Chromosomal mosaicism: underlying mechanisms and consequences for early human embryo development

Margarida da Avó Ribeiro dos Santos

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Mozaïeke chromosoompatronen in humane embryo's: onderliggende mechanismen en gevolgen voor de ontwikkeling
(met een samenvatting in het Nederlands)

Mosaicismo cromossómico em embriões humanos: mecanismos adjacentes e consequências para o desenvolvimento embrionário
(contém resumo em Português)

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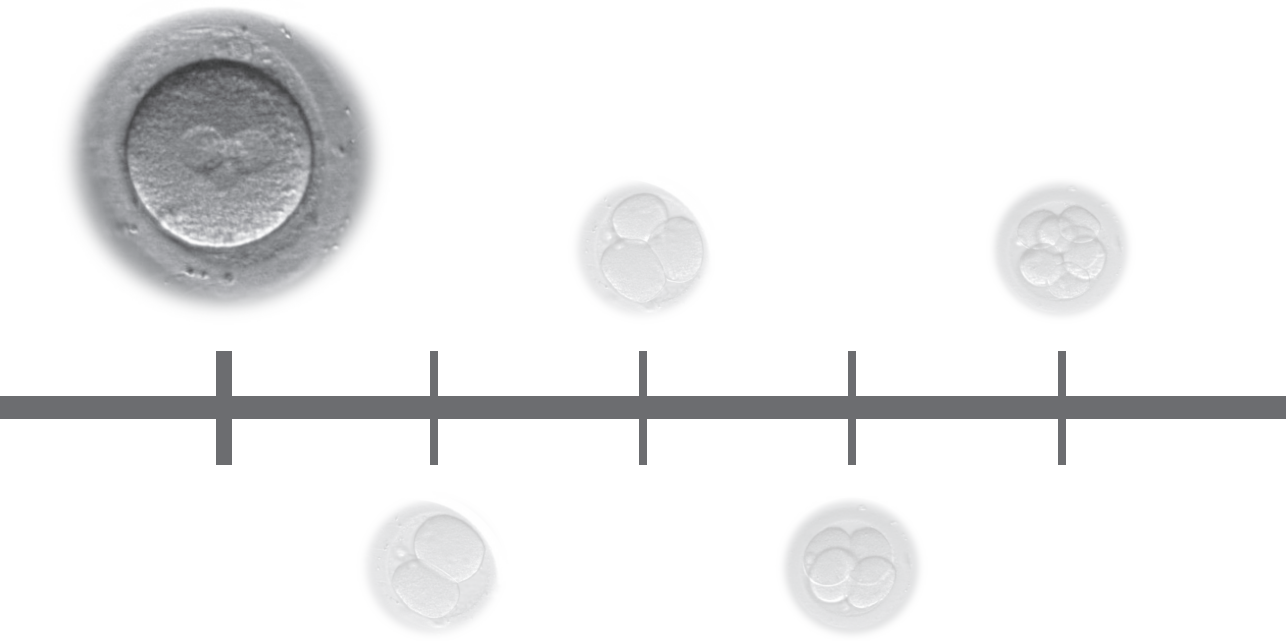
Para os meus pais e avós

Contents

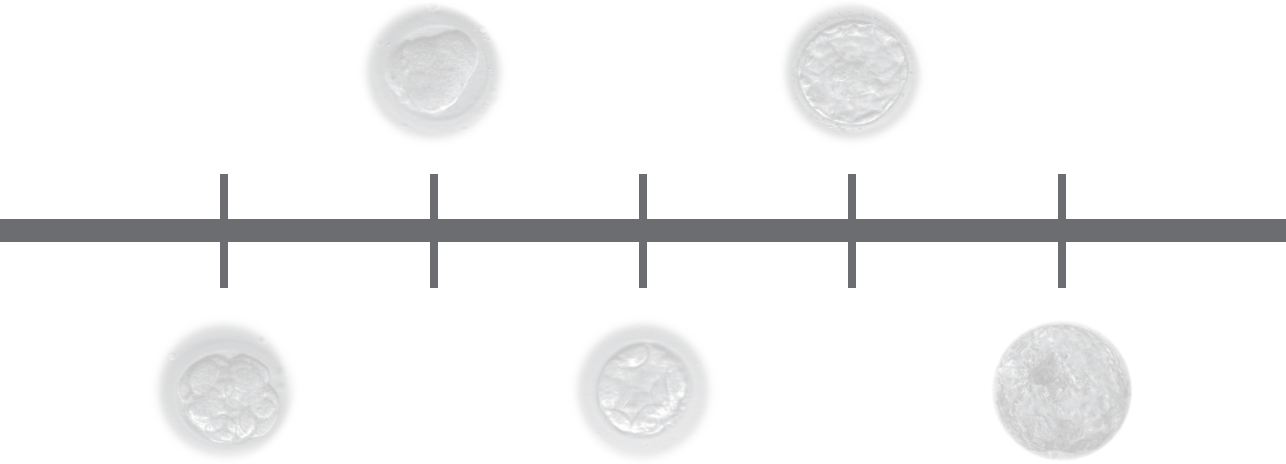
Abbreviations

APC/C	anaphase promoting complex/cyclosome
ART	assisted reproductive technology
CGH	comparative genome hybridization
CPC	chromosomal passenger complex
CSF	cytostactic factor
E_2	estradiol
EGA	embryonic genome activation
FISH	fluorescence *in situ* hybridization
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
H2ApT120	phosphorylation of histone H2A at Thr 120
H3K9me3	trimethylation of histone H3 at Lys 9
H3pT3	phosphorylation of histone H3 at Thr 3
hMG	human menopausal gonadotropin
HP1	heterochromatin protein 1
ICSI	intracytoplasmatic sperm injection
INCENP	inner centromere protein
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
NEB	nuclear envelope breakdown
LH	luteinizing hormone
OHSS	ovarian hyperstimulation syndrome
PCOS	polycystic ovary syndrome
PGD	preimplantation genetic diagnosis
PRC1/2	polycomb repressive complex 1/2
SAC	spindle assembly checkpoint



Chapter 1

General introduction



Introduction

Reproduction is the fundamental process for the propagation of all mammalian species. It requires the interaction of a sperm and an oocyte to form a zygote, the first step in the development of the embryo. In humans, reproduction is considered a relatively inefficient process, as the chance of achieving a spontaneous pregnancy after timed intercourse is at the most 20-30% (Evers, 2002), much lower than in other species such as the rhesus monkey (70%), the captive baboon (80%), or rodents and rabbits (90%) (Foote and Carney, 1988; Ghosh *et al.*, 1997; Stevens, 1997). The inefficiency of human reproduction is mainly explained by the high incidence of pre-clinical losses, an estimate 60% of all conceptions (Macklon *et al.*, 2002). Early pregnancy loss is mainly explained due to the occurrence of chromosome abnormalities, which have been identified in the majority of spontaneous abortion samples investigated (Boue *et al.*, 1975; Eiben *et al.*, 1987; Fritz *et al.*, 2001; Benkhalifa *et al.*, 2005).

In modern society, the number of couples with reproductive problems has increased over the years, mainly due to changes in life style and delayed childbearing. This has lead to the development of revolutionary techniques to overcome infertility, commonly designated as assisted reproductive technology (ART). This has made the oocyte and preimplantation embryo uniquely accessible to new micromanipulation techniques and has allowed a better insight on human early embryos resulting from *in vitro* fertilization (IVF). Soon it became clear that chromosomal abnormalities identified in abortion material from *in vivo* conceptions are also frequently identified in preimplantation embryos generated by IVF (Angell *et al.*, 1983). This indicates that chromosome instability is an inherent feature of both *in vivo* and *in vitro* human conceptions. Likewise, chromosomal abnormalities have also been described in bovine, equine, porcine and non-human primate embryos, in both *in vitro* and *in vivo* produced embryos (Viuff *et al.*, 2000; Rambags *et al.*, 2005; Zijlstra *et al.*, 2008; Dupont *et al.*, 2010). Aneuploidy seems therefore to be an intrinsic characteristic of preimplantation embryo development in humans and other mammalian species. As most research has focused on identifying causative factors in the oocyte, advanced maternal age remains the only etiological risk factor identified for chromosomal aneuploidy (Hassold and Hunt, 2001; Champion and Hawley, 2002). However, the mechanisms underlying post-meiotic errors in preimplantation human embryos and its consequences for developmental potential are still poorly understood.

Ovarian stimulation for *in vitro* fertilization

Reproductive life starts at puberty, when an increase in circulating follicle stimulating hormone (FSH) level during each reproductive cycle recruits a cohort of follicles to escape atresia and undergo further growth (reviewed by Fauser and Van Heusden, 1997; McGee and Hsueh, 2000) (Figure 1.1b). Within the natural menstrual cycle, gonadotropin-releasing hormone (GnRH) is released from the hypothalamus in a pulsatile manner and stimulates the synthesis and release of luteinizing hormone (LH) and FSH by the anterior pituitary gland (Figure 1.1a). In the early follicular phase, serum FSH levels rise, supporting the growth of follicles that is accompanied by proliferation of granulosa cells. This results in a rapid rise in estradiol (E_2) and inhibin B, which creates a negative feedback at the hypothalamic-pituitary axis. Consequent

downregulation of FSH levels then secures the selection of a single dominant follicle, which becomes less dependent on FSH and continues to grow. The remaining follicles from the recruited cohort cease to grow and undergo atresia. Continuous growth of the pre-ovulatory follicle leads to very high E_2 levels and the switch from a negative to a positive feedback effect, resulting in a rapid rise in LH (LH surge) (Figure 1.1c). The LH surge initiates multiple events such as the commencement of oocyte meiotic maturation and rupture of the dominant follicle allowing ovulation of the oocyte, as well as granulosa cell luteinization and corpus luteum formation. The corpus luteum secretes progesterone and inhibin A, responsible for suppression of FSH at the hypothalamus and pituitary. With no fertilization or implantation of an embryo, regression of the corpus luteum allows for a rise of FSH levels and the next menstrual cycle begins (Speroff and Fritz, 2011).

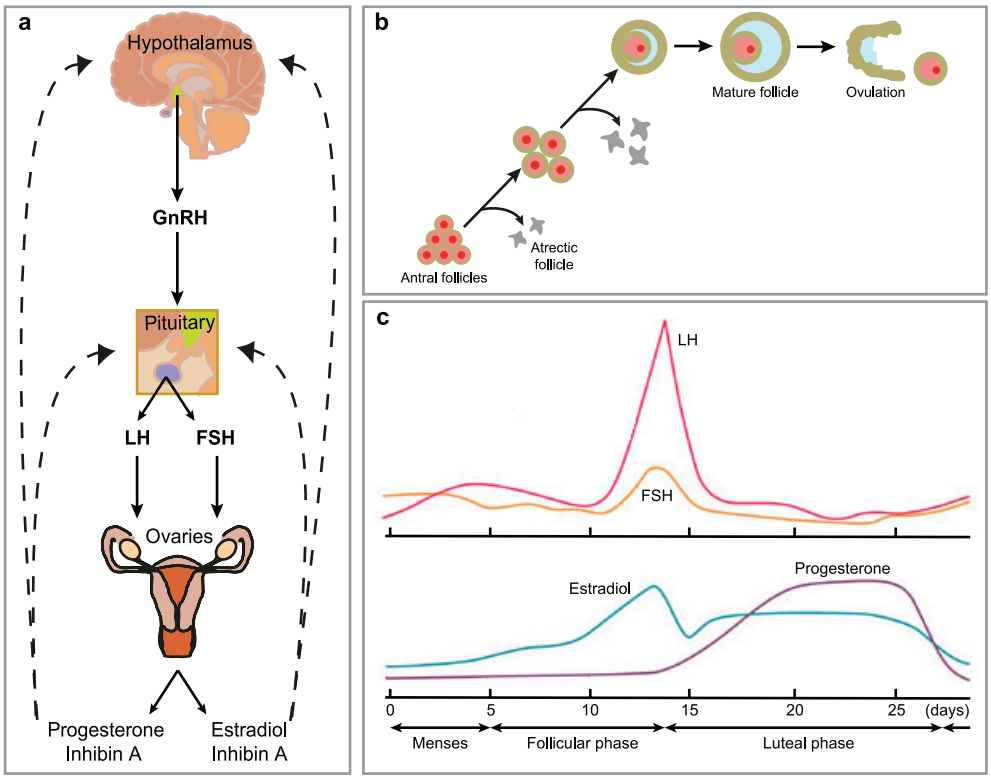


Figure 1.1 – **Schematic representation of the endocrine regulation of the natural menstrual cycle. a)** hypothalamic-pituitary-ovarian axis. **b)** follicle recruitment, selection, maturation and ovulation in the ovaries. **c)** levels of LH, FSH, Estradiol and Progesterone during the menstrual cycle.

To allow retrieval of multiple oocytes in a single menstrual cycle, IVF patients undergo ovarian stimulation with exogenous gonadotropins to promote the development and growth of multiple follicles. Exogenous administration of FSH for an extended period of time prevents the mid-follicular decrease in FSH levels, allowing more than one follicle to gain dominance and continue to grow (Schipper *et al.*, 1998). However, as a result of multiple growing follicles,

E_2 levels will increase more rapidly and may induce a premature LH surge and subsequent premature luteinization. Therefore, to prevent premature LH surge, exogenous gonadotropin treatment is usually supplemented by the administration of GnRH analogues (either GnRH agonists or antagonists), which decreases pituitary release of endogenous FSH and LH. Once pituitary function is suppressed, administration of high doses of exogenous FSH induces multiple follicle growth (reviewed by Macklon *et al.*, 2006).

Thus, during the process of ovarian stimulation for IVF, natural follicle selection is completely overruled, allowing the non-discriminate growth of many follicles. This has led to a growing concern over the possible disturbance of follicle and oocyte growth after ovarian stimulation. Indeed, a few studies indicate an association between ovarian stimulation and the occurrence of chromosomal abnormalities (Munne *et al.*, 1997; Troncoso *et al.*, 2003; Katz-Jaffe *et al.*, 2005; Haaf *et al.*, 2009), and a correlation between the dosage of FSH and degree of aneuploidy has been established (Baart *et al.*, 2007a). This observation along with concerns over the effect of ovarian stimulation on other aspects such as endometrial receptivity, embryonic implantation, epigenetics and perinatal outcomes call for an evaluation and reflection of the risks associated with this central component of IVF treatments.

Aneuploidy and chromosomal mosaicism in preimplantation embryos

For the past two decades, fluorescence *in situ* hybridization (FISH), and more recently array comparative genome hybridization (CGH), have been used for preimplantation genetic diagnosis (PGD) of IVF-derived embryos, enabling screening of chromosomal aneuploidies, i.e., cells with an abnormal number of chromosomes. This has led to an increasing body of evidence demonstrating that the rates of chromosomal abnormalities in IVF-derived embryos are stunningly high, with an estimated 80% of all preimplantation embryos containing aneuploid cells (Voullaire *et al.*, 2000; Wells and Delhanty, 2000). This high frequency of chromosome instability closely resembles the genetic instability observed in human cancers (Vanneste *et al.*, 2009; Voet *et al.*, 2011), suggesting the lack of chromosome segregation surveillance mechanisms during the early stages of embryonic development (Los *et al.*, 2004).

Research into the origin of embryo aneuploidy has so far focused on the contribution of the oocyte, and a link between maternal age and an increased risk for meiotic errors has been established. However, meiotically-derived aneuploidy does not explain the high rate of chromosomal mosaicism in human embryos, characterized by the presence of two or more cell lines with different chromosomal constitution within the same embryo (Baart *et al.*, 2006; Vanneste *et al.*, 2009; Santos *et al.*, 2010b).

Most studies evaluating human embryo aneuploidy rates have looked at cleavage stage embryos (mainly at the 8 cell stage) (Mantzouratou and Delhanty, 2011). Thus, the chromosomal status of embryos during the final stage of preimplantation development, the blastocyst stage, is poorly characterized. The blastocyst (day 5 or 6 post-fertilization) corresponds to the stage at which point *in vivo*-generated embryos typically arrive at the uterus ready to implant (reviewed in Fragouli and Wells, 2011). Therefore, embryos that reach the blastocyst stage are considered to have good implantation potential.

A number of chromosomal abnormalities that are extremely rare in clinically recognized pregnancies are commonly identified at the cleavage stage. However, the frequency of aneuploidies and mosaicism found at term birth (Hassold *et al.*, 1996) and in first trimester chorionic villi samples from viable pregnancies (Los *et al.*, 2004) is extremely low compared to cleavage stages. What happens to aneuploidy and chromosomal mosaicism between the cleavage stage and embryo implantation? Are mosaic embryos capable to form blastocysts, or are they eliminated prior to this stage of development? The majority of the studies investigating the chromosomal constitution of human day 5 blastocysts suggest persistence of most aneuploidies identified in cleavage stage embryos (Benkhalifa *et al.*, 1993; E*de novo*ikov and Verlinsky, 1998; Magli *et al.*, 2000; Ruangvutilert *et al.*, 2000; Sandalinas *et al.*, 2001; Bielanska *et al.*, 2002a; Coonen *et al.*, 2004; Baart *et al.*, 2006; Fragouli *et al.*, 2008). However, the percentage of these cells seems to decline towards the blastocyst stage (Ruangvutilert *et al.*, 2000; Bielanska *et al.*, 2002a). This suggests elimination of aneuploid cells as embryos develop into blastocysts.

In recent years, optimization of culture media and development of embryo-endometrial cells co-culture systems has allowed extended *in vitro* culture of human embryos, which has been associated with increased implantation and pregnancy rates (Mercader *et al.*, 2003). Extended culture of embryos beyond day 5 has therefore the potential to allow a further insight on how aneuploidy and chromosomal mosaicism evolve from cleavage stages to the peri-implantation stage.

Cell division and cell cycle regulatory mechanisms

Aneuploidy in somatic cells is a relatively rare condition. To maintain genetic integrity in all the cells in the organism, it is important that all chromosomes are duplicated before each division and equally divided over the two daughter cells, as unequal division of chromosomes results in aneuploidy.

Each round of cell division, the cell cycle, is divided into four main phases: G1 (cell growth), S (DNA replication), G2 (preparation for cell division) and mitosis (cell division). Mitosis is the final stage of the cell cycle during which there is segregation and separation of the duplicated genome into two daughter cells. To produce a pair of genetically identical daughter cells, the DNA must be faithfully replicated and the replicated chromosomes must be segregated into two separate cells. Mitosis can be divided into several cytogenetically and molecularly distinct phases (Figure 1.2). During prophase, chromosomes condense and the mitotic spindle assembles between the two centrosomes. The transition from prophase to prometaphase is characterized by nuclear envelope breakdown. During prometaphase, kinetochores are captured by the spindle microtubules. The attachment of kinetochores to the spindle allows the movement of sister chromatids back and forward until they align at the equatorial plane. During anaphase, the mitotic spindle separates sister chromatids simultaneously at their centromeres, allowing chromatid segregation to opposite sites of the cell. During telophase, chromatids start to decondense and the nuclear envelope forms again, resulting in the formation of two new daughter nuclei. Along with telophase, the cell undergoes

division of the parental cytoplasm into daughter cells, a process called cytokinesis.

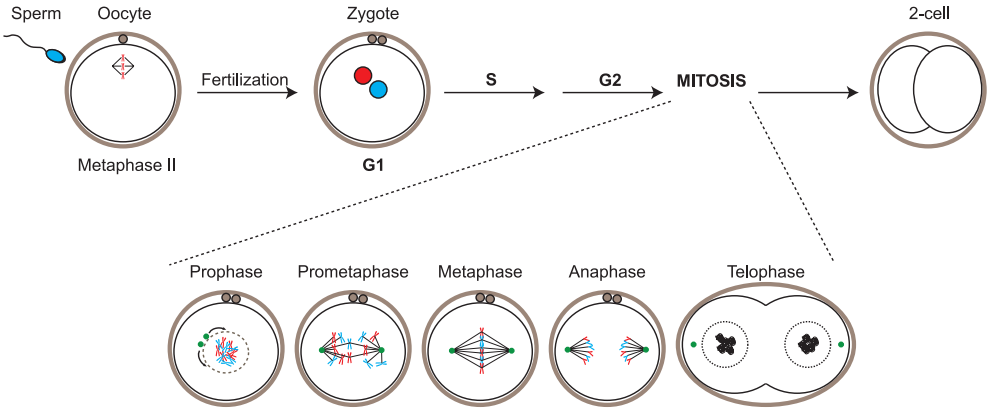


Figure 1.2 – Schematic representation of the different stages of the first cell cycle after fertilization of a mature oocyte by a sperm cell.

Spindle assembly checkpoint

To be accurate, chromosome segregation requires that all sister chromatid pairs are bioriented (bipolar), i.e., with the two sister chromatids of each chromosome bound to opposite spindle poles. To prevent chromosome segregation errors during mitosis, cells have developed a complex surveillance mechanism that is capable of delaying anaphase in the presence of unattached chromosomes. Such surveillance mechanism is known as the spindle assembly checkpoint (SAC), also known as the mitotic checkpoint. The SAC is responsible for inhibition of the Anaphase Promoting Complex/Cyclosome (APC/C) until all chromosomes are bioriented and under tension (Musacchio and Salmon, 2007) (Figure 1.3).

The APC/C is a multisubunit E3-ubiquitin ligase that regulates the metaphase-anaphase transition and exit from mitosis, targeting key mitotic substrates through the addition of a polyubiquitin chain that is used for recognition and degradation by the 26S proteasome (reviewed in Peters, 2006). Two of the main targets of the APC/C are cyclin B and separase. Cyclin B is the activator of the mitotic kinase Cdk1, which is required for mitotic entry. Securin protects cohesion, a protein complex essential for sister chromatid cohesion, from the proteolytic cleavage activity of separase (reviewed in Kim and Yu, 2011).

In mammalian somatic cells, a single unattached kinetochore is sufficient to activate the spindle checkpoint (Rieder *et al.*, 1994). When activated, the SAC interacts with Cdc20, an essential co-factor of the APC/C, which results in impairment of the anaphase onset. The SAC is composed of Mad1, Mad2 and Bub3 proteins and BubR1, Bub1 and Mps1 kinases. These proteins localize to unattached kinetochores, activating the checkpoint through inhibition of the APC/C^{Cdc20} (reviewed in Kim and Yu, 2011). Only when all pairs of sister kinetochores are properly captured by the spindle microtubules and are under tension, the SAC is inactivated and the APC/C^{Cdc20} ubiquitinates securin and cyclin B, which are then degraded. This results in the activation of separase, removal of sister chromatid cohesion, and sister chromatid

separation, so that cells can enter anaphase (reviewed in Kops *et al.*, 2005) (Figure 1.3).

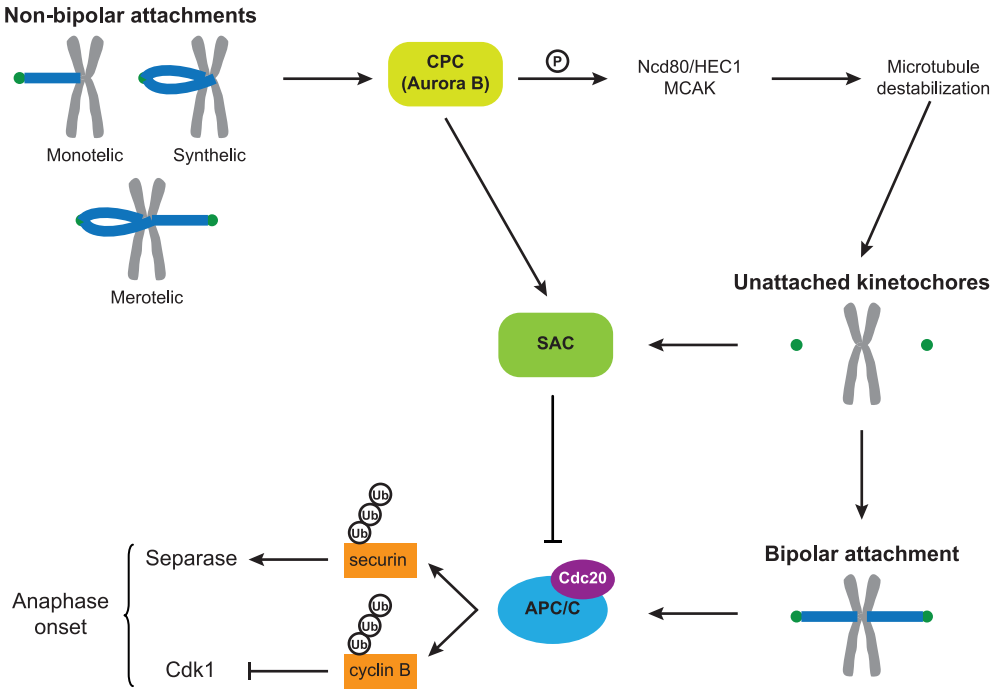


Figure 1.3 – Schematic representation of the mechanisms involved in the surveillance of chromosome segregation. The spindle assembly checkpoint (SAC) and the chromosomal passenger complex (CPC) act together to inhibit the anaphase promoting complex/cyclosome (APC/C), delaying the anaphase onset until all chromosomes establish bipolar attachments.

