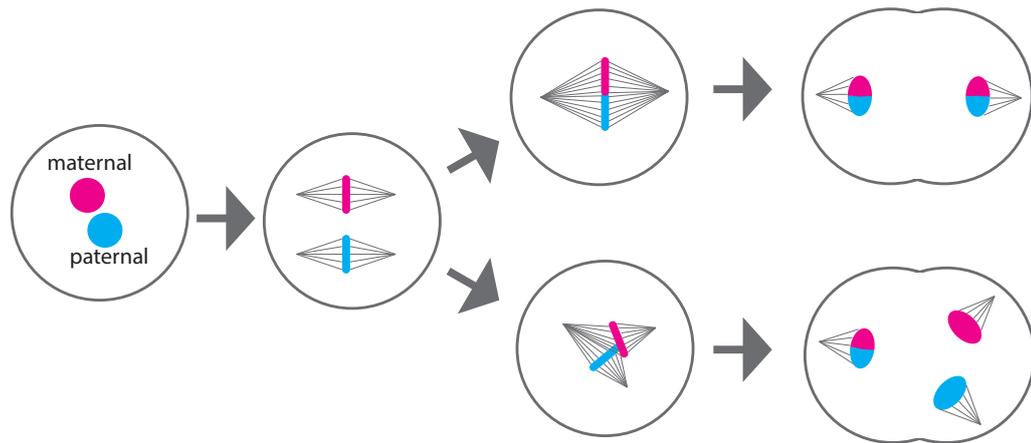


Figure 29: Consequences of the failure to correctly align and fuse dual spindles during mitosis I. The maternal chromosomes are shown in pink and the paternal chromosomes in blue. When alignment and fusion of the two spindles happens correctly, the DNA is segregated into two DNA masses, containing maternal and paternal DNA (top). When two of the spindle poles fuse but the other two fail to do so and remain apart, we expect to see chromosome segregation into three DNA masses, similar to what we observe in multipolar spindles. This would result in one DNA mass containing both parental genome (as a consequence of two spindle poles fusing) and two DNA masses with only one parental genome (as a consequence of two spindle poles remaining apart) (bottom).

3.2. Why are lagging chromosomes occurring during mitosis I?

Our experiments on live human zygotes provide the first observation of lagging chromosomes being present during mitosis I of human embryos. I have previously explained how these can lead to aneuploidies. However, the mechanisms causing lagging chromosomes during mitosis I in humans are largely unknown.

The mitosis I spindle is unique as it has to enclose both parental genomes from two different pronuclei, which will be segregated alongside each other for the first time. The high rate of chromosome segregation errors in mitosis I could be rooted in this unique spindle formation. One hypothesis is that lagging chromosomes could occur from inefficient chromosome capture by the microtubules growing

from the paternally inherited centrosomes. Indeed, the first mitotic spindle in humans was shown to be nucleated from the centrosomes, preferentially capturing chromosomes which are close to the centrosomes (Kai, Kawano et al. 2021). The chromosomes from each parent are enclosed in two pronuclei and they tend to still be physically separated just after NEBD. This is what I observed in our human embryo dataset and what Kai and colleagues observed in their 3PN human zygotes (Kai, Kawano et al. 2021). In some circumstances in humans, the two pronuclei undergo NEBD at different time points from 10 minutes to some hours apart, when observed in clinical embryos (G Hartshorne personal communication) and in bovine embryos (Brooks, Daughtry et al., 2020). It could be that, because of this spatial and possibly temporal separation, two MTOCs are not enough to capture all chromosomes. Indeed, in mice, the same process relies on many MTOCs (Reichmann, Nijmeijer et al. 2018). Moreover, if two distinct spindles are formed during mitosis I in humans, as in cattle and mice (Chapter 1.5.1) (Reichmann, Nijmeijer et al. 2018) (Schneider, de Ruijter-Villan et al., 2020), it could be that nucleation from several poles would cause lagging chromosomes. Indeed, multipolar spindles increase the number of merotelic attachments formed in human somatic cells (Ganem, Godinho et al. 2009). These merotelic attachments were shown to be the primary cause of lagging chromosomes (Cimini, Howell et al. 2001).