Chromosomal passenger complex

The attachment of chromosomes to spindle microtubules, a process mediated by the kinetochores, is a stochastic trial-and-error process. Therefore, before chromosomes achieve bi-orientation in the spindle pole, sister chromatids go through intermediate states of non-bipolar attachment. As mentioned previously, unattached chromosomes can activate the SAC in order to delay anaphase until biorientation is achieved. However, incorrectly attached kinetochores cannot induce SAC activation. Thus, in order to actively destabilize erroneous attachments and induce SAC response, Aurora kinase B specifically phosphorylates kinetochore protein complexes (Ndc80/HEC1) and centromeric MCAK (mitotic centromere-associated kinesin) (Cheeseman *et al.*, 2002; Andrews *et al.*, 2004; Cheeseman *et al.*, 2006). This way, Aurora B is a key player in the mechanism of destabilization of non-bipolar attachments to create unattached kinetochores that activate the SAC. Nevertheless, Aurora B can also interact directly with the SAC by recruiting and activating the SAC kinase Mps1 (Santaguida *et al.*, 2011; Saurin *et al.*, 2011) (Figure 1.3).

Aurora B belongs to a family of serine-threonine kinases that is conserved from yeast to humans (reviewed in Carmena and Earnshaw, 2003). This kinase is a member of

the Chromosomal Passenger Complex (CPC), together with the Inner Centromere Protein (INCENP), Borealin and Survivin. The CPC not only facilitates chromosome alignment during prometaphase, but also delineates the cleavage plane in anaphase and is required for cytokinesis. This complex has therefore a dynamic localization during mitosis. In prophase it localizes along the chromosome arms, where it controls mitotic chromosome structure and organization. During prometaphase, it concentrates at the inner centromeres, in between the kinetochores, to control and regulate proper kinetochore-microtubule attachments. In anaphase, the CPC relocates to the central spindle and the equatorial cell cortex, and in telophase to the mid-body, allowing proper function of the contractile ring and final abscission, ensuring cytoplasmic division (reviewed in Vagnarelli and Earnshaw, 2004).

Regulation of chromosome segregation in preimplantation embryos

Although the mechanisms regulating cell division described above are well characterized in somatic human cells, little is known about chromosome segregation regulation in preimplantation human embryos. In an attempt to explain the high incidence of aneuploidies observed in early human embryonic development, Los *et al.* have suggested that during the first three postzygotic cell divisions, before embryonic genome activation takes place, cell cycle control mechanisms are not fully functional (Los *et al.*, 2004).

Mice with homozygous null mutations in the SAC proteins Mad2 (Dobles *et al.*, 2000), Bub3 (Kalitsis *et al.*, 2000), BubR1 (Baker *et al.*, 2004; Wang *et al.*, 2004) and Bub1 (Perera *et al.*, 2007) die soon after implantation by E6.5-8.5. However these mutations do not seem to impair preimplantation development up to E3.5. Therefore, until recently it remained unclear whether the mitotic checkpoint mechanisms are required to ensure accurate chromosome segregation during mouse preimplantation development. However, in a recent study using RNAi-based silencing of Bub3, BubR1 and Mad2 in cleavage stage mouse embryos, Wei *et al.* have shown that SAC is essential for correct chromosome segregation during the first embryonic mitotic divisions (Wei *et al.*, 2011). The CPC also seems to be required for early mouse development, as embryos degenerate before implantation, by E2.5-3.5, in the absence of the CPC subunits Survivin (Uren *et al.*, 2000a), INCENP (Cutts *et al.*, 1999) or Borealin (Yamanaka *et al.*, 2008a).

Since the incidence of chromosome missegregations in mouse early embryos is very small compared to humans (Tateno *et al.*, 2011), it is not clear yet whether SAC and CPC mechanisms are conserved in human embryos. The lack of a completely functional SAC and/or CPC during the first embryonic divisions in human embryos therefore still offers a valid explanation for the increased incidence of chromosome missegregations compared to mouse early embryo. The investigation of mitotic checkpoint mechanisms in human preimplantation embryos should be the next step towards a better understanding of the etiology of chromosome missegregations taking place during the cleavage divisions.

Aims and outline of the thesis

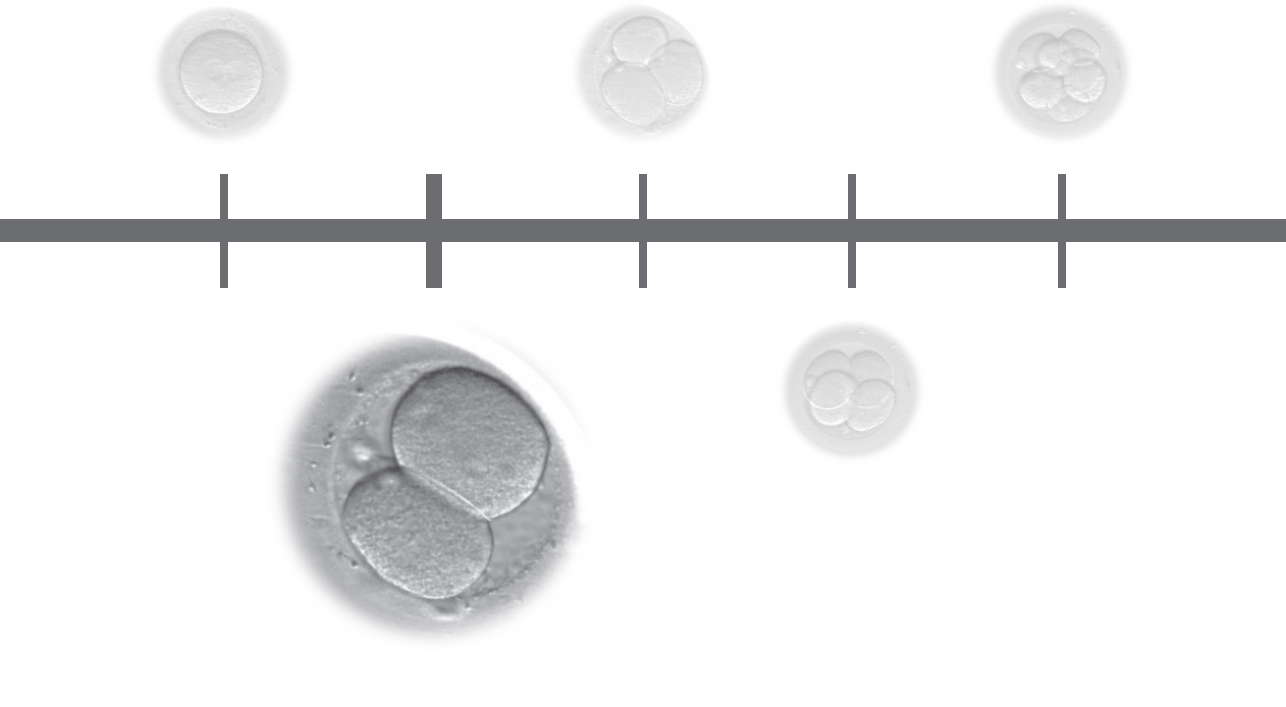
Chromosomal mosaicism, an intrinsic characteristic of human preimplantation embryos, results from chromosome segregation errors taking place during the first embryonic cell divisions. However, the etiology of such missegregations remains elusive. It is also unclear to what extent chromosomal mosaicism impacts on the developmental potential of preimplantation embryos. The research described in this thesis aims at understanding how chromosomal mosaicism influences the fate of embryonic development and identifying mechanisms underlying the increased susceptibility for chromosome missegregation during the first few rounds of cell division in human embryos.

The study of human preimplantation development is limited to the use of IVF-derived human embryos, but it is unknown to what extent they faithfully represent the events of *in vivo* embryonic development. The use of hormonal ovarian stimulation prior to IVF procedures has been suggested to influence embryonic development. In **chapter 2** we review the literature on the impact hormonal ovarian stimulation may have on chromosomal and epigenetic competence of oocytes and embryos, as well as on embryonic implantation and perinatal outcomes.

In **chapter 3** we evaluate how chromosomal mosaicism may influence the fate of development of human preimplantation embryos. For that we used good quality day 4 cryopreserved embryos to assess the chromosomal constitution by FISH analysis in relation to developmental progression to day 5 and day 8 post-fertilization.

Although mosaicism and chromosomal aneuploidy are well recognized to affect human preimplantation development, the etiology of chromosome missegregation remains unknown. Therefore in **chapter 4** we investigate the localization and constitution of the CPC, an important complex involved in regulation of chromosome segregation, in human preimplantation embryos. Although we identified Aurora kinase C to be the main enzymatic subunit in the CPC during the first cleavage divisions, this in itself does not explain the increased incidence of chromosome segregation errors in human early embryos. As correct localization of the CPC is crucial to ensure proper attachment error-correction during mitosis, in **chapter 5** we investigated the epigenetic mechanisms functioning upstream from the CPC that are involved in targeting the complex to the inner centromere. In **chapter 6** we review the differences in the overall mechanisms regulating the first embryonic cell division compared to those described in meiosis and somatic mitosis. We discuss possible implications of these differences for the high incidence of aneuploidy described in early human embryos and identify new research avenues.

Finally, **chapter 7** summarizes the most important conclusions from the conducted studies and discusses the implications for clinical practice and future research.



Chapter 2

The impact of ovarian stimulation for IVF on the developing embryo

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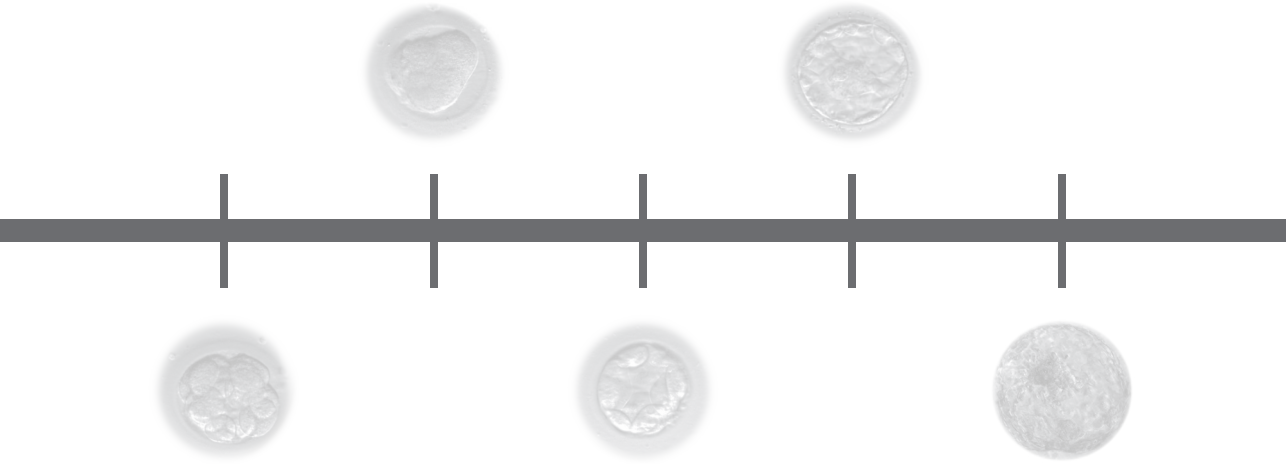


Abstract

The use of assisted reproductive technologies (ART) has been increasing over the past three decades and in developed countries, ART accounts for 1-3% of annual births. In an attempt to compensate for inefficiencies in *in vitro* fertilization procedures, patients undergo ovarian stimulation using high doses of exogenous gonadotropins to allow retrieval of multiple oocytes in a single cycle. Although ovarian stimulation has an important role in ART, it may also have detrimental effects on oogenesis, embryo quality, endometrial receptivity, and perinatal outcomes. In this review we consider the evidence for these effects and address possible underlying mechanisms. We conclude that such mechanisms are still poorly understood and further knowledge is needed in order to increase the safety of ovarian stimulation and to reduce potential effects on embryo development and implantation, which will ultimately be translated into increased pregnancy rates and healthy offspring.

Contents

1. Introduction

Some thirty years after the birth of the first "test-tube" baby, *in vitro* fertilization (IVF) has become a widely available treatment for most causes of subfertility. Despite ongoing advances in the associated assisted reproductive technologies (ART), pregnancy rates remain around 20-30% per started cycle. In order to compensate for inefficiencies in IVF procedures, high doses of exogenous gonadotropins are administered to stimulate the development of multiple oocytes to maturation in a single cycle. The use of such ovarian stimulation protocols enables the selection of one or more embryos for transfer, while supernumerary embryos can be cryopreserved for transfer in a later cycle (Macklon *et al.*, 2006). In recent years it has become evident that ovarian stimulation, although a central component of IVF, may itself have detrimental effects on oogenesis, embryo quality, endometrial receptivity, and perhaps also perinatal outcomes. In this article the impact of ovarian stimulation and underlying mechanisms will be reviewed. Strategies for reducing the impact of ovarian stimulation on IVF outcomes are also addressed.

2. Current regimens for ovarian stimulation

Ovarian stimulation with exogenous gonadotropins promotes the growth of multiple follicles to the preovulatory stage by interfering with the physiological mechanisms, which normally ensure single dominant follicle selection. It is important to distinguish this from ovulation induction treatment, which aims to restore normal follicular growth in anovulatory women.

Current regimens are based on the administration of high doses of either urinary derived or recombinant follicle-stimulating hormone (FSH). The aim is to raise serum FSH levels above the threshold required for follicle development for a prolonged period, in order to enable the growth and maturation of not just one, but the complete cohort of follicles which have reached the FSH dependent stage of development (Figure 2.1) (Fauser *et al.*, 2005). Starting doses of FSH usually vary between 150 and 450 IU/day (Verberg *et al.*, 2009). In addition to FSH, luteinising hormone (LH) may also be administered. However, LH has been shown not to be absolutely necessary for follicular development (Macklon *et al.*, 2006).

Stimulation of the growth of multiple follicles leads to their production of supraphysiological serum estradiol (E_2) levels, which by means of positive feedback at the pituitary may cause a premature LH peak and hence premature luteinization and ovulation. In order to prevent this, exogenous gonadotropin treatment is usually supplemented by the administration of gonadotropin-releasing hormone (GnRH) analogues. In the commonly employed "long protocol", GnRH agonists are commenced in the midluteal phase of the preceding cycle leading to an initial "flare" of gonadotropin hypersecretion, followed by desensitization of the pituitary, resulting in gonadotropin suppression and prevention of a premature LH surge. Although associated with hypoestrogenic side effects and a considerable patient burden, this protocol still remains the most widely used stimulation regimen in contemporary practice (Macklon *et al.*, 2006).

In recent years, GnRH antagonists have become established in clinical practice. In contrast to GnRH agonists, antagonists are immediately effective in reducing endogenous

gonadotropin production and their administration can hence be limited to the mid-to-late follicular phase of the menstrual cycle. They do not therefore suppress the endogenous intercycle rise in FSH, and as a result, less exogenous FSH may be required in association with GnRH antagonist *versus* agonist co-treatment (Figure 2.1) (Macklon *et al.*, 2006).

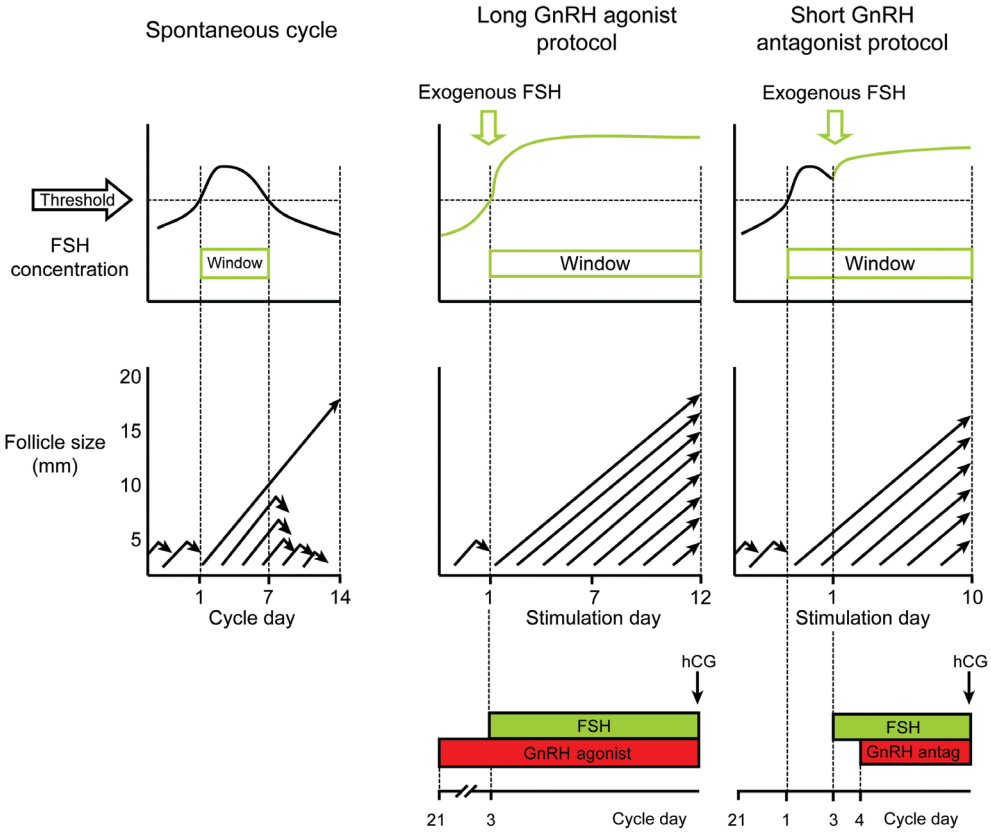


Figure 2.1 – Concentration of FSH, number and size of follicles during the follicular phase of the menstrual cycle in three different situations: natural cycle; long GnRH agonist regimen; and GnRH antagonist regimen. The threshold represents the concentration of FSH in serum above which ongoing gonadotropin-dependent follicle development is stimulated. The window represents the duration of time FSH concentrations are above the threshold. Each arrow represents a developing follicle. Adapted from Macklon *et al.* 2006.

An alternative approach advocated by some is "natural cycle IVF". In contrast to the aims of ovarian stimulation, this treatment is aimed at aspirating the single oocyte, which has developed during a spontaneous cycle. Although appealing in terms of cost and burden of treatment, frequently, no oocyte will be obtained. To reduce the risk of losing the oocyte to premature ovulation, "modified" natural cycle IVF employs GnRH antagonists together with a low dose of exogenous gonadotropins aimed at maintaining development of the follicle despite GnRH antagonist suppression of endogenous gonadotropins. Pregnancy rates using this approach are just 7% per cycle (Pelinck *et al.*, 2002). However, it has been suggested that women who respond poorly to exogenous gonadotropins may be good candidates for

natural cycle IVF (Schimberni *et al.*, 2008). Approximately 10% of women undergoing ovarian stimulation for IVF will demonstrate a poor response, defined as the production of fewer than four follicles (Pellicer *et al.*, 1987), and low levels of serum estradiol (Hanoch *et al.*, 1998). Although more frequent in older women (40 years old or older), poor ovarian response can also occur unexpectedly in younger women. Natural cycle IVF may be therefore an alternative to ovarian stimulation or egg donation, as it has been shown to be as effective as ovarian stimulation in terms of pregnancy rates in this group of patients (Schimberni *et al.*, 2008). Furthermore, in older poor responders, natural cycle allows the retrieval of the dominant follicle only, allowing fertilization of the putatively most competent oocyte available for retrieval.

3. How does ovarian stimulation affect early oocyte and embryo development?

In recent years the previously prevailing paradigm of stimulating hard to obtain large numbers of oocytes for IVF has been increasingly questioned. A number of studies have demonstrated the high burden, risk and costs of this approach, and a detrimental effect of ovarian stimulation on oocyte development. Pellicer and co-workers showed that the retrieval of >10 oocytes in women was correlated with oocytes of lower quality, as decreased fertility rates were reported in this group, when compared with two other groups of women in whom 1 to 5 or 6 to 10 oocytes were retrieved (Pellicer *et al.*, 1989). Similarly, our group has recently shown that the optimum chance of conceiving after the long protocol occurs associated with a harvest of 13 oocytes, and that a fall in pregnancy rates was observed when more than this number were obtained (Figure 2.2) (van der Gaast *et al.*, 2006). This could be indicative of a detrimental effect of supraphysiogical E_2 levels on oocyte quality or indeed endometrial receptivity, as is discussed later. A potentially lethal complication of ovarian stimulation, which is encountered in 1-2% of women undergoing IVF treatment, is the so-called ovarian hyperstimulation syndrome (OHSS). OHSS is associated with excessively high E_2 serum concentrations, which could explain the significantly lower percentages of good-quality oocytes and fertilization rates observed in cycles complicated by OHSS compared with control groups (Aboulghar *et al.*, 1997). In contrast, Ng *et al.* have reported normal nuclear maturity of oocytes and fertilization in patients with high E_2 serum concentrations (Ng *et al.*, 2003).

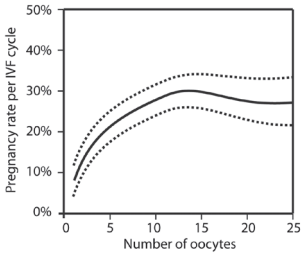


Figure 2.2 – Number of retrieved oocytes in relation to pregnancy rate per started IVF cycle. Adapted from van der Gaast *et al.* 2006.

The detrimental effects of exogenous gonadotropins on embryo development have been best characterized in rodent models. *In vitro* studies showed that ovarian stimulation disrupts (Ertzeid and Storeng, 1992) and delays (Van der Auwera and D'Hooghe, 2001) the development of 1- or 2 cell mouse embryos into blastocysts. Likewise, embryos from superovulated hamsters had significantly reduced mean cell numbers than the controls (McKiernan and Bavister, 1998). *In vivo* studies are concordant, indicating that ovarian stimulation delays embryo development (Ertzeid and Storeng, 2001; Van der Auwera and D'Hooghe, 2001). Furthermore, analysis of the surface architecture of mouse embryos showed a reduction

in the number of cells and of microvilli on blastocysts from gonadotropin-treated females, compared to those from spontaneously ovulating females (Champlin *et al.*, 1987). However, the results of human studies assessing possible effects of ovarian stimulation protocols on embryo development are inconsistent with mouse studies. A retrospective study comparing human embryo quality in the natural *versus* long GnRH agonist stimulated IVF cycle revealed no differences in cleavage rates, developmental capacity (number of blastomeres) or degree of fragmentation of the embryos (Ziebe *et al.*, 2004). Additionally, an excessive response to ovarian stimulation was shown to have no negative impact on embryo quality as assessed by morphology (Ng *et al.*, 2000; Ng *et al.*, 2003).

The discovery of extra-pituitary GnRH receptors in tissues such as the uterus, endometrium, oocytes-cumulus complex, preimplantation embryos and placenta (Casan *et al.*, 1999; Raga *et al.*, 1999; Grundker *et al.*, 2002) has lead to growing concern about possible detrimental effects GnRH antagonist may have on embryo development and implantation. *In vitro* studies have shown that GnRH antagonist is responsible for an inhibitory effect on preimplantation development of mouse embryos (Raga *et al.*, 1999). In an attempt to explain such results, Yang *et al.* (2009) has recently hypothesized that GnRH antagonists could interfere with cell growth by decreasing the synthesis of insulin-like growth factor (IGF) and epidermal growth factor (EGF) receptors, which are involved in the MAP kinase (MAPK)-mediated mitogenic cascade. However, the developmental potential of human preimplantation embryos does not seem to be limited by putative detrimental effects of GnRH antagonist (Yang *et al.*, 2009). Additionally, high doses of GnRH antagonist were shown not to harm the implantation potential of embryos in frozen-thawed cycles (Kol *et al.*, 1999) and a recent meta-analysis showed no significant differences in live-birth rates following co-treatment with GnRH agonists *versus* GnRH antagonists (Kolibianakis *et al.*, 2006).