Recent work in bovine embryos has uncovered a further potential mechanism causing chromosome segregation errors during the first mitotic division. Cavazza and colleagues have shown that zygotes cluster their DNA at the interface of the two pronuclei. This clustering is believed to promote rapid parental genome unification. Indeed, zygotes which successfully clustered their genomes were more likely to divide without chromosome segregation errors. However, it was shown to be an erroneous process, with embryos often failing to cluster their genomes, resulting in higher segregation error rates and micronuclei formation. There has been a longstanding debate as to whether clustering of pronucleoli

visible in pronuclei using time lapse in human IVF laboratories is associated with embryo quality and subsequent potential. It is, however, it is well known that embryos with a range of suboptimal configurations can still produce normal pregnancies and babies. Hence, the importance of this finding is questionable.

Furthermore, the cause of chromosome segregation errors could be rooted in processes which happen long before the first mitotic division even starts. Indeed, the potential of an oocyte to develop into a healthy embryo is decided before fertilisation and before meiosis I resumptions. The oocyte needs to undergo a series of changes, known as cytoplasmic maturation, to allow meiosis, fertilisation and early embryogenesis to be completed (Conti, Gul et al. 2019). Microtubule and ER complexes are formed and constitute the main store of calcium, allowing the oocyte to respond appropriately to sperm entry by triggering calcium oscillations (Trebichalska, Kyjovska et al. 2021). Moreover, the oocyte relies heavily on mitochondria to respond to the high energy demands of mRNA translation prior to meiosis resumption but also to support the dynamic microtubule network which allow chromosome segregation during meiosis and mitosis. We can hypothesise that failure to rearrange the organelle in the cytoplasm properly and to translated maternal mRNAs efficiently could lead to incorrect chromosome segregation. Indeed, if not enough mRNAs are translated due to improper, this could lead to low levels of key mitotic proteins, such as those forming the kinetochore, proteins involved in SAC signalling and proteins involved in error correction. Moreover, a suboptimal mitochondria-ER network which is unable to support the dynamic structures which are microtubules, could lead to the failure to form a bipolar spindle which captures all chromosomes. Indeed, enlarged ER networks with few mitochondria have been observed in the oocytes of infertile patients (Trebichalska, Kyjovska et al. 2021), highlighting the important of optimal cytoplasmic maturation to support fertilisation.

In conclusion, this data shows that mitosis I differs from other divisions as the parental genomes are initially separated into two pronuclei. Moreover, because embryonic genome activation (EGA) has not yet happened, the oocyte relies entirely on maternal mRNAs and on organelles which have been rearranged before the resumption of meiosis I for fertilisation and the completion of mitosis. This has implications for chromosome capture, spindle nucleation and genome unification. Therefore, it seems that it is these unique features of mitosis I which cause this division to be so error prone.

4. Is there a functional spindle assembly checkpoint in the zygote?

4.1. The SAC does not set the timing of anaphase onset in human zygotes

Human preimplantation embryos have high levels of aneuploidy (see Chapter 1.4.2, Chapter 6.2) (Vanneste, Voet et al. 2009, Chavez, Loewke et al. 2012, Fragouli, Alfarawati et al. 2013, Vera-Rodriguez, Chavez et al. 2015, Starostik, Sosina et al. 2020). Cells usually have mechanisms to ensure that aneuploidies do not occur. Error correction mechanisms are responsible for destabilising erroneous kinetochore-microtubule attachment and the SAC causes an arrest in metaphase until all of the chromosomes are attached correctly to the spindle poles (Stukenberg and Burke 2015, Kataria and Yamano 2019). Therefore, I wanted to look at the SAC's activity because a weak SAC could be at the origin of these high aneuploidy rates.

In this study, I have shown that the SAC is not responsible for setting the duration of metaphase during mitosis I of human embryos, i.e. maintaining the zygote in metaphase until all chromosomes are aligned. I silenced the SAC by treating embryos with reversine, which inhibits Mps1 – a kinase essential for both SAC signalling and error correction (Stukenberg and Burke 2015, Kataria and Yamano 2019) (Chapter 5). If there is an active SAC in first mitosis then I would expect anaphase onset to take place prematurely, without correct alignment. This would result in many chromosome segregation errors as well as a short metaphase time, similar to what is seen when human somatic cells are treated with reversine (Santaguida, Tighe et al. 2010). However, the fact that reversine-treated embryos did not display shortened timings from NEB to anaphase onset compared to control embryos (135 and 150 for control and 1 μ M reversine respectively, Chapter 5, Fig. 24, 25) demonstrates that the SAC does not cause a delay in

metaphase until the chromosomes are attached during mitosis I in human embryos. Instead, our data suggests that there is an intrinsic timer which sets the timing from NEB to anaphase onset. This is based on the observation that, in our deselected embryo dataset, prometaphase and metaphase timings were inversely proportional (Chapter 3, Fig. 9). This finding is important as it provides an explanation of why aneuploidies are so prevalent in early human embryos. It also highlights the need to study this enigmatic mitosis further in human embryos as this mitosis I happens very differently to the mitoses of somatic cells. To our knowledge, this is the first time the SAC has been studied in human zygotes.

In contrast to what I observe in human embryos during mitosis I, the SAC has been shown to be functional in mouse zygotes. Indeed, overexpression of the SAC components Bub3, BubR1 and Mad2 in mice zygotes inhibited anaphase. Inhibition of SAC components resulted in biorientation problems, errors in chromosome segregation and importantly, a reduced time to anaphase onset (Wei, Multi et al. 2011). This data provides strong evidence to show that the SAC is functional during the first mitosis of mice embryos. This also suggest that mice embryos are not a good model when it comes to studying SAC activity as SAC inhibition in mouse zygotes resulted in premature mitotic exit, unlike what I observed in human zygotes proving that SAC activity is very different during the mitosis I of in mice and human embryos. This difference in SAC activity could explain why mouse embryos display significantly fewer errors than human embryos: about 75% of human preimplantation embryos are mosaic compared to 25% in mice (Fragouli, Alfarawati et al. 2013, Mashiko, Ikeda et al. 2020, Starostik, Sosina et al. 2020).

However, it is possible that there are other mechanisms, on top of the SAC setting the time of anaphase onset. Indeed, a study in mice aiming to uncover the mechanism behind why the first mitosis is double the length of the second have shown that SAC inhibition with reversine caused a 10-20 minute acceleration in

the degradation of the cell cycle proteins cyclin A2 and B1 compared to control embryos (Ajduk, Strauss et al. 2017). This 10-20 minute decrease in duration in response to SAC inhibition is not sufficient to account for the 30+ minute increase in NEB to anaphase onset timings in mitosis I compared to mitosis II (Ajduk, Strauss et al. 2017). This, added to the fact that SAC proteins were shown to disappear from kinetochores before the metaphase plate formed (Sikora-Polaczek, Hupalowska et al. 2006), provides a compelling argument to suggest that there is a mechanism other than the SAC triggering anaphase onset.

Our data has shown that the SAC does not set the time to anaphase onset. However, this weak SAC appears to be specific to early embryos. Studies in human blastocysts treated with the microtubule poison nocodazole resulted in a higher proportion of cells in metaphase compared to control embryos, suggesting that the SAC is causing mitotic delay in blastocysts (Jacobs, Van de Velde et al. 2017). However, these embryos already had their genome activated, meaning that the proteins controlling the cell cycle at this point are synthesised from the embryo's genome, not from mRNA inherited from the oocyte. Therefore, the fact that the SAC is active in blastocysts does not help us in understanding what is happening in early divisions, hence the need to study the SAC in cleavage embryos.

4.2. Error correction in cleavage embryos

Mps1 has a role both in SAC signalling and error correction, a mechanism where incorrect attachments are destabilised by Ndc80 and Ska phosphorylation (Kataria and Yamano 2019). The fact that I created severe biorientation errors in human embryos undergoing mitosis I but that the embryos did not exit mitosis prematurely, suggest that these errors were created as a result of error correction inhibition and not because they underwent anaphase too early, not having the time to correct these errors. This indeed suggests that error-correction is active in

the first cleavage embryos. The correction of erroneous kinetochore-microtubule attachments during mitosis has not previously been studied in human embryos.

Many proteins of the SAC are also involved in chromosome alignment and error-correction, making it difficult to know for sure if the phenotypes observed are caused by SAC inhibition or by the inhibition of other mechanisms. For example, BubR1 inhibition in bovine embryos resulted in many micronuclei and multiple nuclei as well as chaotic aneuploidies, suggesting that chromosomes were segregated into many different masses with lagging chromosomes (Brooks, Daughtry et al., 2020). However, because BubR1 is also required for chromosome alignment on the metaphase plate in somatic cells, the chromosome segregation errors observed could be due to erroneous kinetochore-microtubules attachments as a result of BubR1 inhibition, not necessarily because the SAC failed to cause a mitotic arrest until all the chromosomes were aligned.