The concern that suppressed LH concentrations in the late follicular phase may be detrimental to clinical IVF outcomes lead to the development of stimulation protocols including exogenous LH (Macklon *et al.*, 2006). Supplementation of LH activity may be advantageous to some patients by accelerating large follicle development, and decreasing the duration of treatment (Filicori *et al.*, 1999). Moreover, LH alone has been shown to be effective in monofollicular stimulation as part of a sequential ovarian stimulation protocol, following initiation with recombinant FSH (recFSH) (Sullivan *et al.*, 1999). Recent studies have indicated that stimulation protocols which include LH may increase the percentage of diploid (Weghofer *et al.*, 2008) and top-quality (Andersen *et al.*, 2006) preimplantation embryos. It has been proposed that such protocols may be beneficial to some women who respond poorly to standard "FSH only" regimens (Mochtar *et al.*, 2007). On the other hand, elevated follicular phase LH levels have been associated with reduced fertility and an increased risk of miscarriage (Regan *et al.*, 1990), which has been confirmed by recent data showing treatment with recLH alone in the late follicular phase to be detrimental to preovulatory follicle development (Hugues *et al.*, 2005; Rao and Tan, 2005). The contradictory findings regarding LH supplementation to ovarian stimulation protocols supports the concept of a "window" for LH, since there seems to be a threshold LH level below which estradiol production is inadequate, and a "ceiling" level above which LH may be detrimental to follicular development (Shoham, 2002).

In order to reduce the exposure of the patient to the risks and side effects of exogenous gonadotropin treatment, the maturation of oocytes can be performed *in vitro*. This approach normally involves the administration of a short period of low dose gonadotropins sufficient to stimulate multiple follicles to grow to a diameter of 12 mm at which the stage the oocytes are aspirated. Women with anovulatory infertility due to polycystic ovary syndrome (PCOS) are known to be at increased risk of developing OHSS and may therefore benefit from *in vitro* maturation (IVM) as an alternative to conventional IVF (Reinblatt and Buckett, 2008). An early case-control study comparing IVF and IVM in PCOS patients showed lower implantation rates with IVM (Child *et al.*, 2002). However, a recent meta-analysis comparing live birth rates after IVM to conventional IVF or intracytoplasmatic sperm injection (ICSI) in women with PCOS emphasized the need for controlled trials in this field (Siristatidis *et al.*, 2009). It therefore remains unclear whether IVM is beneficial for women with PCOS as an alternative to conventional IVF. Once concern with the approach is the relatively high rate of developmental incompetence observed in oocytes subject to IVM. Li *et al.* raised concerns regarding possible deleterious effects IVM might have on the organization of the meiotic spindle and chromosomal alignment (Li *et al.*, 2006). Although at present there are no indications of increased risk of congenital malformations in children conceived by IVM, the processes involved and the long-term outcomes are still poorly understood (Reinblatt and Buckett, 2008) and data from ongoing follow-up studies is awaited. Although there seems to be some evidence that IVM could provide a promising alternative to conventional IVF, particularly in women with PCOS, or others at increased risk of developing OHSS, prospective randomized controlled trials are needed before it can be recommended for clinical practice.

4. Does ovarian stimulation disrupt chromosomal competence of the oocyte and embryo?

Bidirectional signaling between oocytes and granulosa cells is essential for follicular development and the acquisition of oocyte competence (Eppig, 2001). The nuclear and cytoplasmic maturity of the oocyte that accompanies follicular development plays a crucial role in facilitating fertilization and the early stages of embryonic development (Albertini *et al.*, 2003). Exposure of the developing oocyte to supra-physiological concentrations of gonadotropins may disturb oocyte maturation and the completion of meiosis leading to chromosomal aneuploid oocytes and/or embryos (Hodges *et al.*, 2002).

Several studies in the mouse have investigated whether ovarian stimulation could induce chromosomal malsegregation during meiotic maturation. Early studies showed no increase in the incidence of non-disjunction in mouse oocytes obtained after ovarian stimulation *versus* spontaneous ovulation (Hansmann and El-Nahass, 1979; Golbus, 1981). However, more recent studies indicate that exogenous gonadotropin treatment contributes to increased frequency of chromosomal abnormalities. Mouse embryos originating from stimulated females showed a four-fold increase in sister chromatid exchange frequency than embryos from spontaneous ovulations, which is suggestive of induced DNA lesion by ovarian stimulation (Elbling and Colot, 1985). Moreover, when compared to zygotes derived from spontaneous ovulation,

mouse zygotes obtained after ovarian stimulation showed an increased rate of chromosomal aberrations in the female pronucleus and compromised embryo development (Vogel and Spielmann, 1992). Likewise, *in vitro* matured mouse oocytes exposed to high concentrations of FSH showed accelerated nuclear maturation and increased aneuploidy (Roberts *et al.*, 2005).

Although advanced maternal age is the only clearly identified risk factor for chromosomal aneuploidy in the human embryo (Hassold and Hunt, 2001; Champion and Hawley, 2002), a number of studies have reported particularly high rates of chromosomal aneuploidy and mosaicism in early human IVF embryos (Munne *et al.*, 1997; Katz-Jaffe *et al.*, 2005; Baart *et al.*, 2007a). Recently, post-zygotic chromosome instability has been observed to be a common feature of early human embryogenesis, leading to chromosomal disorders such as mosaicism and uniparental disomies in the majority of cleavage stage embryos (Vanneste *et al.*, 2009). Although the mechanisms underlying aneuploidy are still poorly understood, it has been hypothesized that increased rates of embryo aneuploidy could also result from the interference of ovarian stimulation with the natural selection of good-quality oocytes or from exposure of growing follicles to detrimental effects of hyperstimulation on oocyte maturation (Verberg *et al.*, 2009). In order to investigate the role of ovarian stimulation as a possible cause of chromosomal malsegregation in human IVF cleavage stage embryos, Baart *et al.* carried out preimplantation genetic screening (PGS) for aneuploidy using fluorescent *in situ* hybridization (FISH) for 10 chromosomes in two blastomeres biopsied from viable embryos derived from two different stimulation protocols. A significantly higher proportion of aneuploid embryos following conventional high FSH dose long protocol was observed compared with that found after exposure to a mild, lower FSH dose ovarian stimulation protocol (Baart *et al.*, 2007a). The increased number of abnormal embryos was mainly due to a higher incidence of mitotic segregation errors, leading to mosaicism. These findings supported both previous reports of an association between ovarian stimulation regimens and chromosomal mosaicism in human embryos (Munne *et al.*, 1997), as well as reports indicating an association between meiotic and mitotic chromosome 21 cell division errors with significantly higher daily FSH doses (Katz-Jaffe *et al.*, 2005). Thus, milder ovarian stimulation regimens seem to be less detrimental to the vulnerable process of nuclear maturation and chromosomal segregation.

As mentioned previously, the value of LH supplementation to FSH stimulation protocols remains unclear. In an attempt to address this question from a cytogenetic viewpoint, Weghofer *et al.* evaluated the effect of ovarian stimulation on the ploidy of cleavage stage embryos after long agonist down-regulation combined with either recFSH or human menopausal gonadotropin (hMG) (Weghofer *et al.*, 2008). In this small study, a higher rate of diploidy and ongoing pregnancies per cycle was seen in women treated with hMG, suggesting that LH-containing ovarian stimulation protocols may be beneficial for achieving higher diploidy rates in preimplantation embryos.

A significant increase in the proportion of morphologically abnormal oocytes after repeated rounds of ovarian stimulation has been reported both in the cow and the mouse (Lubbadeh *et al.*, 1980; Kanayama and Osada, 2000). In attempt to determine whether repeated ovarian stimulation affected oocyte competence also at the nuclear and cytoplasmic

levels, Van Blerkom and Davis used a mouse model to study the effects of four rounds of ovarian stimulation on cytoplasmic and spindle organization. *In vivo* matured oocytes were reported to suffer a progressive and significant increase in the frequency of spindle defects with each additional round of ovarian stimulation (Van Blerkom and Davis, 2001). In humans, a number of studies confirmed the results from animal studies, with pregnancy and implantation rates reported to significantly decline in cycle 2 compared with cycle 1, (Shapiro *et al.*, 2001; Silberstein *et al.*, 2005; Wang *et al.*, 2008), reaching a plateau for cycles 3-5 at a rate lower than in cycle 2 (Silberstein *et al.*, 2005). However, other studies do not show significant declines on ovarian response to gonadotropin stimulation with repeated cycles, either in terms of the number of oocytes retrieved or in the quality of the embryos based on morphological criteria (Hoveyda *et al.*, 2002; Kolibianakis *et al.*, 2002; Kolibianakis and Devroey, 2004; Doldi *et al.*, 2005). None of these studies looked into the cytogenetic outcomes of the embryos generated, and it therefore remains unclear whether repeated cycles of ovarian stimulation may interfere with oocyte and/or embryo chromosomal competence.

5. Ovarian stimulation and epigenetics

Epigenetic mechanisms regulate gene activity in a hereditary fashion without affecting the genetic constitution (Lucifero *et al.*, 2004). Gene imprinting is an epigenetic process which allows a subset of genes to be expressed in a monoallelic parent-of-origin manner (Lawrence and Moley, 2008). Imprinting occurs in genes that have been shown to be essential for embryonic growth and development, placental function and postnatal behaviour (Isles and Holland, 2005; Fowden *et al.*, 2006; Smith *et al.*, 2006). The main epigenetic mechanisms controlling imprinting are DNA methylation and histone modification. DNA methylation is the best characterised epigenetic modification and in many cases occurs in a differentially methylated region (DMR) (Lucifero *et al.*, 2004).

In the mouse, methylation patterns of imprinted genes are erased in the germ line. In the male, remethylation starts early during embryonic development in the gonocytes and continues up to the spermatogonia stage, whereas in the female it begins after birth, early in the oocyte growth phase, continuing throughout oocyte growth (Zamudio *et al.*, 2008). In humans little information is available on imprinting dynamics, but existing data suggest some conservation of the epigenetic mechanisms described in the mouse (Lucifero *et al.*, 2004).

Genes that acquire their imprints late in oocyte development are believed to be the most susceptible to perturbations on their imprints (Gosden *et al.*, 2003; Fortier *et al.*, 2008). Ovarian stimulation regimens promote the development of many oocytes in a non-physiological endocrine milieu. Therefore it is possible that the acquisition of methylation imprints in oocytes may be disturbed by ovarian stimulation. Methylation defects at the DMRs of *SNRPN* (Angelman syndrome), *KCNQ1OT1* (Beckwith-Wiedemann syndrome) and *PEG1*/*MEST* (Silver-Russel Syndrome) have been identified in affected children conceived with ART (Lawrence and Moley, 2008). Two cell mouse embryos from superovulated female mice showed a correlation between the number of abnormally methylated embryos and embryo loss during preimplantation, indicating that ovarian stimulation may lead to epigenetic abnormalities (Shi

and Haaf, 2002). Determination of DNA methylation profiles of the DMRs of maternally (*PEG1*) and paternally (*H19*) imprinted genes in both mouse and human oocytes demonstrated imprinting reversal upon ovarian stimulation (Sato *et al.*, 2007). Monoallelic expression of *Snrpn* and *H19* imprinted genes in the mouse placenta seems particularly susceptible to perturbation following ovarian stimulation (Fortier *et al.*, 2008). These and previous results from Mann *et al.* suggest that trophectoderm-derived tissues are more susceptible to imprinting disruption (Mann *et al.*, 2004; Fortier *et al.*, 2008).

Ovarian stimulation has also been suggested to have an epigenetic effect on folliculogenesis and gametogenesis by possibly interfering with the homocysteine pathway. A number of intermediates of this pathway are directly involved in processes such as protein and DNA synthesis, and oxidative stress balance, which have important roles in gametogenesis (Ebisch *et al.*, 2007). Ovarian stimulation has been shown to alter folate metabolism in the follicle, which may be a further mechanism by which normal folliculogenesis is disrupted (Boxmeer *et al.*, 2008).

6. Endometrial receptivity and embryonic implantation

Increasing evidence points to pre-clinical pregnancy loss rather than failure of conception as the principal cause for the relatively low fecundity observed in humans. In natural cycles, up to 55% of conceptions are estimated to be lost due to implantation failure or pre-clinical miscarriage (Figure 2.3a) (Macklon *et al.*, 2002). In a recent study by Boomsma *et al.* it was shown that in stimulated cycles, the contribution of implantation failure for the numbers of conception losses is higher (50%) than described for natural cycles (30%) (Figure 2.3b) (Boomsma *et al.*, 2009). This suggests that in patients undergoing ART, not only the quality of the embryo is crucial for achieving successful implantation and clinical pregnancy, but the endometrium also plays an important role.

There are some indications that high E_2 levels resulting from ovarian stimulation may impair endometrial receptivity (Pellicer *et al.*, 1989; Paulson *et al.*, 1990; Simon *et al.*, 1995; Pellicer *et al.*, 1996; Simon *et al.*, 1998). Once the threshold level of E_2 is exceeded, progesterone receptors may be prematurely induced, leading to an increased sensitivity to progesterone and thus early endometrial secretory advancement. This has been described to occur not only during GnRH agonist/gonadotropin protocols in the preovulatory phase, but also during GnRH antagonist/recFSH stimulation (Macklon *et al.*, 2006; Hayden, 2008). In mice, levels of E_2 have been shown to have a critical role in regulating the window of uterine receptivity (Ma *et al.* 2003). Using a delayed-implantation model, low levels of E_2 were shown to maintain uterine receptivity for a longer period of time, whereas high E_2 levels lead to a refractory state, leading to implantation failure (Ma *et al.*, 2003). Moreover, E_2 was shown to have a detrimental effect on embryonic adhesion in mice, with both embryo and endometrium being affected (Gidley-Baird *et al.*, 1986; Ng *et al.*, 2000), although the latter was affected at higher E_2 concentrations only (Valbuena *et al.*, 2001). Ertzeid and Storeng also observed reduced implantation and increased embryo mortality in superovulated recipient mice compared with controls. These authors proposed that decreased uterine receptivity

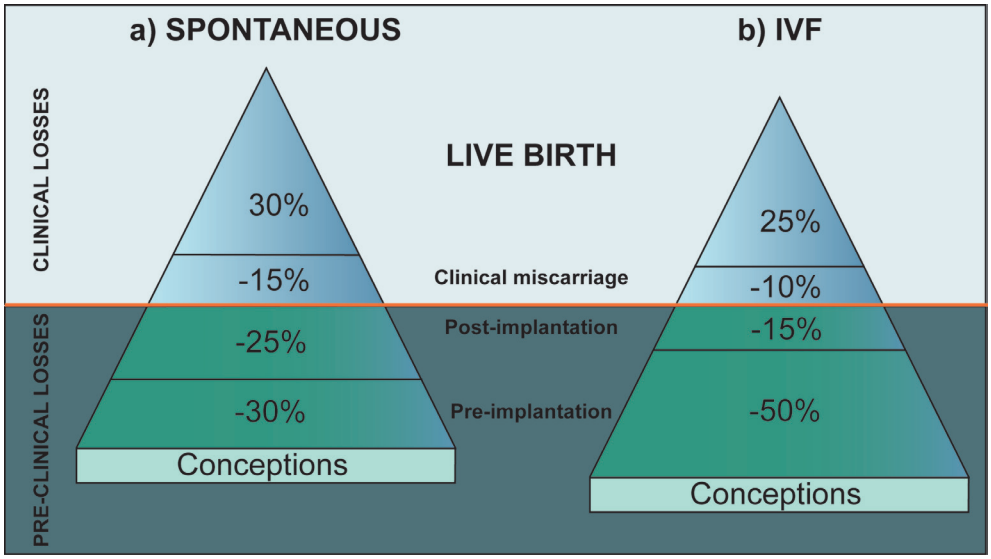


Figure 2.3 – **The pregnancy loss iceberg: an overview of the outcomes of spontaneous versus IVF pregnancies. a)** A total of 70% of conceptions are lost prior to live birth. The majority of these losses occur prior to the time of the missed menstrual period, and are not revealed. **b)** In stimulated cycles, the iceberg "sinks" mainly due to increased preimplantation losses, which results in 75% of conceptions being lost prior to live birth. Adapted from Macklon *et al.* 2002.

after exogenous administration of gonadotropins could be caused by altered expression of cytokines in the endometrium of superovulated mice. Additionally, they have also shown that embryos from superovulated donors transferred to control recipients had a lower implantation rate when compared with that of embryos from control donors (Ertzeid and Storeng, 2001). Therefore, it seems that gonadotropin stimulation compromised not only uterine receptivity but also oocyte/embryo developmental competence.

According to Simon *et al.*, low implantation rates in high responders can be improved by the use of a step-down regimen in a subsequent cycle, which has been shown to result in lower E_2 levels (Simon *et al.*, 1998). An *in vitro* mouse model mimicking early and late embryonic transfers supports these findings, showing that reduction of embryonic exposure to E_2 in late embryo transfers seems to attenuate the toxic effect of E_2 on embryo implantation (Valbuena *et al.*, 2001). Thus, implantation rates in high responders may be improved either by reducing E_2 levels (Simon *et al.*, 1998) or by reducing the time of exposure of the embryo to E_2 (Valbuena *et al.*, 2001).

7. Perinatal outcomes

The evaluation of the use of gonadotropins for ovarian stimulation as a risk factor for perinatal outcomes is complex due to the difficulty of eliminating other confounding risk factors such as maternal age, parity and *in vitro* procedures. Furthermore, ART patients with a history of subfertility have been associated with several fetal and neonatal abnormalities (Lambert, 2003;

Shiota and Yamada, 2005). Subfertility might therefore partially contribute for the association between assisted conception and poor perinatal outcome of singletons. However, several studies seem to indicate that ART itself, including ovarian stimulation, also has an important effect (Kapiteijn *et al.*, 2006).

The use of gonadotropins for ovarian stimulation is the most important cause of multiple pregnancies in ART patients in the United States, with a third of multiple pregnancies being caused by non-IVF ovarian stimulation (Ombelet *et al.*, 2006). Multiple pregnancies are associated with increased risk of miscarriage, growth retardation and preterm delivery (Fauser *et al.*, 2005). However, even singletons are at higher risk of low birthweight, premature birth and perinatal mortality and morbidity in the subfertile population using ART (Schieve *et al.*, 2002; Helmerhorst *et al.*, 2004; Jackson *et al.*, 2004; Kapiteijn *et al.*, 2006; Ombelet *et al.*, 2006). Mouse studies are concordant, as the mean weight of live fetuses was shown to be significantly lower for fetuses obtained from superovulated recipients, compared with that of those obtained from control recipients (Ertzeid and Storeng, 2001). Several studies suggest that low birthweight in IVF singletons is associated with ovarian stimulation (Wennerholm *et al.*, 1997; Kallen *et al.*, 2005b; Wang *et al.*, 2005; Kapiteijn *et al.*, 2006). Nonetheless, a recent study showed no correlation between ovarian stimulation parameters and birthweight (Griesinger *et al.*, 2008). These authors suggest that the results from previous studies indicating an association between ovarian stimulation and low birthweight could be possibly explained due to confounding by the infertility background of the study population. Further studies are therefore needed to confirm the effect of ovarian stimulation on birthweight of IVF babies.

Ovarian stimulation has been shown to lead to imprinting defects in the mouse placenta (Fortier *et al.*, 2008). Elevated expression levels of paternally imprinted gene *insulin-like growth factor 2 (IGF2)* in the placenta have been correlated with fetal growth restriction in humans (Street *et al.*, 2006) and sheep (de Vrijer *et al.*, 2006) and with early embryonic lethality of somatic cell nuclear transfer derived cows (Oishi *et al.*, 2006). Since low birthweight in humans may be an important risk factor for the development of neurological disorders and adult-onset diseases such as coronary heart disease, stroke, hypertention, type II diabetes and osteoporosis, ovarian stimulation could even have adverse effects in adult life (Fleming *et al.*, 2004).

Confined placental mosaicism (CPM), has also been associated with intrauterine growth retardation (Lestou and Kalousek, 1998). Although our group hypothesized that increased rates of CPM may occur after ovarian stimulation due to the persistence of chromosomal mosaicism present in preimplantation embryos into later gestation, and that this mechanism may underlie the reported increase in intrauterine growth retardation in IVF singletons, a large review of national databases were unable to confirm this (Jacod *et al.*, 2008).

A large Swedish cohort study comparing the risk of congenital malformations in infants born after IVF with that of controls showed an association between birth defects and ART (Kallen *et al.*, 2005a). More recently, a multicenter American case-control study corroborated these observations (Reefhuis *et al.*, 2009). Nevertheless, none of the studies looked into a possible association between the administration of drugs used for ovarian stimulation and the incidence of congenital diseases. Data from a meta-analysis by Elizur and Tulandi suggest that

the risk of congenital diseases caused by drugs commonly used in infertility treatments such as aromatase inhibitors, GnRH agonists and antagonists, estrogen and progesterone may be null or minimal (Elizur and Tulandi, 2008). Clomiphene treatment was the only exception, as it might be associated with a slightly higher risk of neural tube defect and hypospadia.

In order to determine the details of adverse birth events in children conceived by ART, the majority of studies mentioned above consulted national or regional registries (Wennerholm *et al.*, 1997; Kallen *et al.*, 2005b, a; Wang *et al.*, 2005; Griesinger *et al.*, 2008; Jacod *et al.*, 2008). The studies by Kapiteijn *et al.* and Reefhuis *et al.* were based was predominantly based on interviews of mothers, who were asked to recall information regarding the preconceptional and pregnancy periods (method of conception, ethnicity, parity, duration of gestation, birth weight, etc). This method of data collection can lead to significant biases and therefore extrapolations based in this kind of analysis have to be moderate.

Overall however, from the studies done so far it seems that the risk of birth defects in children conceived by ART is very small, just 1-2% greater than reported in naturally conceived children (Elizur and Tulandi, 2008). However, follow up studies in adulthood are crucial for a real evaluation of possible long-term effects of ART.

8. Conclusions and future perspectives

Since the birth of Louise Brown in 1978, significant advances have been made in both clinical and laboratory aspects of IVF treatment. However, pregnancy rates remain relatively low, showing there is still much to be learned about the endocrinology of follicle development, oocyte maturation and ovulation, as well as embryo development and implantation. New advances in molecular biology (genomics, epigenetics, proteomics and pharmacogenomics) will contribute to increase our knowledge on ovarian and endometrial physiology and the impact of stimulation regimens at the molecular level, which is still poorly understood. With this knowledge, milder ovarian stimulation regimens can be designed which reduce the potentially adverse effects (Table 2.1) on embryo development. Furthermore, as different patients show distinct responses to the same stimulation protocol, a better understanding of the mechanisms that are affected by ovarian stimulation will help in the development of patient-specific treatments.

A major determinant of IVF success is the accurate selection of the most competent embryos for embryo transfer. Morphology and development rate remain the cornerstones of embryo selection, but are a limited measure of embryo competence. Other techniques such as FISH have been employed to assess the chromosomal constitution of embryos prior to selection for transfer. This is termed preimplantation genetic screening (PGS). Although a number of observational and uncontrolled studies have suggested higher pregnancy rates and reduced miscarriage rates could be achieved after PGS (Devroey and Fauser, 2007), large randomised trials have shown no benefit for pregnancy and delivery rates (Staessen *et al.*, 2004; Mastenbroek *et al.*, 2007). This could be explained by possible damage to the embryo during blastomere biopsy; limitations of FISH technology (only a few chromosomes can be analyzed); and the phenomenon of chromosomal mosaicism (Devroey and Fauser, 2007).