4.3. What could cause this weakening of the SAC?

The SAC has not previously been studied during mitosis I of human zygotes. Therefore, I wanted to study what causes its inefficiency in early human embryos to discover why it is unable to cause mitotic arrest. One possibility is that SAC genes are downregulated in early human embryos. This is based on a study by Wells and colleagues who reported low levels of SAC mRNA transcripts in 2 to 3 cell human embryos (Wells, Bermudez et al. 2005). At this early stage in development, all the mRNAs present in the embryo are maternally inherited and were transcribed in the oocyte. Later in development, at the 4-8 cell stage, the embryo's genome will become activated, leading to a major wave of transcription (Chapter 1.3.1), meaning that SAC genes could become expressed at a higher level, perhaps explaining why blastocysts harbour fewer aneuploidies than cleavage stage embryos (Fragouli, Alfarawati et al. 2013). Blastocysts have been shown to

express SAC genes such as MAD2 and BUB1 at much higher levels than earlier embryos (Wells, Bermudez et al. 2005).

Another hypothesis is that the zygote's very large cell volume (110-120 μm diameter) could explain why the SAC is unable to cause mitotic delay. Indeed, it has been shown in human oocytes that an increase in cell volume decreases the SAC's stringency, whilst a reduction in cell volume has the opposite effect (Kyogoku and Kitajima 2017). It could be that SAC proteins as well as anaphase inhibitors are too diluted in this large cytoplasmic volume and thus unable to produce a signal strong enough to cause a mitotic arrest. Whether this is the case in human early embryonic divisions is unknown. However, the fact that in mouse embryos, the SAC is active during mitosis II (large cells) but not in the 4-8 cell (smaller cells), but then active again at the blastocyst stage which has even smaller cells suggests that there is no obvious link between cell size and SAC activity in early mouse development (Vazquez-Diez, Paim et al. 2019).

In conclusion, the SAC's inability to cause a mitotic arrest in human embryos is no doubt contributing to the high levels of aneuploidies observed in preimplantation embryos. However, what causes the SAC to be inactive/weak is unknown but it is likely to be due to low levels of SAC gene expression possibly coupled with a high cytoplasmic volume, further diluting the already low number of SAC mRNAs and proteins. A hypothesis explaining the very error prone chromosome segregation mechanisms in early embryonic development observed in species that produce a small number of offspring (humans, non-human primates, bovines) is that it has been conserved throughout evolution as it provides genomic diversity (Fragouli, Alfarawati et al. 2013, Daughtry, Rosenkrantz et al. 2019, Cavazza, Takeda et al. 2021).

5. Conclusions and Future Directions

In summary our results provide the first analysis of chromosome segregation errors in the first mitotic division of human embryos. Crucially this has revealed how uniquely error-prone this division is. These errors included: multipolar spindles, lagging chromosomes and micronuclei or multinucleated cells. In addition, I showed that the spindle assembly checkpoint is unlikely to be responsible for setting the timing of anaphase onset. Instead, our data indicates that the zygote has an intrinsic timer that sets the timing from NEB to anaphase onset.

The next challenge is to better understand the role of the SAC during mitosis I. To do so, I will need to destabilise kinetochore-microtubule attachments without inhibiting the SAC. This would allow us to see if biorientation errors can cause a mitotic arrest induced by the SAC. I can achieve this either by using microtubule destabilising drugs such as Nocodazole or Taxol or by physically severing the microtubules using laser ablation. Alternatively, inhibiting the kinesin protein Cenp-E with a small molecule inhibitor has been shown to prevent chromosome alignment and a SAC-mediated mitotic arrest in human somatic cells (Bennett, Bechi et al. 2015) so this could also be used. Indeed, several chromosome alignment errors were created in mouse morula stage embryos by treating them with a combination of Nocodazole and a small molecule Cenp-E inhibitor (Vazquez-Diez, Paim et al. 2019). This shows that the SAC is “leaky” at this stage of mouse embryonic develop, being unable to cause an arrest when a few chromosomes were unaligned but also causing an arrest as a result of severe alignment issues (Vazquez-Diez, Paim et al. 2019). Treating human embryos in such a way will determine if the SAC is active, leaky or completely inactive in the early mitotic divisions of human embryos.