Future studies focusing on a better understanding of the mechanisms and clinical significance of chromosomal mosaicism in early stage embryos may aid interpretation of PGS data, while alternative techniques to FISH such as comparative genomic hybridization (CGH) offer the ability to analyse of the complete set of chromosomes. An alternative to the invasive PGS approach is provided by the analysis of cumulus cell gene expression, which has been proposed as a non-invasive way of accessing embryo quality. Cumulus cells are closely associated with oocytes, and oocyte-cumulus cell communication has been shown to be essential to oocyte development (Hutt and Albertini, 2007). Therefore, the study of differential expression of genes involved in key cumulus cell regulatory pathways using the real-time quantitative polymerase chain reaction offers a promising approach to increase our understanding of the factors controlling follicular development. This would allow not only identification of the most competent oocytes but also monitoring the consequences of different stimulation protocols on the cohort of oocytes retrieved, ultimately contributing to a better understanding of the impact of ovarian stimulation on embryo development.

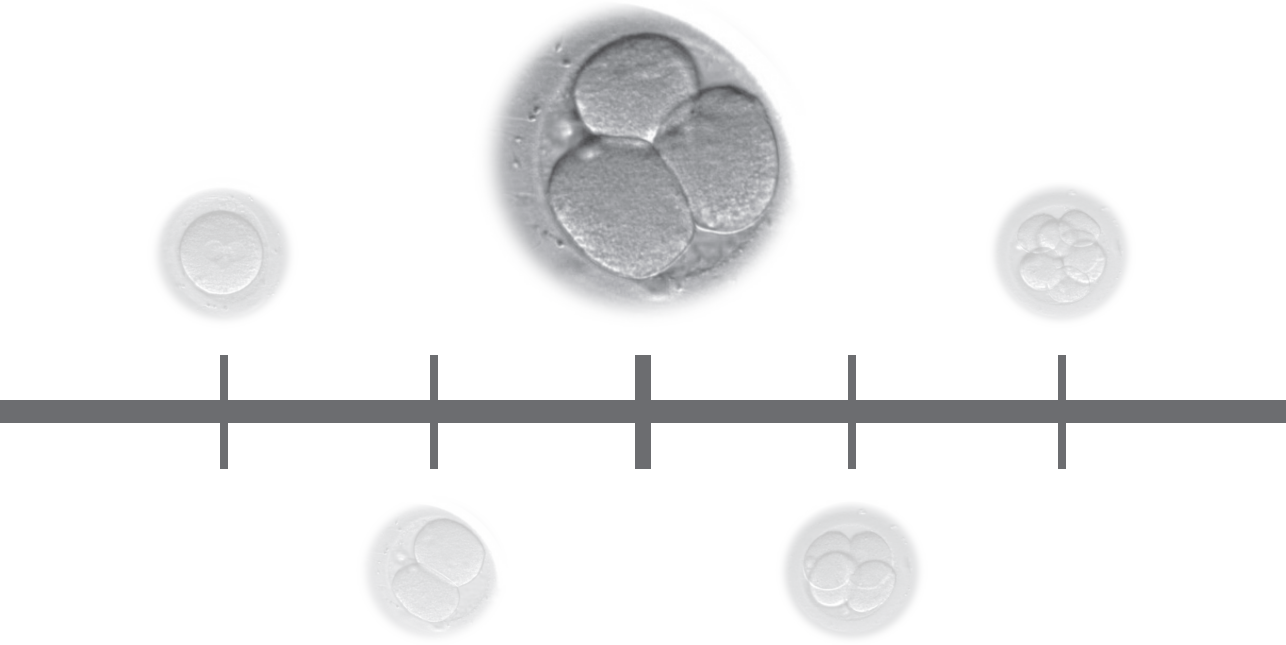
Although most of the research has been mainly focusing in investigating oocyte and embryo development, in an attempt to explain relatively low pregnancy rates, the success of ART does not depend solely on the quality of the embryo, as pre-clinical losses rather than failure of conception are suggested as the main limiting factor (Macklon *et al.*, 2002). The cross talk between the embryo and the endometrium seems to be of major importance for achieving implantation and successful pregnancy. However, there is still poor knowledge of the mechanisms involved in such communication. Therefore new studies exploring the molecular interactions occurring at the embryo-endometrial interface will be crucial to explain low implantation rates and hopefully improve pregnancy rates in patients undergoing ART (Teklenburg and Macklon, 2009).

According to the studies published so far, most medications used in ART appear to be safe. However, it is necessary to carefully reassess the safety of ovarian stimulation on the first generation of ART generated children that is now reaching adulthood. The true impact of ovarian stimulation on development of the offspring will only become clear when the offspring of this generation have reached maturity. Until then, while major detrimental effects appear to be limited, caution continues to be required when developing and administering novel ovarian stimulation regimens for IVF.

Table 2.1 – Summary of the possible impact of ovarian stimulation used for IVF on embryo development.

Parameters correlated with ovarian stimulation	Main outcomes	References
Oocyte development	Retrieval of high numbers of oocytes correlated to lower quality and lower chances of conceiving.	Pellicer *et al.* 1989 Van der Gaast *et al.* 2006
Embryo development	No affect of ovarian stimulation administration on normal cleavage rates, morphology, developmental capacity or degree of fragmentation.	Ng *et al.* 2000 Ng *et al.* 2003 Ziebe *et al.* 2004
	No effect of high doses of GnRH antagonist on implantation potential of embryos in frozen-thawed cycles.	Kol *et al.* 1999
	Early human IVF embryos show high rates of chromosomal aneuploidy and mosaicism.	Baart *et al.* 2007 Katz-Jaffe *et al.* 2005 Munne *et al.* 1997
	Possible association between ovarian stimulation protocols and chromosomal mosaicism.	Baart *et al.* 2007 Munne *et al.* 1997
	Higher doses of FSH used in the long GnRH agonist stimulation protocol lead to increased proportion of aneuploid embryos.	Baart *et al.* 2007
	Higher doses of FSH associated with meiotic and mitotic chromosome 21 cell division errors.	Katz-Jaffe *et al.* 2005
	Repeated rounds of ovarian stimulation may compromise oocyte competence. Implantation and pregnancy rates decline from cycle 1 to cycle 2 and reach a plateau after cycle 2.	Shapiro *et al.* 2001 Silberstein *et al.* 2005 Wang *et al.* 2008
Epigenetics	Methylation defects at the DMRs of *Snrpn* (Angelman syndrome), *Kcnq1ot1* (Beckwith-Wiedemann syndrome) and *Peg1*/*Mest* (Silver-Russel Syndrome) identified in affected children conceived with ART.	Lawrence & Moley 2008(Lawrence and Moley, 2008)
	Correlation between the number of abnormally methylated embryos and embryo loss during implantation.	Shi & Haaf 2002
	Loss of *Peg1* and gain of *H19* methylation in oocytes obtained after ovarian stimulation.	Sato *et al.* 2007
	Trophectoderm-derived tissues more susceptible to imprinting disruption following ovarian stimulation.	Fortier *et al.* 2008 Mann *et al.* 2004
	Putative folliculogenesis disruption due to alteration of folate metabolism after ovarian stimulation.	Boxmeer *et al.* 2008

Endometrial receptivity	Estrogen levels above the threshold lead to endometrial secretory advancement.	Hayden *et al.* 2008 Macklon *et al.* 2006
	The reduction of embryo exposure to high levels of estrogen using a step-down regimen in a subsequent cycle improves implantation rates.	Simon *et al.* 1998
Perinatal outcomes	ART enhances the risk of multiple pregnancies, which are associated with increased risk of miscarriage, growth retardation and preterm delivery.	Fauser *et al.* 2005
	Singleton IVF babies are at higher risk of low birth weight, premature birth and perinatal mortality and morbidity in the infertile population.	Helmerhorst *et al.* 2004 Jackson *et al.* 2004 Kapiteijn *et al.* 2006 Ombelet *et al.* 2006 Schieve *et al.* 2002
	Low birth weight in IVF singletons associated with ovarian stimulation.	Kallen *et al.* 2005a Kapiteijn *et al.* 2006 Wang *et al.* 2005 Wennerholm *et al.* 1997
	Infants born after IVF have a higher risk of developing congenital malformations.	Kallen *et al.* 2005b Reefhuis *et al.* 2009
	The risk of congenital diseases caused by aromatase inhibitors, GnRH agonists, GnRH antagonists, estrogen and progesterone may be null or minimal. Clomiphene was the only drug associated with a slightly higher risk of neural tube defect and hypospadia.	Elizur & Tulandi 2008
	The overall increased risk of congenital diseases in children conceived by ART is 1% to 2%.	Elizur & Tulandi 2008



Chapter 3

The fate of the mosaic embryo: chromosomal constitution and development of day 4, 5 and 8 human embryos

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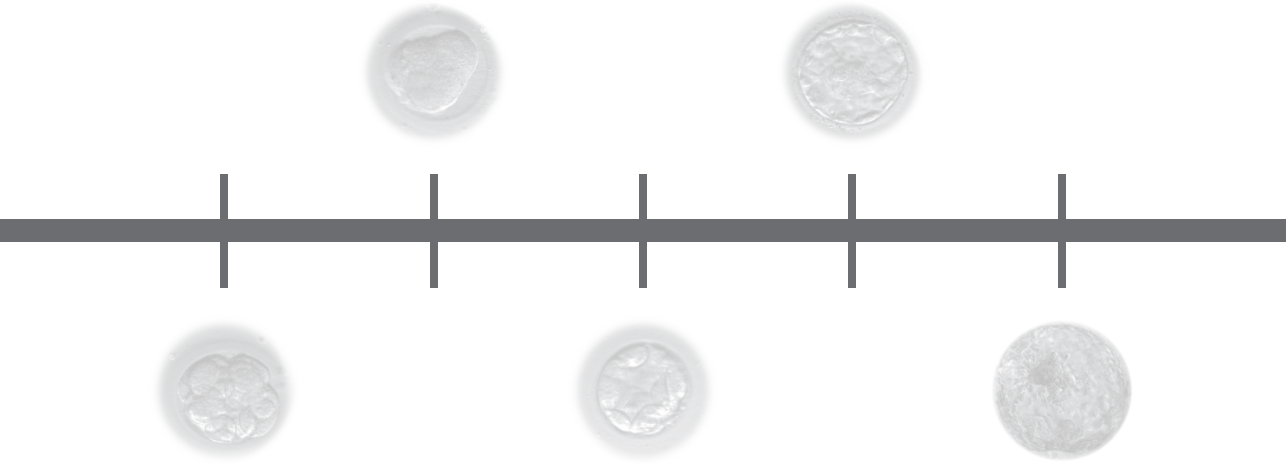

Abstract

BACKGROUND: Post-zygotic chromosome segregation errors are very common in human cleavage stage embryos after *in vitro* fertilization, resulting in chromosomally mosaic embryos. Little is known regarding the significance of mosaicism for the developmental potential of early embryos. We assessed chromosomal constitution and development of embryos from compaction to the peri-implantation stage at day 8 after fertilization.

METHODS: From 112 cryopreserved day 4 human embryos donated for research, 21 were immediately fixed and all cells analyzed by fluorescent *in situ* hybridization (FISH). The remaining 91 embryos were thawed, with 54 embryos suitable for biopsy of one or two cells. Biopsied cells were fixed and analyzed by FISH for chromosomes 1, 7, 13, 15, 16, 18, 21, 22, X and Y. Biopsied embryos were kept in standard culture conditions for 24h. Embryos arrested before cavitation (n=24) were fixed. Developing day 5 blastocysts (n=24) were submitted to co-culture for a further 72 h on a monolayer of decidualized endometrial stromal cells, followed by fixation. Cell numbers were counted and all nuclei analyzed by FISH. Data derived from a previous FISH analysis done by our group on cryopreserved good quality day 5 blastocysts (n=36) were included in the present study.

RESULTS: FISH analysis was successfully performed on 18 day 4 embryos. According to our definition, 80% of embryos were mosaic and 11% showed a chaotic chromosomal constitution. FISH analysis of two blastomeres from morula stage embryos showed that 54% of the embryos were mosaic, 40% normal, and 6% abnormal. Analysis of day 4, 5 and day 8 embryos showed a decrease in incidence of mosaic embryos over time, from 83% on day 4 to 42% on day 8. A significant positive correlation was observed between the total cell number and the percentage of normal cells in developing day 5 and day 8 embryos but not in developing day 4 or embryos arrested before cavitation.

CONCLUSIONS: These data suggest that both the developmental arrest of a significant proportion of mosaic embryos on day 4, and the cell death or reduced proliferation of aneuploid cells within an embryo may be responsible for the observed decrease of aneuploid blastomeres from compaction to the peri-implantation stage.

Introduction

Most of our current knowledge concerning the chromosomal constitution of human preimplantation embryos comes from the analysis of cleavage stage embryos by preimplantation genetic screening (PGS) performed 3 days after fertilization, when embryos are usually composed of 6-10 cells (blastomeres). Molecular cytogenetic analysis of interphase nuclei by fluorescence *in situ* hybridization (FISH) has been the most frequently used technique for the analysis of chromosomal abnormalities in human embryos. Data obtained by such studies have indicated that more than 50% of human cleavage stage embryos generated by *in vitro* fertilization (IVF) contain chromosomally abnormal cells (Delhanty, 2005). These abnormalities may arise from an error during meiosis, resulting in a uniform abnormality present in all cells, or from segregation errors occurring during the first mitotic divisions. The latter event results in chromosomal mosaicism, defined as the coexistence of karyotypically distinct cell lineages derived from a single zygote.

Mosaic embryos can be composed of a mixture of chromosomally normal and abnormal cells or of abnormal cells with different abnormalities. Mosaicism has been reported to affect up to 91% of human preimplantation embryos if all cells are investigated (Voullaire *et al.*, 2000; Wells and Delhanty, 2000; Bielanska *et al.*, 2002a; Clouston *et al.*, 2002; Baart *et al.*, 2006; Mantzouratou *et al.*, 2007; Vanneste *et al.*, 2009). Studies using comparative genomic hybridization (CGH) and array CGH in human preimplantation embryos, allowing the screening of all chromosomes, have confirmed the high prevalence of chromosomal mosaicism at this early stage of development and also demonstrated the high incidence of structural abnormalities (Voullaire *et al.*, 2000; Wells and Delhanty, 2000; Vanneste *et al.*, 2009).

These recent findings have changed our understanding of the cytogenetic processes occurring at the cleavage stage. However, the implications of chromosomal mosaicism for further embryonic development and implantation remain unclear. So far, the majority of the studies investigating the chromosomal constitution of human blastocysts have suggested no definite selection against most of the chromosomal abnormalities observed at the cleavage stage (Benkhalifa *et al.*, 1993; Evsikov and Verlinsky, 1998; Magli *et al.*, 2000; Ruangvutilert *et al.*, 2000; Sandalinas *et al.*, 2001; Bielanska *et al.*, 2002a; Coonen *et al.*, 2004; Baart *et al.*, 2006; Fragouli *et al.*, 2008). Although there are reports of an increase in the proportion of blastocysts showing chromosomal mosaicism, compared to early cleavage stage embryos (Sandalinas *et al.*, 2001; Bielanska *et al.*, 2002a), the proportion of aneuploid cells within an embryo seems to decline towards the blastocyst stage (Ruangvutilert *et al.*, 2000; Bielanska *et al.*, 2002a).

In spite of the high frequency observed in preimplantation embryos, a low percentage (0.3%) of aneuploidy is found at term birth (Hassold *et al.*, 1996). Up to 91% of preimplantation embryos are mosaic (Vanneste *et al.*, 2009). However, the incidence of mosaicism in spontaneous abortion specimens is significantly lower (<10%). First trimester diagnoses in chorionic villi from viable pregnancies show an even lower incidence of mosaicism (1 to 2%) (Los *et al.*, 2004). It seems that the majority of mosaic embryos disappear prior to the period of first trimester, either due to a selection against mosaic embryos, or "normalization", due to

selection against abnormal cells within the embryo (Los *et al.*, 2004). Therefore, a "black box" remains concerning events surrounding implantation (Macklon *et al.*, 2002).

At present, our knowledge of the fate of the mosaic human embryo is limited to the blastocyst stage at day 6 post-fertilization. Clearly, if the significance of mosaicism for subsequent development is to be understood, events subsequent to this stage need to be elucidated. The aim of the present study was therefore to assess the prevalence of mosaic embryos and how the chromosomal constitution of human embryonic cells evolves from compaction to the peri-implantation period at day 8 post-fertilization, using an *in vitro* implantation model.

Material and methods

Embryos

Surplus cryopreserved preimplantation embryos were donated with written consent by 25 couples undergoing routine IVF at the University Medical Center Utrecht in the period between April 1997 and October 2003. Women participating in this study were aged between 29-41 years-old and all became pregnant in the fresh cycle. The use of these embryos for this study was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO – NL12481.000.06) and the local institutional ethics committee.

Ovarian stimulation, oocyte retrieval and IVF procedures, including assessment of embryo morphology, were performed as described previously (Heijnen *et al.*, 2007). A maximum of two day 4 embryos were transferred per cycle. Supernumerary good quality embryos showing some degree of compaction and <20% fragmentation were cryopreserved. Cryopreservation was performed in straws using a slow freezing standard protocol of 1.5 M dimethyl sulfoxide (DMSO) in HTF medium containing 10% GPO (human plasma solution, CLB, Amsterdam, The Netherlands). The embryos were cooled to -6°C for seeding and subsequently slowly cooled to -40°C (rate 0.3°C/min). Finally, they were cooled rapidly at -25°C/min to -140°C, before immersion in liquid nitrogen.

Figure 3.1 summarizes the experimental setup of this study. A total of 112 day 4 cryopreserved embryos were used for this study, after storage for an average of 8.6 years (range: 4.7-11.9). Twenty-one randomly selected day 4 embryos were thawed and all the cells fixed within 10 minutes after removal from liquid nitrogen. Whole embryos were fixed on slides as described for blastomere spreading (see section "Biopsy and fixation of blastomeres and embryos"). From the remaining 91 embryos, a total of 57 survived the thawing procedure, consisting of consecutive washes in decreasing DMSO concentrations. These embryos were then transferred to culture medium supplemented with 14.2% GPO and biopsied within 2h, making use of the spontaneous decompaction embryos demonstrated shortly after thawing and when washed in calcium/magnesium-free medium (G-PGD medium, Vitrolife, Sweden). Before the biopsy procedure, the embryos were scored for quality and number of blastomeres, according to previously described criteria (Huisman *et al.*, 2000; Hohmann *et al.*, 2003). Embryos at an advanced and irreversible stage of compaction (n=3) were not biopsied and therefore excluded from this study.

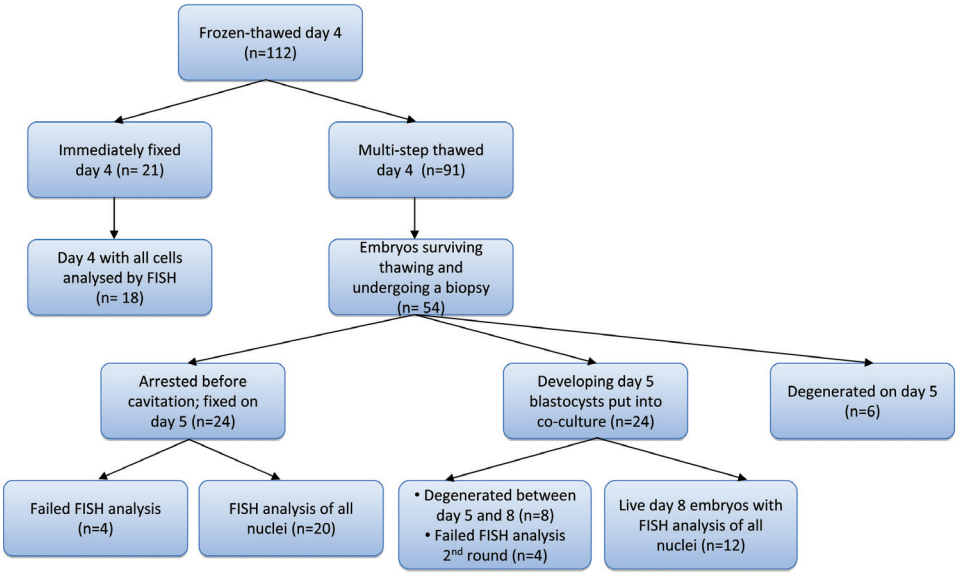

Figure 3.1 – Flow chart summarizing the experimental design of the study.

Embryo-endometrial stromal cell co-culture

Biopsied embryos (n=54) were cultured for a further 24h under standard embryo culture conditions (Heijnen *et al.*, 2007). Six of these had degenerated and were therefore not analyzed. On day 5, embryos arrested before cavitation (n=24) were fixed, whereas developing blastocysts (n=24) were co-cultured on a monolayer of decasualized endometrial stromal cells for a further 72h, as described previously (Carver *et al.*, 2003). In short, endometrial tissues at different stages of the menstrual cycle were obtained from fertile patients undergoing hysterectomy for benign conditions. Samples of surplus endometrial tissue were collected from the Pathology department of the UMCU, with consent from the patients. Endometrial stromal cells were isolated as described previously (Carver *et al.*, 2003) and decasualization was induced by culturing confluent endometrial stromal cells in the presence of 0.5 mM 8-Br-cAMP and 1 µM MPA (both Sigma, UK) for 3-5 days. Day 5 embryos showing signs of cavitation were subjected to 0.1% Pronase in HEPES buffered HTF to remove the zona pellucida and co-cultured with confluent monolayers of decidualized endometrial stromal cells for 72h in the presence of DMEM/F-12 complete medium (Invitrogen, USA). At the end of the co-culture period, embryos were photographed by phase-contrast microscopy, using a Zeiss Axio Observer inverted microscope and the AxioVision imaging system (Zeiss, Germany) (Figure 3.2-c). Finally, day 8 embryos were removed from the co-culture and fixed on a slide as described below, the number of blastomeres counted, and all nuclei analyzed by FISH (Figure 3.2-d,e). The proportion of chromosomally normal cells per embryo was calculated. Statistical analysis using Pearson correlation coefficient allowed testing for statistical significance, with $p<0.05$ considered significant.

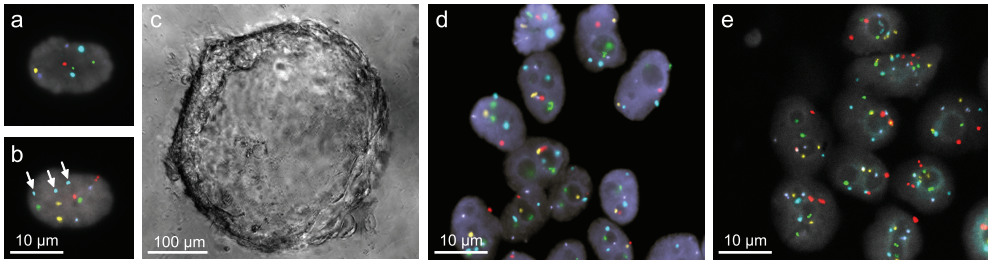

Figure 3.2 – **(a)** Nucleus of a blastomere after the first round of FISH showing chromosomes 1 (aqua), 7 (blue), 15 (green), X (red) and Y (yellow). DNA is stained with DAPI. **(b)** Same nucleus after the second round of FISH showing chromosomes 13 (red), 16 (aqua), 18 (blue), 21 (green) and 22 (yellow). An extra signal for chromosome 16 is shown (arrows). **(c)** Day 8 embryo attached to a monolayer of stromal decidualized cells. **(d)** Spread of nuclei of a day 8 embryo after the first round of FISH (DNA stained with DAPI) and **(e)** after the second round of FISH.

Biopsy and fixation of blastomeres and embryos

Prior to biopsy, embryos were washed twice in calcium/magnesium-free medium (G-PGD medium) and then incubated in the same medium for 5 min at 37°C, allowing decompaction. The biopsy was performed on the heated stage of a Nikon IX-70 microscope, equipped with micromanipulation tools. An infrared diode laser system (OCTAX Laser Shot, OCTAX Microscience GmbH, Germany) with appropriate software (OCTAX EyeWare) was used for dissection of the zona pellucida prior to biopsy. The retrieved blastomeres were dissolved in lysis buffer (0.01 N HCl, 0.1% Tween 20) and the nuclei fixed on poly-L-lysine coated slides using methanol:acetic acid (3:1), as described before (Dozortsev and McGinnis, 2001; Baart *et al.*, 2004). Non-biopsied day 4 and biopsied embryos that either were arrested at day 5 or developed until day 8 were also dissolved using lysis buffer (0.01 N HCl, 0.1% Tween 20) to remove the zona pellucida and the cytoplasm. Nuclei were washed by gentle agitation of the lysis solution until clear and good spreading of nuclei was evident to minimize overlapping. Finally, a drop of methanol:acetic acid (3:1) was added for fixation. Fixed nuclei from biopsied blastomeres and whole embryos were viewed using a phase contrast microscope and their location marked with a diamond pen. Preparations were air-dried and stored at -20°C for up to 6 months prior to FISH analysis.

FISH

Two rounds of five colour FISH were applied to single blastomeres or embryos. In the first round, FISH was performed for chromosomes 1, 7, 15, X and Y and in the second round for chromosomes 13, 16, 18, 21 and 22. The DNA probes used in the first round were centromere probes for chromosomes 1 (pUC 1.77; (Cooke and Hindley, 1979), 7 (pa7t1; (Waye *et al.*, 1987)), 15 (pTRA-20; (Choo *et al.*, 1990)), X (pBamX5; (Willard *et al.*, 1983)) and a Y chromosome heterochromatin probe (RPN1305; (Lau, 1985)). These were labelled as described previously (Baart *et al.*, 2007b), but using a BioPrime DNA labeling kit (Invitrogen), according to the instructions of the manufacturers. The second round of FISH was performed using a commercial ready-to-use probe mix containing centromere probes (Multivysion PB kit; Vysis, USA). The efficiency of the FISH probes was tested on cultured and uncultured

peripheral lymphocyte spreads from two men and two women with normal karyotypes. Slides were prepared according to standard protocols and hybridised using the same protocol as for the embryonic cells. Chromosome localization of the probes was verified on metaphase spreads and FISH signals were counted in 100 interphase nuclei. In addition, the positions of 10 individual nuclei were recorded and images were obtained after each round to check for persisting signals from the first round.

Hybridization was performed as described previously (Baart *et al.*, 2007b). Per slide, 0.2 µl (single blastomeres) or 0.4 µl (whole embryos) of hybridization mixture was applied. Slides were examined with a Zeiss Axio Observer epifluorescence-equipped inverted microscope, using appropriate filters. An "embryo map" was drawn for whole embryos, marking the location and attributing a number to the individual nuclei. Images of representative nuclei were captured with AxioVision imaging system. After the second round, images of the same nuclei were recorded and compared with those from the first round to ensure these had not persisted. Overlapping nuclei were excluded from the FISH analysis.

FISH signal analysis and interpretation

For both rounds, we used the scoring criteria previously published (Munne *et al.*, 1998). Based on the analysis of two blastomeres per embryo, we classified day 4 embryos as normal (both nuclei showing the normal amount of signals for the chromosomes investigated), mosaic (one normal nucleus and one abnormal or each nucleus showing a different abnormality) (Figure 3.2-a,b) or abnormal (both nuclei carrying the same abnormality). Day 4 embryos in which only one blastomere was analyzed were classified as chromosomally normal or abnormal. After analysis of all the cells from each embryo on days 4, 5 or 8, we used the following definitions on the basis of the results obtained. To distinguish between true aneuploidy and FISH artefact, an abnormal cell line was defined as at least 10% of the nuclei showing the same chromosome abnormality. This threshold is frequently used in cytogenetics, if control material is lacking. Applying this criterion resulted in embryos being classified as normal if at least 60% of nuclei showed a normal chromosome constitution, and more importantly, if less than 10% of the nuclei showed the same chromosome abnormality. Embryos were classified as aneuploid if >90% of nuclei showed the same abnormality and if <10% of the nuclei showed a normal or different abnormal chromosome constitution. Embryos were classified as mosaic, if composed of cells with either a normal or an abnormal chromosomal constitution, with between 10 and 90% of the cells showing the same chromosomal abnormality. Chaotic embryos were classified as such when almost all the cells showed different and complex chromosomal abnormalities. Embryos with >90% haploid, tetraploid or triploid nuclei were classed as such. However, we considered the occurrence of some tetraploid cells as a normal phenomenon of *in vitro* cultured embryos (E*vs*ikov and Verlinsky, 1998; Bielanska *et al.*, 2002a) and counted them as normal cells.

After reanalysis on day 5 and 8, confirmation rates were calculated to investigate the reliability of the day 4 diagnosis. The chromosomal constitution diagnosed at day 4 was considered to be cytogenetically confirmed when the chromosome constitution of the investigated blastomeres was reflected in at least 10% of the cells within the embryo analyzed

on day 5 or 8 (Baart *et al.*, 2006).

Reanalysis of data collected in a previous study

In the present study, the FISH analysis performed at day 5 post-fertilization was done on selected embryos showing signs of developmental arrest before reaching the blastocyst stage. Therefore, this group of embryos is unlikely to be representative of the chromosomal constitution of good-quality day 5 embryos (blastocysts). In a study previously published by our group (Baart *et al.*, 2007b), cryopreserved good quality day 5 blastocysts (n=36) were analyzed by FISH for 15 chromosomes. In order to gain insight into the changing chromosomal constitution of embryos between days 4, 5 and 8, we included in the analysis data from Baart *et al.* (2007), excluding the information regarding 5 extra chromosomes not analyzed in the present study. The proportion of normal cells was then calculated. Statistical analysis using Pearson correlation coefficient allowed testing for statistical significance, with $p<0.05$ considered significant.

Results

Control lymphocytes

Home-labelled probes used were the same as previously described (Baart *et al.*, 2007b). These probes were tested on 10 metaphase and 100 interphase nuclei of lymphocytes to check localization and test for FISH efficiency. The hybridization efficiencies for the individual probes were calculated as the percentage of nuclei showing the expected number of signals and ranged between 94-100%, depending on the individual DNA probe. This was comparable to what was described previously (Baart *et al.*, 2007b).

Analysis of non-biopsied day 4 embryos

In order to assess the chromosomal constitution and incidence of mosaicism of compacting embryos, cryopreserved day 4 embryos were thawed (n=21) and fixed within 10 minutes after removal from liquid nitrogen. None of the day 4 embryos successfully analyzed by FISH (n=18) consisted of only normal cells (Supplementary Table 3.1). Only one (6%) embryo was classified as normal (embryo 1) according to our definition, with 3/15 (20%) differently abnormal cells (Figure 3.3 and Table 3.1). Fifteen (83%) of the thawed day 4 embryos were diagnosed as mosaic and two (11%) as chaotic. The average percentage of chromosomally normal cells per embryo was 55% (range 0-83%).

FISH analysis of blastomeres and biopsied embryos

In total, 91 blastomeres from 53 compacting embryos were analyzed by FISH. From 18 embryos, one cell was available for analysis and for 33 embryos the analysis was based on two blastomeres. Embryos 38 and 50 showed partial compaction by the time of biopsy, and therefore 3 cells were inadvertently biopsied. From the group of embryos where a single blastomere was biopsied, 39% were normal. In the group of embryos with two or three blastomeres biopsied 40% were normal, 54% mosaic and 6% abnormal (Tables 3.2 and 3.3). FISH analysis of two

blastomeres is consistent with the analysis of all cells from non-biopsied morulas, revealing a high rate of mosaicism, with more than half (54%) of the embryos identified as mosaic. These findings are also comparable to our earlier report on day 3 biopsy of cryopreserved embryos, where 24% of embryos with two cells biopsied (n=21) were chromosomally normal, 57% mosaic and 19% abnormal (Baart *et al.*, 2004).

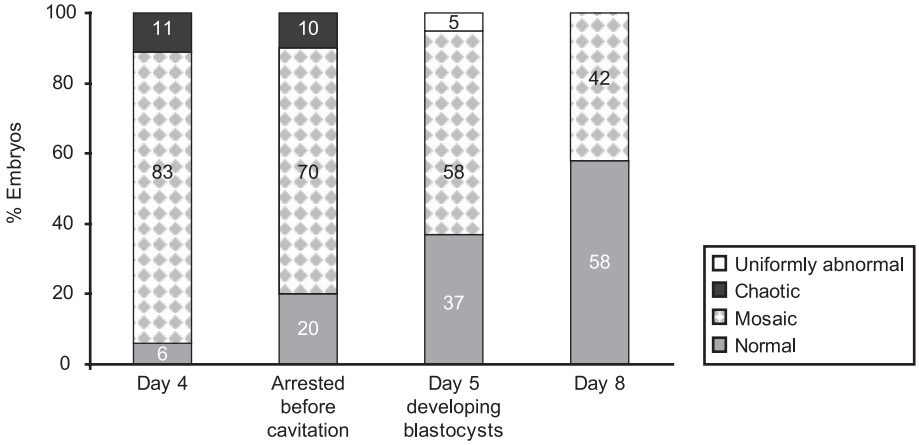

Figure 3.3 – **Incidence of mosaicism on days 4, 5 and 8 post-fertilization.** Percentage of day 4 morulas, embryos arrested before cavitation, developing day 5 blastocysts and day 8 peri-implantation embryos, diagnosed as normal, mosaic, chaotic, and uniformly abnormal after FISH analysis of chromosomes 1, 7, 13, 15, 16, 18, 21, 22, X and Y.

Reanalysis of biopsied day 5 and day 8 embryos

After embryo biopsy and FISH analysis, embryos were reanalyzed at day 5 if arrested at that time or at day 8 and the diagnosis compared to the day 4 analysis. From the 24 embryos arrested before cavitation that were fixed on day 5, 20 were successfully analyzed after two rounds of FISH, showing that 14 (70%) were mosaic, 4 (20%) normal, and 2 (10%) chaotic (Figure 3.3 and Table 3.2). From the 24 blastocysts put into co-culture, 8 had degenerated by day 8 and could not be retrieved. The remaining 16 embryos were successfully fixed on that same day. However, 4 embryos were excluded from further analysis due to failed hybridization for the second round of FISH. In total, 2859 blastomere nuclei from 12 embryos were analyzed for 10 chromosomes (Supplementary Table 3.2). In 8% of the nuclei, some overlap was observed and these were excluded from the FISH analysis. We diagnosed seven day 8 blastocysts (58%) as normal and 5 (42%) as mosaic. No day 8 embryo consisted of only normal cells, nor did we find an embryo with uniform aneuploidy. In each embryo, a range of cells with different abnormalities was found, each abnormality present in cell numbers not reaching the 10% threshold of our definition for a mosaic cell line. For instance in embryo 43, several cells with either a monosomy 1, monosomy 15, monosomy 16, monosomy 18, trisomy 13, monosomy 13, trisomy 21, monosomy 21 or monosomy 22 were present (Supplementary Table 3.2). These nine different abnormalities cannot all have originated from errors arising during the first cleavage divisions. This may therefore indicate that new segregation errors

have occurred in this embryo after day 4. The average percentage of chromosomally normal cells per embryo was 66% (range 35-84%).

Table 3.1 – FISH analysis of non-biopsied frozen-thawed day 4 embryos using probes for chromosomes 1, 7, 13, 15, 16, 21, 22, X and Y.

NON-BIOPSIED DAY 4			
Embryo no.	No. cells analyzed	% normal cells	FISH interpretation
1	15	80	Normal
2	8	88	Mos +1 / 2N
3	15	67	Mos -7 / 2N
4	13	69	Mos -13 / 2N
5	11	45	Mos -15 / 2N
6[3]	10	30	Mos +15 / 2N
7	19	53	Mos -16 / 2N
8	6	83	Mos -18 / 2N
9	13	69	Mos -X / 2N
10	9	78	Mos -1 / +15 / 2N
11	14	57	Mos -7 / -21 / 2N
12	14	57	Mos -15 / -13 / 2N
13	9	56	Mos -15 / -1 / -16 / 2N
14	13	31	Mos -22 / -15 / -18 / 2N
15	8	38	Mos -22 / -18 / -15 / +21,+22,+22 / 2N
16	8	63	Mos +21 / -7,+22 / +1,+1,+13,+15,+15,+16,+18,+22 / 2N
17	9	22	Chaotic[1]
18	12	0	Chaotic[2]

[1] Mos -1,-13,-X,null 21 [3] / -18,-Y,null 22 [2] / -1,-13,-18,-21 [1] / +13,+22 [1] / 2N [2].
[2] Chaotic embryo, where all the cells had different combinations of abnormalities. However, 58% of the cells showed errors in common: +1,+7,+15,+X.
2N = normal copy number for the chromosomes investigated; Mos = mosaicism; null = nullisomy. In the mosaic cases the different abnormal cell lines are presented according to their size with the largest first. A normal diploid cell line is always listed last (ISCN, 2009).
[3] Case where the chromosomal abnormality might have had a meiotic origin followed by a subsequent mitotic error.

Tables 3.2 and 3.3 summarize the interpretation of the FISH analysis on days 5 (embryos arrested before cavitation) and 8 (developing blastocysts) and compare observations with the results from the biopsy on day 4. The day 4 diagnosis was not predictive of the potential of the embryo to develop until day 8. Reanalysis of biopsied embryos that either arrested before cavitation or developed until day 8 showed similar cytogenetic confirmation rates (40% for arrested embryos and 36% for day 8 embryos). However, the proportion of embryos with a false positive diagnosis on day 4 (i.e. embryos that were diagnosed as abnormal or mosaic on day 4 but classified as normal after reanalysis) was higher on day 8 embryos (4/7=57%) than on arrested embryos analyzed at day 5 (2/12=17%) (Tables 3.2 and 3.3). Conversely, the incidence of a false negative diagnosis was higher on day 5, where 6/8 (75%) embryos

diagnosed as normal on day 4 were found to be mosaic on day 5, whereas 1/4 (25%) day 8 embryos were falsely diagnosed as normal. This was largely due to the increased incidence of chromosomally normal embryos on day 8, with the percentage of embryos diagnosed as mosaics falling from 70% on day 5 to 42% on day 8 (Figure 3.3). Reanalysis of the FISH results of day 5 good-quality blastocysts analyzed in a study by Baart *et al.* (2007) showed a lower percentage of chromosomally mosaic day 5 blastocysts (58%) than observed in the present study (70%), where embryos arrested before cavitation were analyzed at day 5. Overall, we identified seven cases (7/50=14%) where at least one of the abnormalities observed likely originated during meiosis.

Discussion

In the present study we performed FISH analysis of human embryos in order to assess the frequency of chromosomal mosaicism at three different stages of development (morula, preimplantation blastocyst and peri-implantation blastocyst). Good quality, frozen-thawed morula stage embryos were either fixed and all cells analyzed, or biopsied and allowed to develop *in vitro*. Reanalysis was performed either at day 5, in case of developmental arrest, or at day 8. Data derived from a previous study done by our group on cryopreserved good quality day 5 blastocysts were included. This approach allowed evaluating the chromosomal constitution of human embryos during different stages of development and determining how chromosomal constitution may influence the developmental capacity of embryos.

Consistent with previous findings (Baart *et al.*, 2007b), we found almost all embryos to be mosaic at the morula stage. This high rate of mosaicism is reflected in our results obtained after biopsy and FISH analysis of one or two cells from day 4 embryos. We found that the incidence of mosaic embryos decreased over time, with a significant decrease between day 4 and day 8 blastocyst stage (Figure 3.3). Moreover, we found a positive correlation between the total number of cells in the embryo and the proportion of chromosomally normal cells in developing day 5 and day 8 blastocysts, but not in day 4 morulas and embryos arrested before cavitation. Finally, we observed that FISH diagnosis on 1 or 2 blastomeres of day 4 embryos was not predictive of subsequent developmental potential.

The high incidence of chromosomal abnormalities in cleavage stage embryos has been brought to light by several studies employing PGS-FISH over the past years (Delhanty, 2005). However, it is still unclear which mechanisms lead to such high aneuploidy rates. The inefficiency of the cell cycle checkpoints during the first cleavage divisions (before embryonic genome activation at around the 8 cell stage) (Tesarik *et al.*, 1986; Braude *et al.*, 1988) has been suggested as a possible cause for improper chromosome segregation (Los *et al.*, 2004). According to this hypothesis, activation of the embryonic genome and initiation of compaction could lead to the establishment of functional cell cycle checkpoints, resulting in prevention of new errors and the developmental arrest of chromosomally abnormal cells and/ or the entire embryo. In the present study we investigated the frequency of chromosomal abnormalities after compaction, to ascertain whether the incidence of chromosomal abnormalities decreases after presumable activation of the embryonic genome.

Table 3.2 – FISH analysis of blastomeres from frozen-thawed day 4 embryos and reanalysis of corresponding embryos on day 5, using probes for chromosomes 1, 7, 13, 15, 16, 21, 22, X and Y.

DAY 4				ARRESTED BEFORE CAVITATION (DAY 5)			Confirmation
Embryo no.	No. biopsied cells	FISH results (nucleus 1/ nucleus 2)	FISH interpretation	No. cells analyzed	% normal cells	FISH interpretation	
19	1	2N	Normal	18	67	Mos -7 / -18 / 2N	-
20	1	2N	Normal	21	24	Mos -13 / +13 / +13,+16 / 2N	-
21[5]	1	-7,-22	Monosomy 7, 22	5	0	Mos -22 / -21,-22 / -7, -15,-18,-22	+
22[5]	1	+21	Trisomy 21	12	50	Mos +21 / 2N	+
23	2	2N / NA	Normal	15	80	Normal	+
24	2	2N / 2N	Normal	5	100	Normal	+
25	2	2N / 2N	Normal	15	80	Mos -16 / 2N	-
26	2	2N / 2N	Normal	8	88	Mos +1 / 2N	-
27	2	2N / 2N	Normal	13	54	Mos -18 / +13 / +16 / 2N	-
28[1]	2	2N / 2N / 2N	Normal	21	29	Mos -18 / -15 / 2N	-
29	2	2N / -18	Mosaic	21	76	Normal	-
30	2	2N / -13	Mosaic	16	81	Normal	-
31	2	2N / -18,+21	Mosaic	24	67	Mos +13 / 2N	-
32	2	2N / 4N	Mosaic	8	75	Mos -X / -7, -18 / 2N	-
33[5]	2	3N,-7,-22 / 3N,-1,-7,-22	Mosaic	15	0	Mos 3N / -13, -22 / -22	+
34	2	2N / -18	Mosaic	8	50	Mos -13,-18,-21 / -18 / XYY / 2N	+
35	2	-1,-7,-13 / -1,-7	Mosaic	6	0	Chaotic[2]	-
36[5]	2	-18 / NA	Monosomy 18	32	47	Mos -18 / -1 / 2N	+
37[5]	2	-16,+7 / -16,+7	Monosomy 16, Trisomy 7	10	30	Chaotic[3]	+
38[4]	3	-13 / 2N / 2N / 2N	Mosaic	10	60	Mos -18 / -16,-18/ XYY / 2N	-

[1] Two blastomeres biopsied, with one of them being binucleated.
[2] +13,-18 / +17,+13,-18 / +7 / +13 / +7,+13,+18 / +1,+1,+7,+7,+13.
[3] Confirmation of the trisomy 7 in two cells: +1 [2] / -1,-22,+7,+7,XYY [2] / -4,-7,+13,+15,+22 [1] / +1,+15,+15 [1] / +13,+1,+1,+15,+15,XYY [1] / 2N [3].
[4] Three blastomeres biopsied, with one of them being binucleated.
2N = normal copy number for the chromosomes investigated; Mos = mosaicism; NA = not analyzed. In the mosaic cases the different abnormal cell lines are presented according to their size with the largest first. A normal diploid cell line is always listed last (ISCN, 2009).
[5] Cases where the chromosomal abnormality might have had a meiotic origin followed by subsequent mitotic errors.

FISH analysis of morula stage embryos revealed that the great majority of day 4 embryos (83%) are mosaic according to our definition, and no embryo consisted of normal cells only.

Thus, the compaction stage does not provide a developmental barrier for chromosomally abnormal or mosaic embryos. These results are concordant with data from a previous study by our group, where FISH analysis of 15 chromosomes in cryopreserved compacted morulas (n=12) revealed all embryos to be mosaic (Baart *et al.*, 2007b). Cryopreservation has been suggested to possibly induce chromosomal abnormalities after thawing and subsequent culture (Iwarsson *et al.*, 1999; Baart *et al.*, 2004; Salumets *et al.*, 2004). Therefore, both in the present and in the previous study by Baart *et al.* (2007), embryos were fixed immediately after thawing to avoid changes in chromosome constitution. Work done by Bielanska *et al.* using fresh embryos showed that more than half of morula stage embryos are mosaic (58%), when screening for 9 chromosomes (Bielanska *et al.*, 2002a). We therefore believe our results to be a good representation of the chromosomal constitution of morula stage embryos.

When comparing the percentage of mosaic embryos at day 4 *versus* day 5 blastocysts, we observed a significant decrease by day 5 of development (Figure 3.3). This suggests that a proportion of mosaic day 4 embryos do not reach the blastocyst stage. We report a decrease in the incidence of mosaic embryos over time, with the lowest incidence observed at day 8. The decrease of mosaic cases over time may be partially caused by our definition of mosaicism, which requires at least 10% of the cells to carry the same chromosome abnormality. This criterion is currently the best available method to distinguish true aneuploidy from FISH artifacts, when control material is lacking. However, it may lead us to underestimate the proportion of mosaic embryos at day 8 and overestimate at day 4 and day 5. An example is embryo 46 (Table 3.3), where only 63% of the cells were found to be normal, but none of the abnormalities reached the 10% threshold. It is currently not known if this embryo could be diagnosed as normal, since the minimal proportion of normal cells needed for further normal development is yet unknown.

The presence of chromosomally abnormal cells does not exclude blastocyst development, as seen in this and previous studies (Sandalinas *et al.*, 2001; Coonen *et al.*, 2004; Bielanska *et al.*, 2005; Fragouli *et al.*, 2008). However, our data also suggest that a significant proportion of mosaic embryos undergo developmental arrest before reaching the blastocyst stage. According to the model proposed by Evsikov and Verlinsky (1998), if the number of aneuploid cells at the morula stage reaches a certain threshold level, there is self-elimination (arrest) of the whole embryo. However, embryos with a number of aneuploid cells below the threshold level develop further and reach the blastocyst stage. So far this hypothesis of a threshold has not been directly investigated, but mouse knockout models have shown that up to 30% of aneuploid cells can be tolerated in apparently healthy animals (Li *et al.*, 2009).

Contrary to somatic cells, human and mouse embryonic stem cells (ESCs) containing chromosomal abnormalities do not initiate apoptosis. However, upon differentiation of ESCs, there is apoptosis of chromosomally abnormal cells (Mantel *et al.*, 2007). A similar mechanism has been suggested to be involved in human preimplantation embryos, where differentiation is initiated with the formation of the blastocyst. Thus, selection against chromosomally abnormal cells may be initiated at the blastocyst stage, via the elimination and/or non-proliferation of such cells (Ambartsumyan and Clark, 2008).

In this study we aimed at finding indirect evidence supporting the model where apoptosis

Table 3.3 – FISH analysis of blastomeres from day 4 embryos and reanalysis of corresponding embryos on day 8.