It will also be important to determine the dynamics of SAC proteins, such as Mad2 or Bub1, in live human embryos and establish if they localise to unattached kinetochores. SAC signalling is initiated at unattached chromosomes, following the recruitment of SAC kinase Mps1, which leads to the recruitment of many SAC proteins and phosphorylation cascades, eventually leading to the assembly of the MCC, which will cause the APC/C to remain inactive, leading to a mitotic arrest (Kataria and Yamano 2019). It is important to see if these proteins do actually assemble at unattached kinetochores. If the SAC is weak or inactive in early human embryos, is it because these proteins do not localise to unattached kinetochores, because they leave too early or because even when they do localise, due to low expression of SAC proteins and/or the large volume of the embryo, they are not able to cause an arrest? These proteins could be stained by microinjecting mRNAs coding for fluorescently tagged proteins and fluorescently tagged proteins directly in human oocytes and embryos. mRNA injections have previously been used to stain chromosomes, kinetochores and microtubules in live human oocytes (Schuh and Ellenberg 2007). I successfully synthesised and injected Histone H2B mRNA tagged with mCherry in live GV stage oocytes in some preliminary experiments. I aim to extend this to human embryos and to different mRNAs and proteins, including proteins of the SAC. Moreover, Trim-Away methods, which deplete already synthesised proteins could potentially be used to target SAC proteins. Inhibiting these could provide valuable insight into how the SAC machinery assembles and functions in embryos, a technique which has been shown to work in mouse oocytes (Clift, McEwan et al. 2017). Very little is known about the SAC during early human development. It is crucial to study its activity as this could uncover the causes of the high aneuploidy rates reported in human preimplantation embryos.

Another aspect that is important to investigate in human embryos is spindle nucleation. Indeed, how the spindle is nucleated during the first cleavage division is enigmatic. Studies in mice and bovine embryos have shown that the maternal

and paternal genomes are separated into two distinct spindles that align and fuse before anaphase onset (Reichmann, Nijmeijer et al. 2018) (Schneider, de Ruijter-Villan et al. 2020, preprint). Staining the microtubules, possibly using mRNA or protein injection, would allow us to see if, in early metaphase, there are two distinct spindles. Furthermore, staining the parental genomes would allow us to determine if the maternal and paternal DNA remain spatially separated into two spindles. This has been done in live mouse embryos by crossing two transgenic mouse lines (Reichmann, Nijmeijer et al. 2018). Staining the parental genomes differently could potentially be done in human embryos by injecting a fluorescently tagged histone protein in a mature oocyte, in which a sperm stained with SiR-DNA could be injected by ICSI. Moreover, because the paternal genome undergoes demethylation just after fertilisation, the parental genomes can be differentially stained in fixed embryos using an antibody against 5' methylcytosine, something that has been done in human and mouse embryos (Barton, Arney et al. 2001, Beaujean, Hartshorne et al. 2004)

Another important avenue for research, when looking at spindle assembly, would be to study the role of centrosomes in the first cleavage division. Unlike mice and like bovines, human zygotes inherit a centrosome from the sperm. The centrosomes are the key microtubule organising centres (MTOCs) from which the microtubules are nucleated to form the mitotic spindle in somatic cells (Bettencourt-Dias and Glover 2007). This raises the question of the centrosomes' role in the first mitotic division of human embryos: if there are two distinct spindles, how can they nucleate both spindles? Studies in bovine embryos have shown that the initial stages of spindle nucleation during mitosis I happen without the centrosomes and that it is only later on that the centrosomes become incorporated in the spindle (Schneider, de Ruijter-Villan et al. 2020). They also showed that spindles could be nucleated, and anaphase onset could take place even in cases where the centrosomes were incorrectly positioned (Schneider, de Ruijter-Villan et al. 2020). This could suggest that the centrosomes' role in the first

embryonic mitotic division is not as central as in the mitoses of somatic cells. Looking at centrosome function and localisation would help understand this unique division.

Studying the SAC, spindle assembly and the role of centrosomes, would help uncover some of the mechanisms which cause aneuploidies in human embryos. This is important as aneuploidies have long been associated with poor embryo development, infertility and pregnancy loss (McCoy 2017, Kahraman, Cetinkaya et al. 2020). In this thesis, I have identified the first mitotic division as being uniquely error prone. Imaging human embryos during a longer time frame would allow for a better understanding of the fate of aneuploid cells. Do they cause an arrest in embryonic development? Are they eliminated by apoptosis? Are these errors corrected in later divisions or passed on to daughter cells? Early embryonic development is still very much a mystery but studying the cause of aneuploidies, their fate in future divisions and the impact of specific error types on embryonic development could positively impact fertility treatments in the future.

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Appendix

A. Preprint on bioRxiv

Ford, E., Currie, C. E., Taylor, D. M., Erent, M., Marston, A. L., Hartshorne, G. M., McAinsh, A. D. (2020) "The First Mitotic Division of the Human Embryo is Highly Error-prone." bioRxiv.

B. Records of all embryos donated to research presented in this thesis

Table 1: All embryos imaged undergoing mitosis I on a widefield microscope.

Table 2: All embryos imaged undergoing mitosis II on a widefield microscope.

Table 3: All egg sharer embryos imaged undergoing mitosis I on a widefield microscope.

Table 4: Embryos of the first egg sharer patient undergoing interphase and mitosis II.

Table 5: Mitotic timings of 10 μ M Reversine treated and control embryos on a widefield microscope.

Table 6: Mitotic timings of 1 μ M Reversine treated and control embryos on a spinning disk confocal microscope.

Table 7: Mitotic timings of 1 μ M Reversine treated and control embryos from an egg sharer patient on a widefield microscope

Embryo number	NEB?	PN	Prometaphase	Metaphase	Anaphase onset to furrow ingression	Cytokinesis	NEB to 2-cell (Total time)	Chromosome segregation	Lagging chromosomes?	Maternal age	Treatment
3004 (ii)	NO		60	90	70	50	270	Bipolar	YES	37	ICSI
3028 (i)	NO		N/A	40	30	60	130	Multipolar	NO	35	ICSI
3034 (vii)	NO		N/A	90	30	30	150	Multipolar	YES	28	IVF
3045 (ii)	NO		N/A	N/A	20	30	50	Bipolar	NO	31	IVF
3067 (ii)	NO		150	210	60	105	525	Bipolar	YES	23	ICSI
3068 (iii)	YES	2PN	75	N/A	N/A	N/A	75	N/A	N/A	29	ICSI
3088 (iv)	NO		30	90	75	150	345	Bipolar	YES	29	ICSI
3083 (vii)	YES	2PN	15	135	30	30	210	Bipolar	NO	45	IVF
3101 (ii)	NO		N/A	135	60	60	255	Multipolar	NO	38	IVF
3148 (i)	NO	2PN	15	45	105	45	210	Bipolar	NO	38	ICSI
3158 (vi)	YES	3PN	15	105	30	45	195	Multipolar	NO	30	IVF
3159 (iv)	YES		135	0	30	15	180	Bipolar	NO	38	IVF
3159 (i)	NO	2PN	N/A	0	0	135	135	N/A	N/A	38	IVF
3169 (iii)	NO		N/A	15	30	15	60	Bipolar	NO	35	IVF
3170 (ii)	NO		N/A	N/A	N/A	30	30	N/A	N/A	36	IVF
3172 (ii)	YES	3PN	30	105	30	45	210	Multipolar	YES	39	IVF
3181 (i)	YES	1PN	75	15	15	30	135	Bipolar	NO	31	IVF
3197 (v)	YES	2PN	30	120	45	45	240	Multipolar	NO	30	IVF
3197 (vi)	YES	2PN	105	45	75	60	285	Bipolar	NO	30	IVF
3201 (i)	YES	3PN	90	60	30	60	240	Multipolar	NO	38	IVF
3204 (v)	NO		N/A	N/A	45	60	105	Bipolar	NO	30	IVF
3209 (ii)	NO		N/A	N/A	60	30	90	N/A	N/A	30	IVF
3210 (i)	YES		105	30	75	90	300	Multipolar	YES	37	IVF
3215 (v)	NO		N/A	N/A	0	30	30	Bipolar	YES	31	IVF
3218 (vii)	YES	2PN	120	0	15	15	150	Multipolar	N/A	28	IVF
3222 (ii)	YES	3PN	90	60	15	60	225	Bipolar	N/A	35	IVF
3226 (v)	NO		N/A	N/A	N/A	30	30	N/A	N/A	31	IVF
3227 (vi)	NO		15	135	30	15	195	Multipolar	YES	31	IVF
3233 (i)	NO		N/A	N/A	N/A	30	30	Bipolar	NO	40	ICSI
3233 (v)	NO		225	45	75	75	420	Multipolar	YES	33	IVF
3237 (iii)	YES	2PN	165	N/A	N/A	N/A	165	Bipolar	N/A	35	IVF
3246 (ii)	YES	1PN	75	45	0	75	195	Bipolar	NO	33	IVF
3251b (iii)	NO		N/A	45	30	30	105	Bipolar	NO	32	IVF
3251b (iv)	YES	3PN	135	15	15	45	210	Bipolar	NO	32	IVF
3274 (v)	NO	3PN	N/A	N/A	15	45	60	bipolar	NO	34	IVF
3276 (i)	YES	1PN	30	90	30	45	195	Multipolar	NO	37	IVF
3291 (ii)	YES	2PN	45	30	30	45	150	multipolar	YES	31	IVF
3292 (i)	YES	3PN	75	45	15	60	195	multipolar	YES	33	IVF
3258vii	YES	3PN	N/A	N/A	45	75	120	multipolar	NO	34	IVF

Table 1: All embryos imaged undergoing mitosis I on a widefield microscope. The timings of the different mitosis timings in minutes, whether or not NEB was imaged, the pronucleus status, the types of chromosome segregation (bipolar or multipolar), the presence of lagging chromosome, maternal age and the type of fertility treatment (IVF or ICSI) are displayed.