DAY 4				DAY 8			Confirmation
Embryo no.	No. biopsied cells	FISH results (nucleus 1/ nucleus 2)	FISH interpretation	No. cells analyzed	% normal cells	FISH interpretation	
39	1	2N	Normal	231	74	Normal	+
40	1	+16	Trisomy 16	185	71	Normal	-
41	1	+13	Trisomy 13	424	70	Mos +13 / 2N	+
42	2	2N / 2N	Normal	194	84	Normal	+
43	2	2N / 2N	Normal	863	83	Normal	+
44	2	2N / NA	Normal	134	61	Mos +13 / 2N	-
45	2	-15,-X / -1, -15,-X	Mosaic	86	72	Normal	-
46	2	2N XYY / 2N	Mosaic	176	63	Normal	-
47	2	2N / +13	Mosaic	239	59	Mos -18 / 2N	-
48	2	2N / -15	Mosaic	16	50	Mos 6N / -13 / +13 / 2N	-
49	2	+16 / NA	Trisomy 16	254	75	Normal	-
50[1]	3	NA	NA	57	35	Mos -15 / 2N	NA

2N = normal copy number, and 6N = six copies for each of the chromosomes investigated; Mos = mosaicism; NA = not analyzed. In the mosaic cases the different abnormal cell lines are presented according to their size with the largest first. A normal diploid cell line is always listed last (ISCN, 2009).
[1] Case where the chromosomal abnormality might have had a meiotic origin followed by a subsequent mitotic error.

of chromosomally abnormal cells is initiated upon differentiation at the blastocyst stage, but not before this stage of embryonic development. Therefore, we tested for a correlation between the total cell number and the percentage of normal cells within an embryo at day 4, day 5 (arrested and blastocyst), and day 8 (Figure 3.4). We found a significant positive correlation between the total number of cells and the percentage of chromosomally normal cells per embryo in day 5 blastocysts and day 8 peri-implanted embryos, but not in day 4 morulas or embryos arrested before cavitation. The difference in the results for the two groups analyzed at day 5 (arrested embryos that failed to initiate cavitation and blastocysts) support the model. Thus, our data provide indirect evidence that cavitation may be critical for the onset of a negative selection against abnormal cells (E*vs*ikov and Verlinsky, 1998) and/or for the establishment of a growth advantage of the normal over the abnormal cells (Wells and Delhanty, 2000; Coonen *et al.*, 2004).

Surprisingly, analysis of day 8 embryos showed the persistence of high numbers of cells with different chromosomal abnormalities until this stage of development (Supplementary Table 3.2), although not falling within the range of our definition for mosaicism. The identification of numerous different segregation errors at day 8 indicates that new abnormalities can arise after cavitation. This may explain the reported poor predictive value of FISH diagnosis on 1 or 2 blastomeres of day 4 embryos. Out of the 50 embryos analyzed, we identified seven embryos (14%) where at least one of the abnormalities was likely to have been caused by a meiotic error. All of these cases were combined with additional post-meiotic errors.

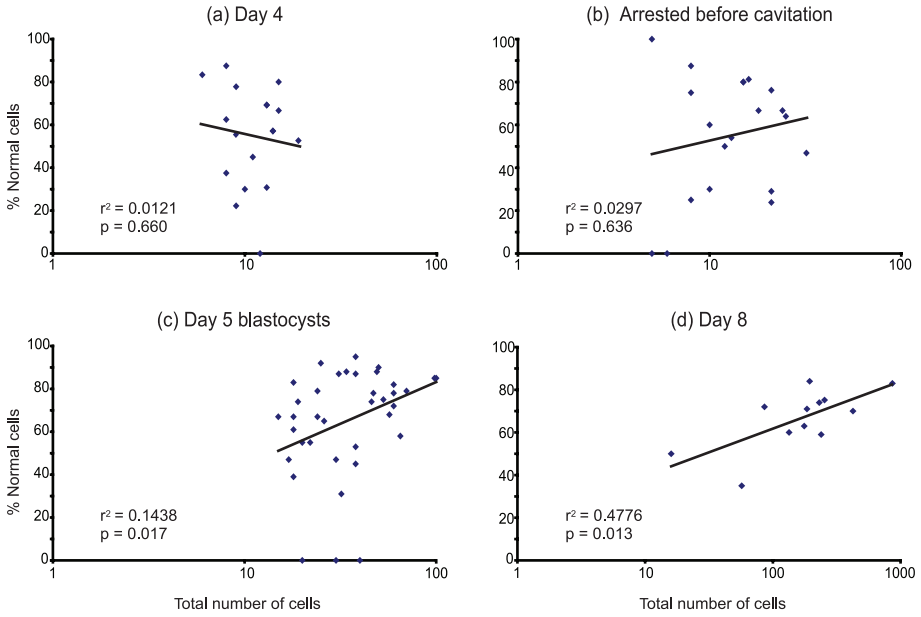

Figure 3.4 – **Correlation between the percentage of chromosomally normal cells and the total number of cells per embryo at different stages of development. (a)** Day 4 morulas **(b)** embryos arrested before cavitation **(c)** Day 5 blastocysts **(d)** Day 8 peri-implanted embryos. a and b: non-significant correlation ($p>0.05$). c and d: significant correlation ($p<0.05$).

This study is the first to provide insight in the fate of chromosomally abnormal cells in human embryos during development from the morula stage up to the peri-implantation stage of day 8. It is however important to note that the embryos studied have been cultured *in vitro* for an extended time period. *In vitro* culture conditions may affect the chromosomal competence of embryos, as demonstrated in a mouse model (Hodges *et al.*, 2002). It is also important to note that the possible effect of embryo-endometrial stromal cell co-culture is unknown. However, co-culture of human embryos and stromal endometrial cells has been established into the routine practice of some IVF centers and associated with increased pregnancy and implantation rates (Mercader *et al.*, 2003). No increased risk of congenital birth defects associated with the co-culture technique have been reported. Furthermore, embryos have been selected (first for cryopreservation and later for co-culture from day 5 to day 8) and therefore may not reflect the general blastocyst population. Additionally, we only analyzed the copy number of 10 of the 23 pairs of chromosomes and we have no information on the incidence of structural abnormalities. These have recently been demonstrated to be highly prevalent in human IVF embryos (Vanneste *et al.*, 2009). However, the embryos used in this study represent the best currently available model accessible for research.

Tetraploidy has been described as a normal phenomenon in embryonic trophoblast cells (Edgar and Orr-Weaver, 2001). Furthermore, the occurrence of some tetraploid cells has been considered as a normal phenomenon of *in vitro* cultured embryos (Evsikov and Verlinsky, 1998; Bielanska *et al.*, 2002b). Therefore, we included tetraploid and near tetraploid

cells into the group of chromosomally normal cells. However, our definition may lead to an underestimation of the incidence of chromosomal abnormalities, as tetraploid cells can arise after aberrant cell division (Brito and Rieder, 2006). We have at the moment no method of distinguishing between these possibilities.

In conclusion, our data suggests that a proportion of mosaic embryos undergo developmental arrest between compaction and cavitation. If the embryo continues to develop, reduced proliferation or cell death of aneuploid cells may be responsible for the increased proportion of chromosomally normal cells throughout development of human embryos. Although the biological implications of chromosomal mosaicism has not been well explored yet, emerging evidence illustrate that we may currently underestimate the impact on embryonic development and disease in later life (Iourov *et al.*, 2008).

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Supplementary tables

Supplementary Table 3.1 – FISH analysis of all the cells from human embryos cryopreserved on day 4, using probes for chromosomes 1, 7, 13, 15, 16, 21, 22, X and Y.

Embryo no.	No. cells analyzed	FISH results day 4	No. cells
1	15	+1, +13	1
		-13, -22	1
		-21	1
2	8	+1	1
		2N	7
3	15	-7, XXY	2
		-7, XXY	1
		+13	1
		+18	1
		2N	10
4	13	-1, +13	1
		-18	1
		+13	1
		+18	1
		2N	9
5	11	-15	2
		~4N	1
		-15, XO	1
		-16	1
		-18	1
		-7, -16	1
		2N	4
6	10	+15	3
		-16, +15	1
		-18, +15	1
		+15, XXY	1
		+13, +15	1
		4N	1
		2N	3
7	19	-16	3
		-1	1
		-13	1
		-22	1
		+1	1
		+13	1
		+15	1

		2N	10
8	6	-18	1
		2N	5
9	13	-X	2
		-16	1
		-21	1
		2N	9
10	9	-1	1
		+15	1
		2N	7
11	14	-16, -21	1
		-21	1
		-7	1
		-7, -16	1
		+16	1
		2N XXYY	1
		2N	8
12	14	-15	1
		-15, XO	1
		-15, -16	1
		-18	1
		+13, +18, +21, +22, +7, +7, +15, +15	1
		+13	1
		2N	8
13	9	-15	2
		-1	1
		-16	1
		2N	5
14	13	-22	3
		-13, -22	1
		-15, -22, XO	1
		-15, -16, -18, -22	1
		-18	1
		-21	1
		-7	1
		2N	4
15	8	-22	2
		-15	1
		-18	1
		+21, +22, +22	1
		2N	3
16	8	-7, +22	1

		+13, +16, +18, +22, +1, +1, +15, +15	1
		+21	1
17	9	-1, -13, -16, -18, XO, null 21	1
		-1, -13, -16, XO, null 21	1
		-1, -13, -18, -21	1
		-1, -13, XO, null 21	1
		-18, YO, null 16, null 22	1
		-18, YO, null 18, null 22	1
		+13, +22	1
		2N	2
18	12	+1, +7, +15, XXX	4
		-1, -13, -16, -21, null 7, null 15	1
		-1, -13, -16, -22, null 7, null 15	1
		-1, -13, -21, -22, null 7, null 15	1
		-7, -16, -22, +13, null X	1
		+1, +15, -7	1
		+1, +7, +15, -16, -22, XXX	1
		+1, +7, +15, +16, +18, XXX	1
		+1, +7, +15, +18, -16, XXX	1

Supplementary Table 3.2 – FISH analysis of all the cells from peri-implanted human embryos on day 8, using probes for chromosomes 1, 7, 13, 15, 16, 21, 22, X and Y.

Embryo no.	FISH interpretation day 4	No. cells analyzed	FISH results day 8	No. cells
39	Normal	231	-16	13
			-1	12
			-18	12
			4N	7
			~4N	5
			-22	5
			-15	4
			+21	3
			+15	2
			+22	2
			-1, -22	1
			-1, -18	1
			-13	1
			-16, 22	1
			-18, +13	1
			-18, -21	1
			-21	1
			+15, +15, XXY	1
			2N	158
40	Trisomy 16	185	-16	6
			-1	5
			-15	5
			-7	5
			+13	5
			+21	3
			4N	2
			-1, -7	2
			-21	2
			-22	2
			+18	2
			2N XO	1
			~4N	1
			-1, +13	1
			-1, XXX	1
			-13, -16	1
			-15, +13	1
			-15, +16	1
			-16, +21, +22	1

			-18, +16	1
			-18, XO	1
			-7, +13	1
			-7, +21, +22	1
			-7, -16	1
			+1, +13	1
			+13, +21	1
			+21, XO	1
			+22	1
			+7	1
			2N	128
41	Trisomy 13	424	4N	38
			+13	31
			~4N	11
			6N	7
			-1	7
			-16	7
			-18	7
			-15	6
			-7	6
			+22	5
			+21	4
			-13	3
			-22	3
			+18	3
			-1, -18	2
			+13, +13	2
			+13, +22	2
			+15	2
			2N XXX	2
			2N XXXX	2
			1N	1
			2N XO	1
			-1, +22, +13, +13, +18, +18, +21, +21, XXX	1
			-13, XXXX	1
			-13, -22	1
			-15, +13	1
			-16, +13	1
			-16, +13, +21, +22	1
			-16, -18, -21, -22, XO	1
			-18, +21	1
			-18, -22	1

			-21	1
			-22, XO	1
			-7, XO	1
			+1, +1	1
			+13, +13, +21, +21, +22, +22	1
			+15, +15	1
			+1	1
			+1, XO	1
			+1, +7, +15, +16, +21, +13, +13, +18, +18, +22, +22, XXX	1
			+13, +15	1
			+13, +15, +22, +21, +21	1
			+13, +21	1
			+18, +22	1
			+21, +22	1
			+21, +22, XXX	1
			+7	1
			2N	247
42	Normal	194	4N	5
			-18	5
			-13	3
			-15	3
			-16	3
			-22	3
			+15	3
			~4N	2
			+18	2
			2N, XO	1
			-1	1
			-1, +18	1
			-15, -16	1
			-15, -22	1
			-18, +13	1
			+13	1
			+21	1
			+22	1
			2N	156
43	Normal	863	4N	39
			-1	21
			-15	20
			-16	19
			~4N	15

			-18	14
			+13	12
			-13	7
			+21	7
			-21	5
			-22	5
			+1	4
			+15	3
			2N, XO	2
			2N, null 22	1
			6N	1
			-1, +15	1
			-1, -13	1
			-1, -15	1
			-1, -16	1
			-1, -18	1
			-13, -16, -18	1
			-13, -18	1
			-13, -15, + 21	1
			-15, -18	1
			-15, -21	1
			-15, -22	1
			-15, -16	1
			-16, -21, null 15, null 18, null X, null Y	1
			-21, -22	1
			-22, +13	1
			-7, -15, -16, null 1	1
			-7, -18, -21	1
			+1, +1, + 15, +18	1
			+7, +7, -18, +13, +15, +21, XYY	1
			+13, +15	1
			+13, +22	1
			+16	1
			+18	1
			+ 21, XXY	1
			+22	1
			+7, +13	1
			2N	662
44	Normal	134	~4N	12
			4N	7
			-18	5
			+13	5

			-1	3
			-13	3
			+15	3
			+21	3
			-15	2
			-15, +13	2
			-16	2
			+13, +13, +22, +22	2
			+13, +21	2
			+22	2
			2N, XO	1
			8N	1
			-1, -16, -22, -21	1
			-1, -18, +21, +13, +13	1
			-15, -18	1
			-15, -22	1
			-16, +13, +15	1
			-16, -18, +1	1
			-21	1
			-22	1
			+15, +15	1
			+16, +16	1
			+21, +21	1
			+1	1
			+1, +7, +13, +13	1
			+13, +15	1
			+13, +21, +22, +15, +15	1
			+22, +13, +13, +21, +21	1
			+22, +15, +15, +13, +13	1
			2N	62
45	Mos -15, -X / -1, -15, -X	86	4N	5
			-16	3
			~4N	2
			-1	2
			-16, -18	2
			-18	2
			+16	2
			+21	2
			2N, XO	1
			6N	1
			8N	1
			-1, -18	1

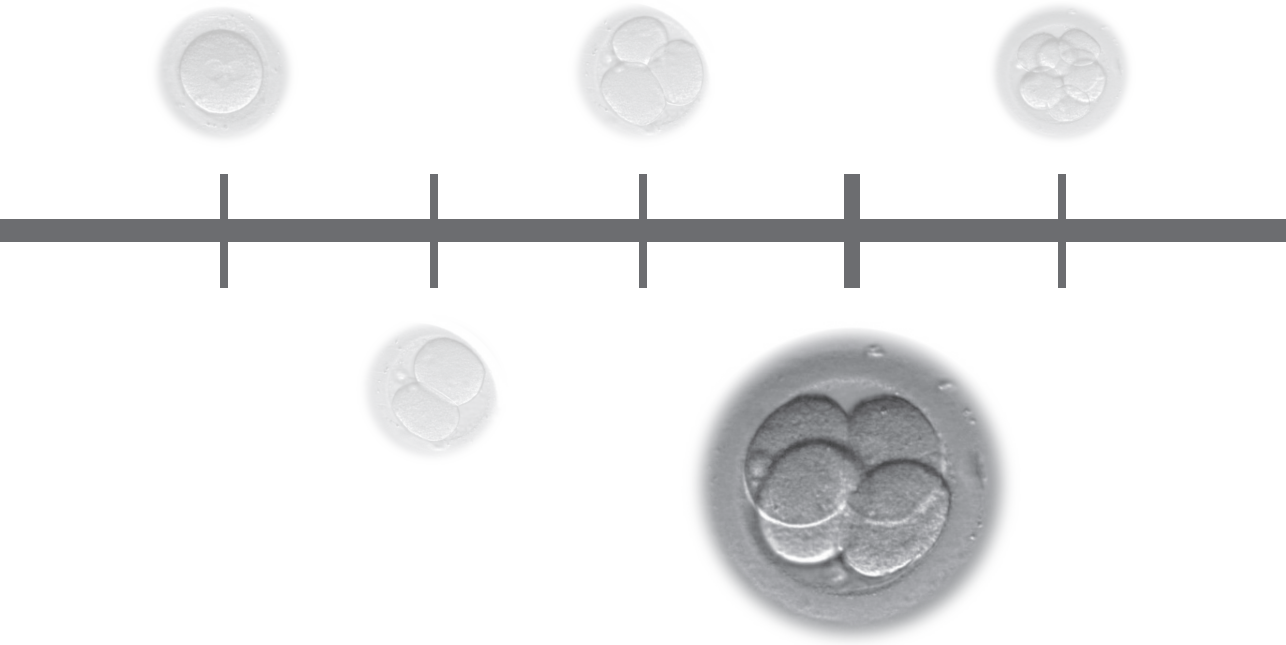
			-15	1
			-15, -21	1
			-21	1
			-22	1
			+1	1
			+13	1
			+22	1
			2N	55
46	Mos 2N XYY / 2N	176	-16	9
			-1	8
			-13	6
			~4N	4
			-21	4
			+13	4
			4N	3
			2N, XO	2
			-13, -16	2
			-13, -18	2
			-18	2
			-22	2
			-7	2
			+22	2
			+7	2
			2N YO	1
			-1, -16	1
			-1, -7	1
			-1, -7, -13, -18, null 21, XO	1
			-15	1
			-15, XO	1
			-15, 22	1
			-16, +21	1
			-18, +13	1
			-18, -21, -22	1
			-7, -13, + 1, +18, +21, +21	1
			null 13	1
			null 16	1
			+15	1
			+15, null 7	1
			+16	1
			+21	1
			+22, XXY	1
			2N	104

47	Mos +13 / 2N	239	-18	24
			4N	21
			2N, XO	9
			+13	8
			-21	7
			-16	4
			-18, XO	4
			6N	3
			+13, +13	3
			+21	3
			~4N	2
			+	2
			+1, XO	2
			-1, +18	1
			-1, -16	1
			-13, XO	1
			-15	1
			-15, + 22	1
			-15, XO	1
			-15, -18, + 13, XO	1
			-16, + 13	1
			-16, + 22	1
			-16, -18	1
			-16, -22	1
			-18, +21, +21	1
			-18, +13	1
			-18, +13, +15, +22, +22	1
			-18, + 21, 22, +13, +13	1
			-18, -22	1
			-21, -22	1
			-22	1
			-22, XO	1
			-7	1
			-7, -13, -18, null 16, null 22	1
			-7, -16, -18	1
			+13, +13, XO	1
			+13, +13, +21, +21	1
			+13, +13, +21, +21, +22, +22, XO	1
			+13, XO	1
			+16	1
			+18, XO	1
			+22	1

			2N	118
48	Mos -15 / 2N	16	6N	2
			-1, YO	1
			-13	1
			-15, -22, null 13, null 21	1
			+13, +13, +22, +22	1
			+13	1
			XXY	1
			2N	8
49	Trisomy 16	254	4N	18
			-16	12
			-18	7
			+13	5
			-1	4
			+15	4
			+21	4
			-13	3
			-15	3
			-21	3
			+13, +13	3
			-22	2
			-7	2
			+22	2
			+1, +1, +1, +1, +1, +1, +1, + 21	1
			-1, -15	1
			-18, XO	1
			-7, -15, XO	1
			-7, -16, -18	1
			null 21	1
			+1, +1, +15, +15	1
			+13, +21	1
			2N XXX	1
			2N	173
50	NA	57	-15	21
			~4N	15
			4N, -15, -15	7
			6N	1
			8N	1
			-15, -18	1
			-15, -18, XO	1
			-15, -16	1
			-22	1

			-1, -15	1
			-15, -16, -18, XO	1
			-15, +13	1
			+1, +16, +18, +21, +13, +13, +22, +22, XXY	1
			2N	4

2N = normal copy number; ~4N = near tetraploid. 4N/ 6N/ 8N = four/ six/ eight copies for each of the chromosomes investigated; null = nullisomy.



Chapter 4

A role for Aurora C in the chromosomal passenger complex during human preimplantation embryo development

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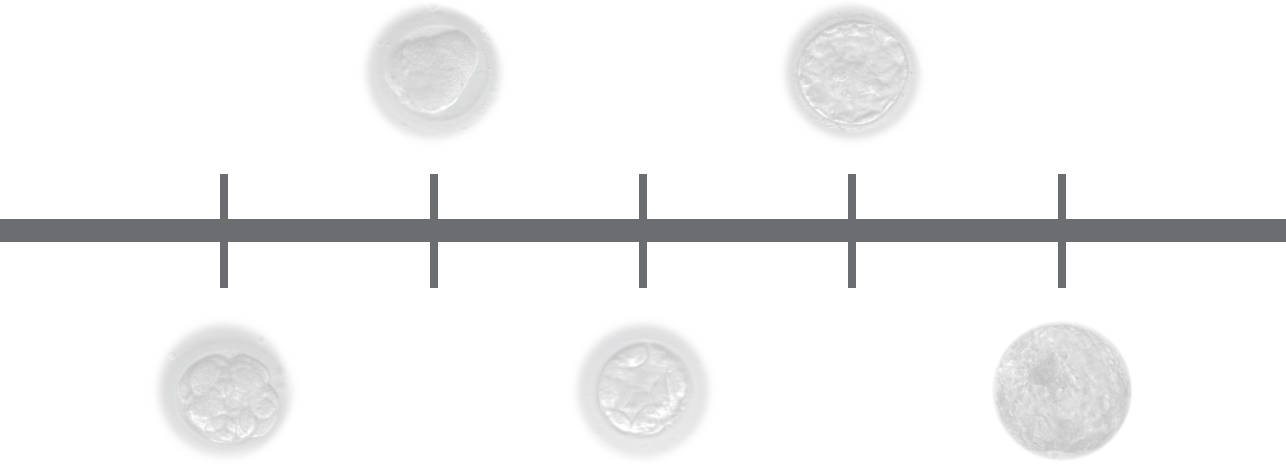

Abstract

BACKGROUND: Human embryos generated by IVF demonstrate a high incidence of chromosomal segregation errors during the cleavage divisions. To analyze underlying molecular mechanisms, we investigated the behavior of the chromosomal passenger complex (CPC) in human oocytes and embryos. This important mitotic regulatory complex consists of INCENP, Survivin, Borealin and Aurora B, or the meiotic kinase Aurora C as a possible alternative subunit.

METHODS: We analyzed mRNA expression by quantitative RT-PCR of all members of the CPC in human oocytes, tripronuclear (3PN) zygotes, 2 cell and 4 cell embryos developed from 3PN zygotes, as well as good quality cryopreserved 8 cell, morula and blastocyst stage embryos. Protein expression and localization of CPC members was investigated by immunofluorescence in oocytes and embryos arrested at prometaphase, with a focus on Aurora B and Aurora C. Histone H3S10 phosphorylation, a known Aurora B kinase target, was investigated as an indicator of a functional CPC.

RESULTS: INCENP, Survivin and Borealin were detected at the inner centromere of prometaphase chromosomes in all stages of preimplantation development investigated. Whereas Aurora B and C are both present in oocytes, Aurora C becomes the most prominent kinase in the CPC during the first three embryonic cell cycles. Moreover, Aurora C mRNA was upregulated together with Aurora B after activation of the embryonic genome and both proteins were detected in early day 4 embryos. Subsequently, only Aurora B was detected in blastocysts.

CONCLUSIONS: In contrast to somatic cells, our results point to a specific role for Aurora C in the CPC during human preimplantation embryo development. Although the presence of Aurora C in itself may not explain the high chromosome segregation error rate, differences between Aurora B and C, as well as regulation of the balance in expression of these kinases before and after activation of the embryonic genome, may help in identifying crucial factors.

Introduction

The introduction of fluorescence *in situ* hybridization (FISH) for PGD has enabled screening of human embryos for chromosomal aneuploidies before transfer in IVF. This has led to an increasing body of evidence demonstrating that, in IVF derived embryos, an estimate of 80% of all preimplantation embryos have chromosomally abnormal cells (Voullaire *et al.*, 2000; Wells and Delhanty, 2000). The majority of the aneuploidies observed at this stage have originated during the first mitotic divisions of early preimplantation development, resulting in chromosomally mosaic embryos (Marquez *et al.*, 2000; Bielanska *et al.*, 2002a; Coonen *et al.*, 2004; Daphnis *et al.*, 2005; Baart *et al.*, 2006; Santos *et al.*, 2010b). A study using an array-based method allowing genome-wide screening of the copy number in single embryonic cells from cleavage stage embryos found the high frequency of chromosomal instability to be similar to human cancers (Vanneste *et al.*, 2009). Furthermore, considerable percentages of chromosomal mosaicism have also been reported for bovine, equine, porcine and non-human primate embryos (Viuff *et al.*, 2000; Rambags *et al.*, 2005; Zijlstra *et al.*, 2008; Dupont *et al.*, 2010), both for embryos produced *in vitro* and *in vivo* (bovine: (Viuff *et al.*, 2000); porcine (Zijlstra *et al.*, 2008)). This indicates that chromosome segregation in preimplantation embryos is more error prone than in other dividing cells. The molecular mechanisms that control chromosome segregation in cleavage stage embryos are not well described (Jones *et al.*, 2008a), raising the question if these differ from somatic mitotic cells.

Correct segregation of chromatids to the two daughter cells during mitosis is crucial for maintaining genomic integrity and cells have a complex cell cycle machinery in place to regulate this process, including a checkpoint mechanism called the spindle assembly checkpoint (SAC) (Vagnarelli and Earnshaw, 2004). An important protein complex contributing to proper SAC functioning is the chromosomal passenger complex (CPC), named after its dynamic localization during mitosis. At the onset of mitosis, the CPC moves from the arms of the condensing chromosomes to the inner centromere. During the metaphase to anaphase transition, the CPC disassociates from the centromeres to localize to the microtubules of the central spindle in anaphase and telophase and to the midbody during cytokinesis. Parallel to its location, the CPC is involved in chromosome condensation, spindle assembly, the correction of erroneous microtubule-kinetochore interactions, signaling to the SAC and the completion of cytokinesis (reviewed by Vagnarelli and Earnshaw, 2004). The complex consists of four subunits: the inner centromere protein INCENP, Survivin, Borealin and the active enzymatic unit Aurora B kinase (Vader *et al.*, 2006b). The role of the non-enzymatic CPC members is localization of the kinase at the correct place and time (reviewed in Vader *et al.*, 2006b; Ruchaud *et al.*, 2007). Aurora B binds to the so-called IN-box region of INCENP and in turn, INCENP regulates the localization of Aurora B by interacting with Borealin and survivin (Klein *et al.*, 2006; Vader *et al.*, 2006a; Jeyaprakash *et al.*, 2007).

Aurora B belongs to a family of serine/ threonine protein kinases, which in mammals also comprises the two other family members Aurora A and C. Although the three proteins share a high sequence similarity, each Aurora kinase has a specific localization pattern and function during cell division (Carmena *et al.*, 2009). Aurora A is involved in centrosome maturation, bipolar spindle assembly and cell cycle progression in somatic cells (Sugimoto *et al.*, 2002;

Brittle and Ohkura, 2005; Barr and Gergely, 2007) and oocytes (Yao *et al.*, 2004). Aurora C is the most recently described and least characterized family member that arose during mammalian evolution through gene duplication of Aurora B (Brown *et al.*, 2004). Expression of Aurora C was first described in the testis (Bernard *et al*., 1998), where it is involved in chromatin condensation and proper attachment of homologous chromosomes during the first meiotic division (Tang *et al.*, 2006). Aurora C knockout mice are viable and males have normal testis weights, but reduced litter sizes with some males being sterile. Observed sperm abnormalities include heterogeneous chromatin condensation, loose acrosomes and blunted heads. However, as multiple copies of the Aurora C gene (*Aurkc*) are present in the mouse genome, a knockout approach is not reliable (Hu *et al.*, 2000). In contrast, male mice expressing a kinase-dead form of Aurora B as a transgene present with decreased testis weights, as well as severely impaired spermatogenesis and reduced litter size (Kimmins *et al.*, 2007). The relative importance of Aurora B *versus* C for male mouse meiosis remains uncertain, awaiting proper Aurora B and C knockout mouse models. Naturally occurring human mutations in *AURKC*, causing a severe truncation of the protein, have been described and are associated with male infertility (Dieterich *et al.*, 2007). This results from defective meiosis leading to the production of polyploid, multi-flagellar spermatozoa with abnormal acrosomes (Dieterich *et al.*, 2009). Two females carrying the same homozygous mutation were reported to be fertile, indicating that Aurora C may be dispensable for completion of meiosis in the human female, but not in the male (Dieterich *et al.*, 2009). However, transcript profiling of human oocytes points to a prominent role for Aurora C (Assou *et al.*, 2006). Moreover, a recent study in mouse demonstrated that microinjection of mRNA coding for a kinase dead form of Aurora C disrupts meiosis I in *in vitro* matured oocytes, suggesting an essential role for Aurora C (Yang *et al.*, 2010). This result is in contrast to previous findings on Aurora B function during mouse female meiosis (Shuda *et al.*, 2009; Vogt *et al.*, 2009), indicating the importance of Aurora B for female meiosis.

Based on gonad-specific expression and the homozygous mutation phenotype in man, Aurora C has been coined the germ cell specific homologue (Shuda *et al.*, 2009). However, Aurora C is also expressed in various tumor lines (Sasai *et al.*, 2004) and its expression can be detected at a low level in other somatic tissues (Lin *et al.*, 2006), including the pineal gland where it is implicated in circadian clock function (Price *et al.*, 2009). Moreover, Aurora C was shown to fully support mitotic progression when replacing Aurora B in somatic cells (Slattery *et al.,* 2009). Aurora C interacts with the other CPC proteins (Li *et al.*, 2004; Yan *et al.,* 2005; Slattery *et al.*, 2008) and shares some substrates with Aurora B (Slattery *et al.*, 2008). Therefore, in addition to a meiotic role, Aurora C may also have a tissue specific role in mitotic cells (Slattery *et al.*, 2009).

To obtain a better understanding of regulation of chromosome segregation in human primary oocytes, the early fertilization stage (female meiosis II), zygotes and preimplantation embryos, we have investigated the expression of CPC subunits, including both Aurora B and C, in a unique series of human oocytes and embryos. The high level of expression of *AURKC* in human and mouse oocytes (Assou *et al.*, 2006; Yang *et al.*, 2010) and the prominent role of Aurora C in human male meiosis lead us to hypothesize that Aurora C could act as the

preferred enzymatic subunit of the CPC at the reductional divisions in oocytes. Moreover, as in the human the first cleavage divisions are maternally directed (Tesarik *et al.*, 1986; Braude *et al.*, 1988), Aurora C might well continue to play a role in the control of mitosis before activation of the embryonic genome.

We studied mRNA and *in situ* protein expression of all CPC members in human oocytes and at all stages of human preimplantation embryo development. We observed that Aurora C can be detected at both the mRNA and protein level in oocytes and cleavage stage embryos. Yet, after the 8 cell stage and up to the morula stage, there was upregulation of both *AURKB* and *AURKC* mRNA and pericentromeric localization of both kinases on prometaphase chromosomes. In blastocysts Aurora C transcripts and protein were undetectable, and only Aurora B was found at the inner centromere of prometaphase chromosomes. Our results point to a specific role for Aurora C as the enzymatic subunit in the CPC during human female meiosis and preimplantation embryo development, until complete replacement by Aurora B at the blastocyst stage.

Material and methods

Collection of human oocytes and spermatocytes

Ovarian stimulation, oocyte retrieval and IVF procedures were performed as described previously (Hohmann *et al.*, 2003; Heijnen *et al.*, 2007). At the day of oocyte retrieval (day 0), immature oocytes (metaphase I [MI]) were donated by couples undergoing ICSI treatment at the IVF laboratory of the University Medical Center Utrecht. Some mature oocytes (metaphase II [MII] stage) were donated in a case where on the day of oocyte retrieval no sperm cells could be obtained. MI oocytes were either processed immediately or allowed to mature in a 5% CO_2 atmosphere at 37°C in G-IVF Plus medium (Vitrolife) overnight (18 h) and fixed at the MII stage.

Testis material was obtained after testicular spermatozoa extraction from a man (age 47 years) of proven fertility with previous vasectomy and subsequent vaso-vasostomy that did not succeed. Remnant cellular material was used. A Johnson score of 9.5 (range 9 – 10) was determined at the pathology department of the Radboud University Nijmegen Medical Center. The patient signed an informed consent for participation in a project approved by the Dutch Central Committee on Research Involving Humans Subjects (CCMO – NL12408.000.06).

Collection of human embryos

Under Dutch law, embryos are not allowed to be created for research. Therefore, human embryos are only available for research after embryo selection for transfer or cryopreservation. Exceptions to this rule are embryos resulting from abnormal fertilization, i.e. oocytes fertilized by two spermatozoa simultaneously or where the second polar body failed to extrude. Evaluation of the number of pronuclei 18-20h after insemination allows identification of such embryos, characterized by the presence of three pronuclei; this is observed in ~4% of all inseminated oocytes. To avoid potential triploid pregnancies, these embryos normally are discarded (Ulmer *et al.*, 1985). We used triploid (3PN) embryos as a model for embryo development during the

first cleavage divisions. Surplus good quality cryopreserved preimplantation embryos were used to study embryo developmental stages from day 3 to day 5 (Figure 4.1). Tripronuclear zygotes and surplus embryos were donated with written informed consent by couples undergoing routine IVF at the Erasmus MC University Medical Center in the period between March and July 2010, and November 2000 and December 2007, respectively. The use of both types of surplus embryos was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO – NL28739.000.09) and the local institutional ethics committee.

Embryo culture and assessment of embryo morphology were performed as described previously (Hohmann *et al.*, 2003). Cryopreservation was performed in straws using a slow freezing standard protocol of 1.5 M dimethyl sulfoxide (DMSO) in culture medium containing 10% GPO (human plasma solution, CLB), as described previously (Santos *et al.*, 2010b). Women donating cryopreserved embryos were aged between 29-41 years. A total of 90 embryos were thawed and 44 survived after consecutive washes in decreasing DMSO concentrations. Day 3 (8 cell) embryos were processed within 2 h after thawing. Early day 4 embryos (12-16 cell embryos) were randomized for either immediate processing, or culture until late day 4 (fully compacted morulas) or day 5 (blastocysts). After randomization, embryo morphology was evaluated. Only those of good morphological quality and showing the stage-appropriate characteristics were used to avoid a selection bias in embryo quality.

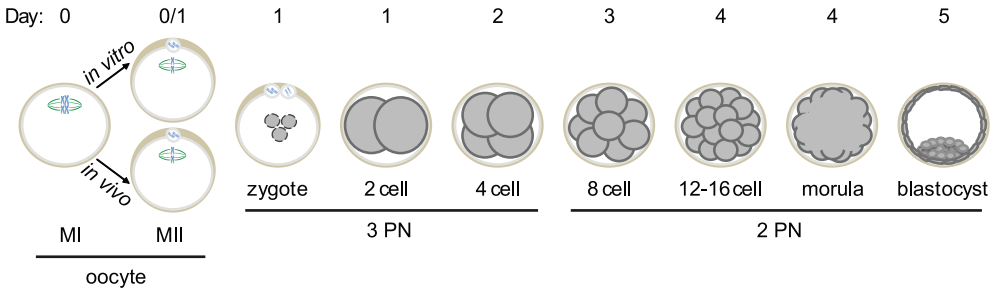

Figure 4.1 – Schematic representation of the stages of oocyte and embryo development used in this study. Metaphase I (MI) and metaphase II (MII) oocytes were fixed on the day of retrieval (day 0), or MI oocytes were left to mature to MII overnight. After fertilization, tripronuclear (3PN) zygotes were fixed at prometaphase of the first embryonic mitotic division or allowed to develop until 2 cell or 4 cell stage. Good quality diploid (2PN) 8 cell embryos or morulas that had been cryopreserved on day 3 or day 4 respectively, were thawed and immediately fixed or left in culture until reaching 12-16 cell, morula, or blastocyst stage.

Single oocyte and embryo RT-qPCR

Quantification of mRNA levels was performed in individual single oocytes and preimplantation embryos at nine developmental stages (Figure 4.1): oocytes at MI (n=2) and MII (n=5), abnormally fertilized oocytes with three pronuclei (3PN; n=13), 2 cell embryos (3PN- 2 cell; n=10) and 4 cell embryos (3PN- 4 cell; n=4), both resulting from tripronuclear zygotes, and 8 cell embryos (n=12), 12-16 cell embryos (n=6), morulas (MOR; n=5), and blastocysts (BLAS; n=10), all good quality embryos cryopreserved at days 3 and 4 (Figure 4.1). For quantitative RT-PCR (RT-qPCR) of single oocyte/embryos, the Taqman® PreAmp Cells-to-Ct Kit (Applied

Biosystems) was used according to the manufacturer's protocol with minor adjustments. The zona pellucida was removed from the oocytes and embryos by incubation in 0.1% protease (Sigma) in G-MOPS Plus medium (Vitrolife) for 3 min, prior to washing in G-MOPS Plus medium and 1X PBS. Lysis was performed for 5 min in 20 µl Taqman® PreAmp Cells-to-Ct lysis solution and terminated by addition of 2 µl of stop solution. After 2 min of incubation the lysate was stored at -20°C until further processing within one week. RNA was reverse transcribed to cDNA within an hour at 37°C by adding 25 µl of 2X RT Buffer and 2.5 µl of 20X RT Enzyme Mix to each lysate, prior to inactivating the enzyme for 5 min at 95°C. For sequence-specific preamplification of cDNA, Taqman Gene Expression Assays (Assays-on-demand, Applied Biosystems) were pooled and diluted 1:100 with 1X TE buffer (10 mM Tris-HCl, 5 mM EDTA; pH 7.5) to a final concentration of 180 nM of each primer. The following assays were used: *HPRT1* (Assay ID: Hs99999909_m1; amplicon size 100bp), *AURKB* (Hs00177782_m1; 130bp), *AURKC* (Hs00152930_m1; 91bp), Borealin (*CDCA8*; Hs00216479_m1; 127bp), Survivin (*BIRC5*; Hs00153353_m1; 93bp), *INCENP* (Hs00220336_m1; 62bp), BRG1 (*SMARCA4*; Hs00231324_m1; 106bp) and *ZP3* (Hs00610623_m1; 74bp). Assays were selected to recognize all validated (RefSeq) splice variants of each gene of interest, except for assays for *INCENP* (recognizing only NM_020238.2) and *BIRC5* (recognizing NM_001012271.1 and NM_001168.2). To 12.5 µl cDNA, 25 µl Taqman® PreAmp Master Mix and 12.5 µl of 0.2X pooled Taqman® Gene Expression Assays were added. After 10 cycles of preamplification (10 min at 95°C, followed by 10X 15 sec at 95°C and 4 min at 60°C), the preamplified cDNA (50 µl) was diluted with 100 µl 0.5X TE buffer. QPCR was performed on an ABI Prism 7000 Sequence Detecting System (Applied Biosystems) using 10 µl 2X Taqman® Gene Expression Master Mix, 1 µl Taqman® Gene Expression Assay and 5 µl nuclease-free water added to 4 µl diluted preamplified cDNA. The 2-step cycling parameters were as follows: one cycle of 2 min at 50°C, followed by one cycle of 10 min at 95°C to activate the polymerase and 40 cycles of 15 sec at 95°C and 1 min at 60°C.

Results were analyzed using Sequence Detection Software version 1.2.3 (Applied Biosystems) and expressed as cycle threshold (Ct) values. Presence of a single PCR-product of expected amplicon size was verified by 2% agarose gel electrophoresis. In order to be able to use a relative quantification approach to compare expression levels of *AURKB* and *AURKC*, we ensured that these commercial assays have similar amplification efficiencies, within the limits set by the supplier (E=100±10%). Additionally, linearity during the preamplification reaction was tested on a series of 1:2 diluted cDNA from oocytes and blastocysts. The averaged expression level at the MII oocyte stage was used as a reference to calculate the relative levels in all other stages, according to the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001). Differences in *AURKB* and *AURKC* expression across developmental stages were analyzed using the Mann-Whitney test in SPSS version 17.0. A p-value of 0.05 was considered statistically significant. To compare expression levels of *AURKB* directly to expression levels of *AURKC*, the relative expression for *AURKB* was calculated using *AURKC* as a reference.

Antibodies

The following antibodies were used in this study: rabbit polyclonal antibodies against AURKC

(ab38299, 1:100; Abcam), H3S10p (1:10,000; Cell Signaling), INCENP (1:1,000; Sigma), Borealin (1:2,000; kindly supplied by S. Wheatley, University of Nottingham Medical School, Nottingham, UK), Survivin (1:2,000, R&D Systems) and GFP (S.M.A. Lens, University Medical Center Utrecht, The Netherlands). Mouse monoclonal antibodies against AURKB (1:250; BD Biosciences) and INCENP (1:1,000, Upstate). Human autoantibodies against the centromere (CREST, HCT-0100, 1:100, Immunovision and 1:2,000, Cortex Biochem). Primary antibodies were detected by labeling with the appropriate secondary antibodies conjugated with Alexa fluor 488, 555, 594 or 647 (Molecular Probes).

Overexpression of Aurora A, B and C kinase in U2OS cells

Human osteosarcoma cells (U2OS) were grown at 37°C with 5% CO2 in DMEM containing 6% FCS, glutamine, penicillin and streptomycin. Transfections were performed using a standard calcium phosphate protocol. U2OS cells were co-transfected with 1 µg empty GFP vector, GFP-AURKA, GFP-AURKB or GFP-AURKC together with an empty pcDNA3 vector (9 µg). Cells were first synchronized at the G1/S transition by incubation for 24 h with 2.5 mM thymidine and later arrested in mitosis by incubation for 18 h with nocodazole (250 or 25 ng/ml; for harvesting or fixation of cells, respectively). Cells were harvested or fixed and used for cell lysis or immunofluorescence. Cell lysis and Western blotting was performed as described previously (Smits *et al.*, 2000) and immunoprobed with anti-Aurora B and -C antibodies (1:250 and 1:500).

For immunofluorescence, U2OS cells were grown on slides and fixed with PFA buffer (4% w/v PFA in PBS). The slides were washed in PBS and dehydrated with ice-cold methanol. Subsequently, cells were blocked with 3% (w/v) BSA in PBS-T (PBS, 0.01% v/v Tween-20) for 30 min at room temperature (RT), and incubated with anti-Aurora C and CREST antibodies overnight at 4°C. Slides were washed three times in PBS-T and then incubated with secondary antibodies for 1-2 h. After rinsing in PBS-T, slides were mounted with vectashield containing DAPI for counterstaining (Vector Laboratories).

Fixation and immunofluorescence of spermatocytes

Nuclear spreads were made as described previously (Peters *et al.*, 1997), with minor modifications (Baart *et al.*, 2000). Briefly, a suspension of spermatogenic cells was made by pulverizing the testis tubuli with two ribbed forceps. After hypotonic treatment, cells were resuspended in 100 mM sucrose, pH 8.2 to obtain a cell concentration of about $15x10^6$ cells/ml. From this suspension 10 µl was applied to a slide dipped in 1% (w/v) PFA solution, containing 0.15% (v/v) Triton X-100. After horizontal drying for 1.5 h in a humid chamber, slides were washed twice with 0.08% Kodak Photo-Flo and air-dried. Slides were stored at -80°C until use.

Surface spread preparations were processed for immunofluorescence as described (Baart *et al.*, 2000). Meiotic prophase I stages were approximated by DAPI staining of annex nuclear morphology as based on previous experience with the synaptonemal complex marker SYCP3 (de Vries *et al.*, unpublished results). Early and late pachytene substages were distinguished by the more prominent DAPI intense sex body present in late pachytene. Metaphase nuclei were recognised by separate bivalent chromosome domains and the absence of a DAPI-

intense sexbody. At least 50 early first meiotic division nuclei (leptotene, zygotene) and 100 pachytene nuclei were studied, as well as 5 first metaphase nuclei. Images were captured with a Zeiss AxioCam MR digital camera with Axiovision 3.1 software, using a Zeiss Axioplan fluorescence microscope (all Carl Zeiss).

Fixation and immunofluorescence of oocytes and embryos

MI and MII oocytes were incubated with nocodazole (500 ng/ml, Sigma) for 30 min before fixation. Tripronuclear zygotes were incubated with colcemid (1,5 µg/ml) to arrest cells at prometaphase until pronuclei had disappeared. Good quality day 4 and 5 embryos were treated with nocodazole (500 ng/ml) for 4 h before fixation.

Oocytes and embryos were incubated in 0.1% protease in G-IVF Plus medium (1-2 min) for removal of the zona pellucida and washed in G-IVF Plus medium. Fixation was performed as previously described, with minor modifications (Puschendorf *et al.*, 2008). In short, cells were fixed in PFA buffer for 15 min at RT. After fixation, oocytes/embryos were rinsed in PBS-T and incubated with 0.2% (w/v) Triton X-100 in PBS for 15 min at RT for permeabilization. Oocytes and embryos were washed in PBS-TB (PBS-T, 2% w/v BSA), blocked with PBS-TB/ 5% NGS for 4h and incubated with primary antibodies at 4°C overnight. After washing with PBS-TB, oocytes and embryos were incubated with the appropriate secondary antibodies conjugated with Alexa Fluor 488, 555, 594 or 647 (Invitrogen), washed with PBS-TB, and mounted on coverslips with vectashield mounting solution containing DAPI for DNA counterstaining (Vector Laboratories). To obtain chromosome spreads, arrested zygotes and day 4 embryos were (after zona removal) submitted to the same hypotonic treatment as spermatogenic cells. After brief transfer to 100 mM sucrose, cells were placed on a slide dipped in a 1% (w/v) PFA solution, containing 0.15% (w/v) Triton X-100. Slides were dried and processed as described for spermatogenic cell surface spreads.

Images were obtained using a Delta Vision microscope and deconvolution software (Applied Precision) or with an AxioCam MR digital camera with Axiovision 3.1 software, using an inverted Axio Observer fluorescence microscope equipped with an ApoTome (all Carl Zeiss) for optical sectioning.

Results

Aurora C kinase is detected in human male and female meiotic cells

A commercially available polyclonal antibody against Aurora C, raised against a synthetic peptide corresponding to amino acids within residues 1-50 of human Aurora C was used. To confirm that this antibody does not cross-react with the other Aurora kinases, specificity was tested by immunofluorescence on U2OS cells overexpressing GFP-AURKA, GFP-AURKB and GFP-AURKC (Supplementary Figure 4.1a). The antibody did not detect Aurora A or B, but co-localized with the GFP-signal for Aurora C. We further tested the specificity of anti-Aurora C antibody using immunoblots of lysates of GFP-AURKA, -B and -C transfected U2OS cells. The antibody did not recognize GFP-AURKA or GFP-AURKB, but detected GFP-AURKC (Supplementary Figure 4.1b).

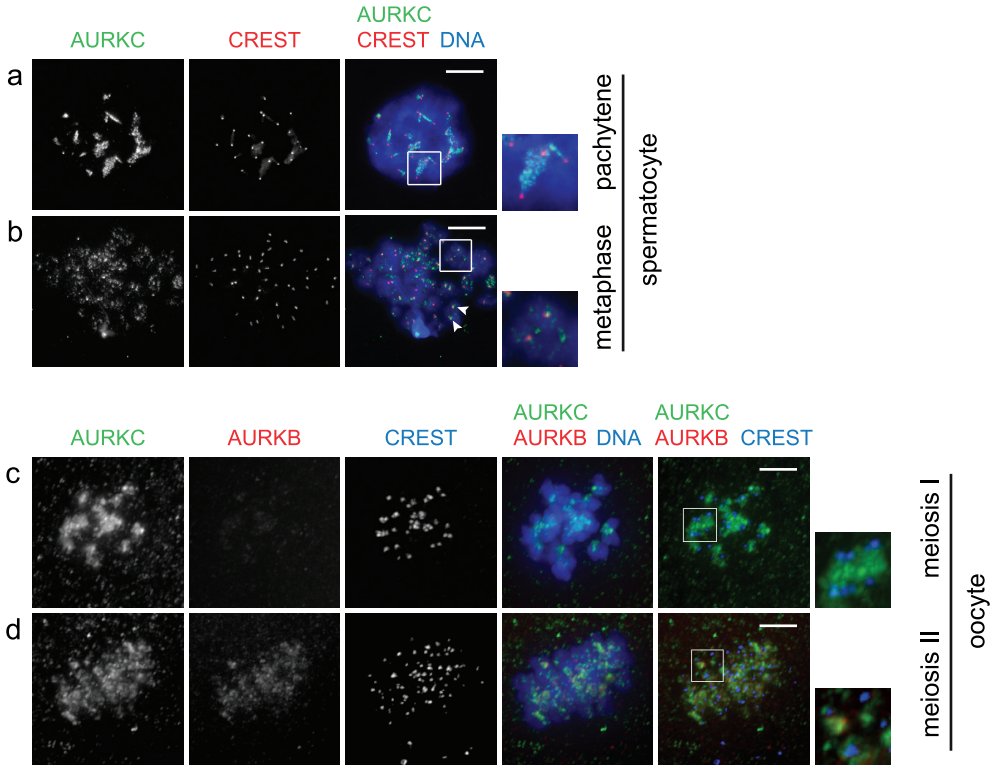

Figure 4.2 – Immunolocalization of Aurora C in human primary spermatocytes and MI and MII oocytes. (a) Late pachytene spermatocyte nucleus, with Aurora C visible in concentrated clouds of various sizes. The merged image shows Aurora C localized adjacent to the centromeres (CREST antigen). **(b)** MI spermatocyte. Aurora C is not only localized to the centromeric heterochromatin regions as in late pachytene, but is also visible throughout the chromosome domains. In most of the dispersed clouds of Aurora C two intense spots colocalize with the centromeres (arrow heads). (c-d) MI and MII oocytes, with antibody staining for Aurora B and C and centromeric regions (CREST). **(c)** In MI oocytes, Aurora C was observed in a diffuse pattern surrounding the centromeres. **(d)** In MII oocytes, Aurora B localized more to the centromere, while Aurora C was observed in a more diffuse pattern surrounding the centromeres. DNA was counterstained with DAPI. Square boxes are blowups of each corresponding smaller box. Scale bars are 5 μm.