Name	NEB?	Prometaphase	Metaphase	Anaphase onset to furrow ingression	Cytokinesis	NEB to 2 cell	Chromosome segregation	Lagging chromosomes?
3040 (ii) cell 1	YES	110	50	60	20	240	Bipolar	NO
3040 (ii) cell 2	YES	160	0	0	20	180	N/A	N/A
3034 (i) cell 1	YES	70	10	30	10	120	Bipolar	NO
3019 (i) cell 1	YES	110	20	40	80	250	Multipolar	NO
3159 (iv) cell 1	YES	70	45	15	15	145	Bipolar	NO
3159 (iv) cell 2	YES	70	45	15	15	145	Bipolar	NO
3174 (iv) cell 1	YES	15	75	15	15	120	Bipolar	NO
3174 (iv) cell 2	YES	30	60	15	15	120	Bipolar	NO
3174 (v) cell 1	YES	30	45	30	15	120	Bipolar	NO
3226 (viii) cell 1	YES	60	45	30	15	150	Bipolar	NO
3226 (viii) cell 2	YES	75	30	15	30	150	Multipolar	NO
3227 (viii) cell 1	YES	60	45	60	60	225	Bipolar	NO
3227 (viii) cell 2	NO	NA	NA	NA	30	30	N/A	N/A
3260 (i) cell 1	YES	45	30	45	15	135	Bipolar	YES
3260 (i) cell 2	YES	60	60	15	30	165	Bipolar	NO
3260 (ii) cell 1	YES	60	30	30	15	135	Bipolar	NO
3260 (ii) cell 2	YES	45	45	30	15	135	Bipolar	YES
3278 (vii) cell 1	YES	60	60	15	15	150	Multipolar	NO
3278 (vii) cell 1	YES	45	45	15	30	135	Bipolar	NO
3278 (vii) cell 2	YES	60	45	30	45	180	Bipolar	NO

Table 2: All embryos imaged undergoing mitosis II on a widefield microscope. The timings of the different mitosis timings in minutes, whether or not NEB was imaged, the types of chromosome segregation (bipolar or multipolar) and the presence of lagging chromosome are displayed.

Embryos number	NEB?	Prometaphase	Metaphase	Anaphase onset to furrow ingression	Cytokinesis	NEB to 2-cell	Chromosome segregation	Lagging chromosomes?
3247 (iii)	YES	15	120	15	45	330	Bipolar	NO
3247 (iv)		N/A	N/A	N/A	15	15	Bipolar	NO
3247 (v)	YES	90	30	45	30	315	Bipolar	YES
3247 (vi)		15	75	30	45	255	Bipolar	NO
3258 (ii)	YES	30	15	90	45	180	Bipolar	N/A
3258 (iii)	NO	N/A	150	0	30	N/A	Bipolar	YES
3258 (vi)	NO	N/A	75	15	45	N/A	Bipolar	NO
3272 (i)	YES	75	75	45	30	225	Bipolar	YES
3272 (v)	NO	N/A	90	45	15	N/A	Bipolar	NO
3272 (vii)	YES	75	60	30	30	195	Bipolar	NO

Table 3: All egg sharer embryos imaged undergoing mitosis I on a widefield microscope. The timings of the different mitosis timings in minutes, whether or not NEB was imaged, the types of chromosome segregation (bipolar or multipolar) and the presence of lagging chromosomes are displayed.

Embryo number	NEB?	Interphase	Prometaphase	Metaphase plate	Anaphase onset to cytokinesis	Cytokinesis	NEB to 2 cells	Chromosome segregation	Lagging chromosomes?
3247 (ii) cell 1	YES	750	60	75	15	15	165	Bipolar	NO
3247 (iv) cell 1	YES	900	45	45	30	15	135	Bipolar	NO
3247 (iv) cell 2	YES	750	15	105	30	15	165	Bipolar	NO
3247 (v) cell 1	YES	825	45	75	15	15	150	Bipolar	NO
3247 (v) cell 2	YES	765	30	210	15	15	270	Bipolar	NO
3247 (vi) cell 1	YES	765	45	60	15	15	135	Bipolar	NO

Table 4: Embryos of the first egg sharer patient undergoing interphase and mitosis I. The embryos were imaged on a widefield microscope. Two embryos had both cells undergoing mitosis II and two embryos had on cell undergoing mitosis II. The timings of the different mitotic stages and interphase in minutes, whether or not NEB was imaged, the types of chromosome segregation (bipolar or multipolar) and the presence of lagging chromosomes are displayed.

Treatment	Embryo number	NEB?	PN	Prometaphase	Metaphase	Anaphase onset to furrow ingression	Cytokinesis	Total time	Chromosome segregation	Lagging Chromosomes?
DMSO	3291 (ii)	YES	2	60	15	30	30	135	Multipolar	NO
DMSO	3292 (i)	YES	3	45	75	15	75	210	Multipolar	YES
Reversine 10 μ M	3292 (ii)	YES	3	75	0	45	30	150	N/A	YES
Reversine 10 μ M	3292 (iii)	YES	3	45	0	30	60	135	N/A	YES
Reversine 10 μ M	3292 (iv)	YES	3	75	45	120	75	315	N/A	YES

Table 5: Mitotic timings of 10 μ M Reversine treated and control embryos on a widefield microscope. The timings of the different mitosis timings in minutes, whether or not NEB was imaged, the pronucleus status the types of chromosome segregation (bipolar or multipolar) and the presence of lagging chromosomes are displayed.

Treatment	Embryo number	NEB?	PN	Prometaphase	Metaphase	Anaphase onset to furrow ingression	Cytokinesis	Chromosome segregation	Lagging Chromosome?
Control	3346 (ii)	YES	2	105	45	30	30	Multipolar	YES
Control	3348 (i)	YES	3	30	75	30	30	Bipolar	NO
Control	3349 (i)	YES	2	60	60	15	60	Multipolar	YES
Control	3349 (iii)	NO	N/A	150	0	0	60	Multipolar	YES
Control	3350 (ii)	NO	N/A	N/A	30	0	645	Bipolar	NO
Reversine 1 μ M	3346 (iv)	NO	N/A	N/A	N/A	15	30	Multipolar	YES
Reversine 1 μ M	3346 (v)	NO	N/A	N/A	N/A	15	30	no alignment	YES
Reversine 1 μ M	3349 (v)	YES	2	30	90	0	60	Bipolar	YES
Reversine 1 μ M	3349 (vi)	YES	2	150	0	0	15	Bipolar	YES
Reversine 1 μ M	3350 (iv)	YES	2	90	75	30	30	Multipolar	YES

Table 6: Mitotic timings of 1 μ M Reversine treated and control daselected on a spinning disk confocal microscope. The timings of the different mitosis timings in minutes, whether or not NEB was imaged, the pronucleus status the types of chromosome segregation (bipolar or multipolar) and the presence of lagging chromosomes are displayed.

Treatment	Embryo number	NEB?	PNs	Prometaphase	Metaphase	Anaphase onset to furrow ingression	Cytokinesis	Chromosome segregation	Lagging chromosomes?
Control	3326 (i)	YES	2	210	0	45	30	Multipolar	NO
Control	3326 (ii)	NO	2	0	150	90	60	Bipolar	YES
Control	3326 (iii)	YES	3	135	120	45	45	Bipolar	NO
Control	3326 (iv)	YES	2	15	225	30	15	Bipolar	NO
Reversine 1 μ M	3326 (v)	YES	2	165	285	60	45	Multipolar	YES
Reversine 1 μ M	3326 (vii)	YES	2	135	240	60	0	Bipolar	YES
Reversine 1 μ M	3326 (viii)	YES	2	75	60	45	45	Multipolar	YES

Table 7: Mitotic timings of 1 μ M Reversine treated and control embryos from an egg sharer patient on a spinning disk confocal microscope. The timings of the different mitosis timings in minutes, whether or not NEB was imaged, the pronucleus status the types of chromosome segregation (bipolar or multipolar) and the presence of lagging chromosomes are displayed.

C. HFEA consenting and information

1. HFEA Licenced centre 0340
2. HFEA Licenced centre 0013
3. Consent form: “deselected” eggs and embryos
4. Patient information sheet: “deselected” eggs and embryos
5. Consent form: egg sharer eggs and embryos
6. Patient information sheet: egg sharer eggs and embryos
7. Consent form: men providing sperm for research
8. Patient information sheet: men providing sperm for research