Since Aurora C is reported to be highly transcribed in mouse spermatocytes (Bernard *et al.*, 1998; Hu *et al.*, 2000), the staining pattern and intensity of Aurora C was first studied in human primary spermatocyte nucleus spread preparations. From leptotene to early pachytene a faint, evenly distributed, dotted Aurora C kinase signal was observed (not shown). In later pachytene nuclei the Aurora C signal localized to chromosome regions adjacent to the centromeres (CREST antigen), probably reflecting the centromeric heterochromatin regions (Figure 4.2a). In early meiotic metaphase nuclei the Aurora C signal was more dispersed over the chromosomes and not solely localized to the centromeric heterochromatin regions as in late pachytene (Figure 4.2b). However, more intense spots surrounded the centromeric signal (Figure 4.2b, see insert).

We next investigated the localization of Aurora C during human female meiosis in MI and MII oocytes. MI oocytes were available 4-6 h after oocyte retrieval if they had failed to progress to the MII stage by the time the ICSI procedure was completed. As a consequence,

synchronization was not optimal and oocytes could be at any stage between early prometaphase and metaphase of meiosis I. MI oocytes (n=13) were treated with nocodazole for 30 min and fixed immediately. We detected Aurora C along the chromosome arms in some oocytes, but in other MI oocytes, Aurora C signal was observed to localize near the centromere (Figure 4.2c). This is consistent with Aurora C staining recently described in mouse oocytes for prometaphase I and metaphase I, respectively (Yang *et al.*, 2010). In all MII oocytes (n=19), Aurora C staining was observed to localize to the chromosome regions adjacent to the centromeres (Figure 4.2d) and only weakly along the chromosome arms.

Aurora B staining was also investigated together with Aurora C in MI and MII oocytes. In all MI oocytes investigated (n=10), Aurora B staining was either not detected, or observed very weakly at chromosome regions adjacent to the centromeres (Figure 4.2c). In three MII oocytes, Aurora B staining showed a more intense signal than Aurora C (data not shown), but in most MII oocytes (n=12), Aurora B staining was less intense than Aurora C (Figure 4.2d), with some oocytes showing no staining for Aurora B. This suggests that during female meiosis, both Aurora B and Aurora C are involved. However, in meiosis I Aurora B is hardly detected, while in meiosis II both kinases are found.

mRNA expression and protein localization of INCENP, survivin and Borealin in human oocytes and embryos

To investigate if the CPC proteins INCENP, Borealin and survivin are expressed and functional in human oocytes and embryos, we first examined mRNA expression of these subunits in multiple individual oocytes and embryos at seven different preimplantation developmental stages (Figure 4.1). Normalization of gene expression is especially challenging in preimplantation embryos. Owing to the absence of active transcription in the early embryo followed by activation of embryonic transcription later on, finding a reference gene with stable expression throughout all stages of development is problematic (Kuijk *et al.*, 2007; Mamo *et al.*, 2008). In rabbit embryos, where embryonic genome expression is activated at the same developmental stage as human embryos, *HPRT1* was shown to be a suitable reference gene for preimplantation development, when *in vivo* and *in vitro* produced embryos are compared (Mamo *et al.*, 2008). We found *HPRT1* to be highly regulated during embryo development (Supplementary Figure 4.2a), similar to the pattern described for rabbit embryos (Mamo *et al.*, 2008). Therefore, resulting gene expression levels were normalized using average expression levels of the gene in the metaphase II oocyte as a reference. As this method of normalization is not informative for the abundance of transcripts, average Ct values are presented for each gene investigated per developmental stage (Supplementary Table 4.1).

The expression levels of zona pellucida protein 3 (*ZP3*) and SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 (*SMARCA4*) were determined to further check the sensitivity of our single oocyte/ embryo RT-qPCR approach. *ZP3* expression is expected to be maternal only (Rajkovic and Matzuk, 2002). Our results demonstrate high expression in oocytes and a steady decrease in transcript levels until barely detectable at the blastocyst stage (Supplementary Figure 4.2b). *SMARCA4* has been described to be present as a maternal transcript (Bultman *et al.*, 2006) and subsequently to be

one of the first genes transcribed after zygotic gene activation in mouse embryos (Hamatani *et al.*, 2004). In our series, expression of this gene was shown to decline from the oocyte to the 8 cell stage and then showed an increased expression from the 8 cell stage onwards (Supplementary Figure 4.2c). This confirms that transcripts detected up to the 8 cell stage are likely maternal, with activation of the embryonic genome occurring from the 8 cell stage on (Braude *et al.*, 1988).

Normalized expression levels of *INCENP*, Survivin (*BIRC5*) and Borealin (*CDCA8*) were compared between the nine different stages of oocyte and preimplantation embryo development (Figure 4.3a-c). Transcript abundance for INCENP was low in oocytes and embryos up to the 8 cell stage, after which an increase occurred, starting at the 12-16 cell stage, with significant upregulation at the blastocyst stage (Figure 4.3a). Survivin and Borealin both showed a high transcript level at the MI oocyte stage, decreasing gradually until the 8 cell stage and increasing towards the morula and blastocyst stage (Figure 4.3b, c).

To investigate expression of these CPC subunits at the protein level, human oocytes and tripronuclear zygotes treated with nocodazole or colcemid were immunostained for INCENP, Survivin and Borealin. In both oocytes (data not shown) and zygotes (Figure 4.3d-f), each CPC protein was detected at the inner centromere in prometaphase and in a zygote escaping the nocodazole block INCENP was also detected at the spindle midzone (Figure 4.3d'). These observations are consistent with findings in somatic mitotic cells. As INCENP forms the binding factor between the enzymatic subunit and Survivin and Borealin (Klein *et al.*, 2006), double staining for Aurora C and INCENP was performed. These two proteins co-localized on the inner centromere of prometaphase chromosomes in zygotes (Figure 4.3g). To investigate CPC function in these human zygotes, immunostaining with a H3S10p antibody was performed. This phosphorylation site is known to be targeted by active Aurora B, as well as Aurora C (Li *et al.*, 2004; Sasai *et al.*, 2004; Yan *et al.*, 2005). Histone H3S10 was phosphorylated along the chromosome arms, as previously described for somatic mitotic cells. These results suggest that a functional CPC is assembled in human oocytes and zygotes.

Expression of AURKB and AURKC in human oocytes and preimplantation embryos

Aurora C has been described to fully compensate for loss of Aurora B as the enzymatic subunit of the CPC (Slattery *et al.*, 2009). In oocytes, we detected both kinases in prometaphase of meiosis II (Figure 4.2). We therefore investigated the presence of Aurora B and Aurora C in human preimplantation embryos. First, mRNA levels for these kinases at nine different stages of oocyte and preimplantation embryo development was examined. *AURKB* mRNA levels were similar in MI and MII oocytes and then steadily decreased until the 8 cell stage, but increased significantly from the morula stage onwards (Figure 4.4a). *AURKC* mRNA levels were highest in MI oocytes and significantly decreased until the 4 cell stage. A gradual increase was observed starting from the 8 cell stage, with the morula stage reaching levels similar to the zygote. Subsequently, expression sharply decreased, until barely detectable at the blastocyst stage (Figure 4.4b). To better visualize differences in *AURKB* and *AURKC* expression patterns, the ratio between *AURKB* and *AURKC* was used. Both assays were verified to yield linear pre-amplification, as well as similar amplification efficiencies, enabling a

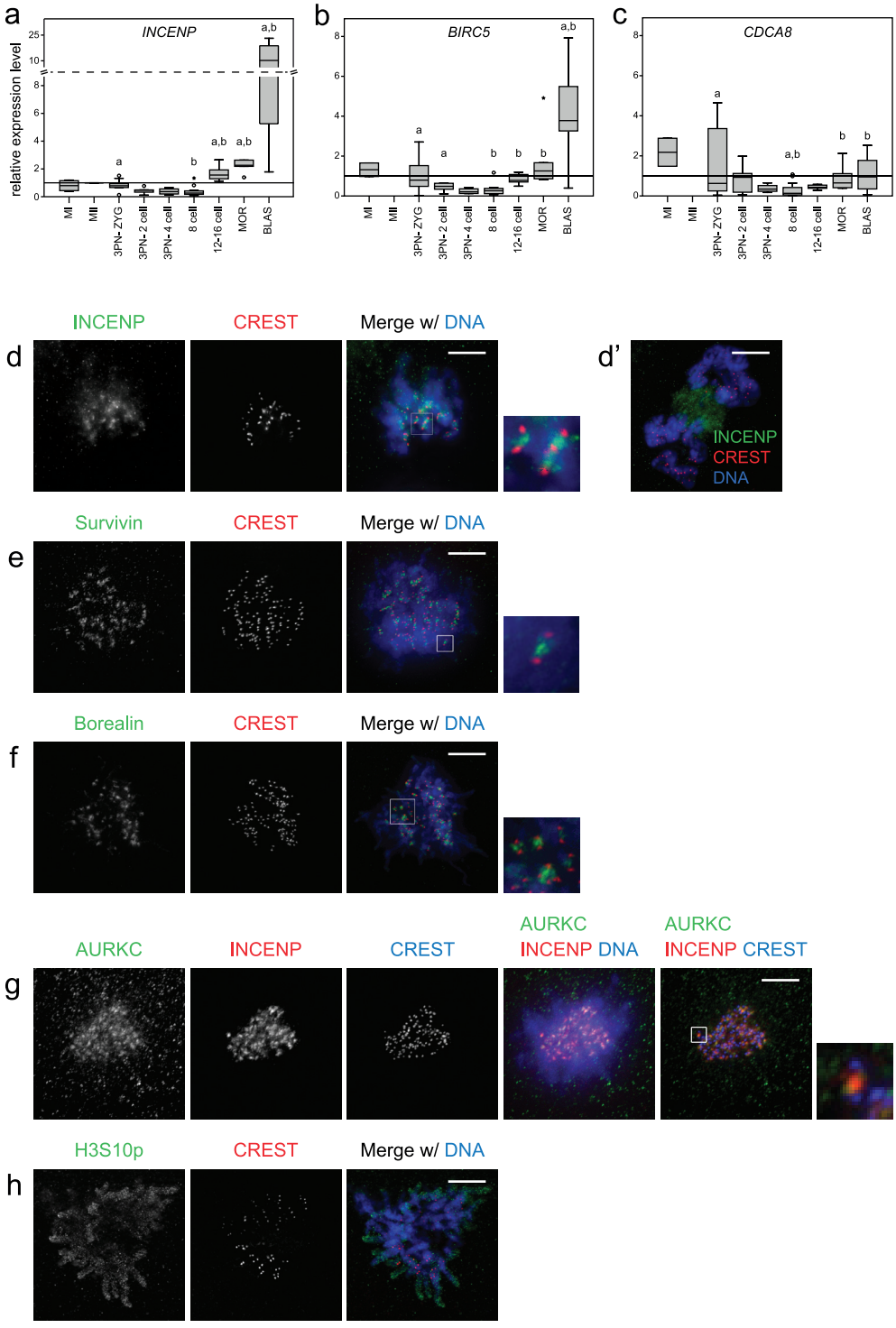

Figure 4.3 – Expression of the chromosomal passenger complex (CPC) members INCENP, Survivin and Borealin in human oocytes and preimplantation embryos. Relative expression levels after RT-qPCR of **(a)** *INCENP*, **(b)** *BIRC5* (Survivin) and **(c)** *CDCA8* (Borealin) in single oocytes and preimplantation embryos at nine developmental stages:

oocytes at MI (n=2) and MII (n=5), zygotes (3PN, n=13), 2 cell embryos (3PN- 2 cell, n=10), 4 cell embryos (3PN-4 cell, n=4), 8 cell embryos (n=12), 12-16 cell embryos (n=6), morulas (MOR, n=5), and blastocysts (BLAS, n=10). The mean expression level at the oocyte MII stage was taken as a reference to calculate the relative levels of the other stages. Note the scale of the y-axis differs. *Boxes* indicate 25th and 75th percentiles, with the horizontal line representing the median value. *Whiskers* span the range observed, *open circles* and *asterisks* represent outliers. Stages with average expression levels significantly different from the zygote stage ([a]) and the 8 cell stage ([b]) are indicated ($p < 0.05$). (d-h) Immunolocalization of CPC proteins in human tripronuclear zygotes at prometaphase: INCENP **(d)**, Survivin **(e)** and Borealin **(f)** relative to centromeres (CREST). **(d')** INCENP relocalization to the midzone during anaphase. **(g)** Immunolocalization of Aurora C and INCENP in human tripronuclear zygotes showing co-localization of Aurora C and INCENP at the inner centromere. **(h)** Immunolocalization of histone H3S10p along the chromosome arms in human tripronuclear zygotes at prometaphase. Zygotes used for immunostaining were arrested in prometaphase after treatment with nocodazole. DNA was counterstained with DAPI. Square boxes are blowups of each corresponding smaller box. Scale bars are 5 µm.

direct comparison of transcript abundance in single oocytes and embryos. Plotting the levels of *AURKB* mRNA relative to levels of *AURKC* mRNA (Figure 4.4c) shows comparable levels of transcripts for *AURKC* and *AURKB* up to day 4 of embryo development, with an exception at the 4 cell stage, where *AURKC* transcripts are more severely depleted. After the 8 cell stage, *AURKB* mRNA increases rapidly, and together with the severe reduction of *AURKC* mRNA by the blastocyst stage, results in a 16-fold higher level of *AURKB* than *AURKC* mRNA on day 5.

We next investigated the presence and localization of Aurora B and Aurora C in human preimplantation embryos. We performed triple immunolabeling for Aurora B, Aurora C and centromeric proteins in tripronuclear zygotes (n=17), 2 cell (n=4) and 4 cell (n=4) embryos of tripronuclear origin, as well as in good quality 8 cell (n=7), 12-16 cell embryos (n=10) and blastocysts (n=4). Aurora C was localized near the centromeres of prometaphase chromosomes from the zygote to the 12-16 cell stage (Figure 4.4d-f). In contrast, Aurora B was barely detectable up to the 8 cell stage. From the 8 cell stage onwards, staining intensity increased, with both Aurora B and C detected at prometaphase (Figure 4.5b) and one example where only Aurora B was detected. In 12-16 cell embryos, the ratio between Aurora B and C signal intensity was variable (Figure 4.4d-h). In most embryos, both kinases were found to co-localize at the centromeric regions (Figure 4.4f), but in one example only Aurora C was detected (data not shown). Other embryos revealed very little or no detectable Aurora C staining but abundant Aurora B around the centromere and in small dispersed amounts along the chromosome arms (Figure 4.4g). In 23 prometaphases from 4 blastocysts, only Aurora B was found and abundantly present (Figure 4.4h).

To investigate in more detail if Aurora B and Aurora C proteins co-localize at prometaphase, chromosome spreads from tripronuclear zygotes, as well as diploid 8 cell and 12-16 cell stage embryos treated with colcemid or nocodazole were prepared, allowing higher resolution images (Figure 4.5a-c). On prometaphase chromosomes from zygotes, Aurora C staining was observed on the chromosome region surrounding the centromere and along the chromosome arms (Figure 4.5a). Aurora B staining was only weakly detected and more localized to the centromeric region. In 8 cell and 12-16 cell embryos, staining intensity of Aurora B and C was observed to be more similar, as was the localization of the signal (Figure 4.5b and c).

Although expression of *AURKB* mRNA was detected from the zygote to the 4 cell stage, Aurora B protein was weakly or not observed on prometaphase chromosomes.

Immunofluorescence analysis from the 8 cell stage onwards confirms our observations at the mRNA level, with the presence of both Aurora B and C from the 8 cell to the morula stage. After cavitation there is a progressive switch to Aurora B and at the blastocyst stage, Aurora B appears to be the only kinase involved in the CPC.

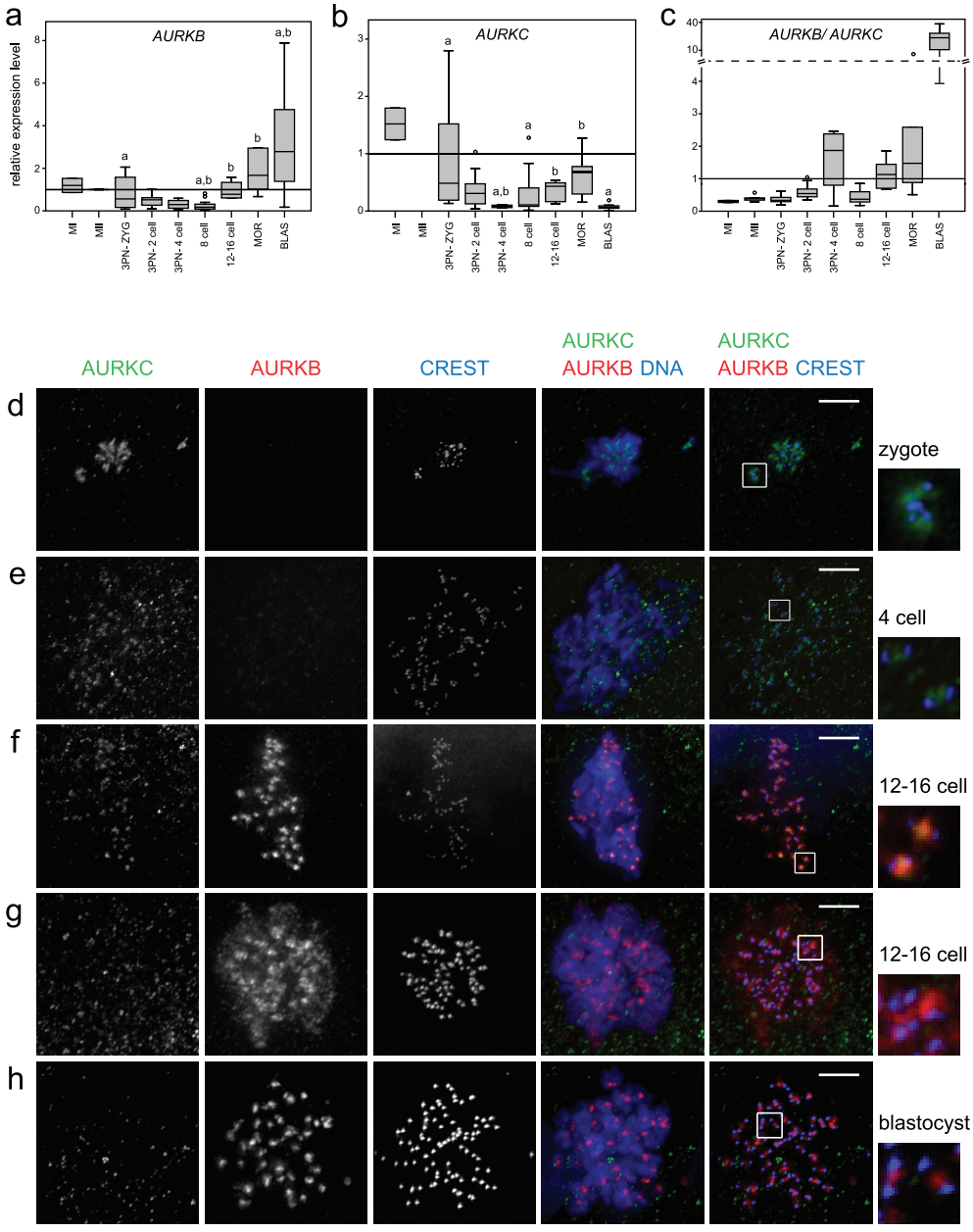

Figure 4.4 – Expression of *AURKB* and *AURKC* in human oocytes and preimplantation embryos. Relative expression levels after RT-qPCR of **(a)** *AURKB* and **(b)** *AURKC* in single oocytes and preimplantation embryos at nine developmental stages: oocytes at MI (n=2) and MII (n=5), zygotes (3PN, n=13), 2 cell embryos (3PN- 2 cell, n=10), 4 cell embryos (3PN- 4 cell, n=4), 8 cell embryos (n=12), 12-16 cell embryos (n=6), morulas (MOR, n=5), and blastocysts

(BLAS, n=10). The mean expression level at the oocyte MII stage was taken as a reference to calculate the relative levels of the other stages. Note the scale of the y-axis differs. Stages with average expression levels significantly different from the zygote stage ([a]) and the 8 cell stage ([b]) are indicated ($p < 0.05$). **(c)** Relative expression of *AURKB* over *AURKC* for all developmental stages. (d-h) Immunolocalization of Aurora B and C relative to centromeres (CREST) in human preimplantation embryos: **(d)** haploid set of chromosomes in a tripronuclear zygote, **(e)** triploid 4 cell embryo, (f-g) diploid 12-16 cell embryo and **(h)** blastocyst. In zygotes **(d)** and 4 cell embryos **(e)** only Aurora C was detected near centromeric regions. In 12-16 cell embryos, relative intensity of Aurora B and C was variable, but in blastocysts only Aurora B was abundant around the centromere and in small amounts on the chromosome arms. All embryos used for immunostaining were arrested in prometaphase. DNA was counterstained with DAPI. Square boxes are blowups of each corresponding smaller box. Scale bars are 5 µm.

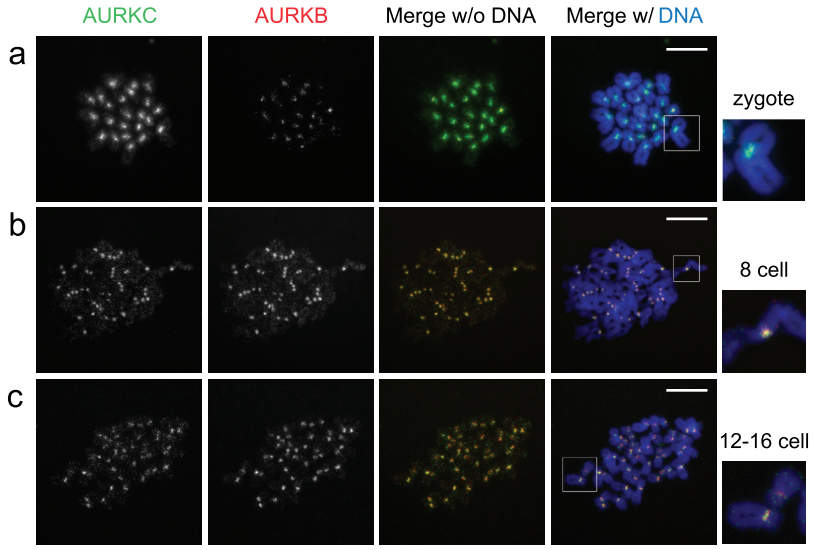

Figure 4.5 – **Immunolocalization of Aurora B and Aurora C on chromosome spreads from preimplantation human embryos: (a)** tripronuclear zygote, **(b)** 8 cell embryo and **(c)** 12-16 cell embryo. In zygotes **(a)**, Aurora C signal was abundant at centromeric regions and dispersed along the chromosome arms, whereas Aurora B was weakly detected at the inner centromere. In 8 cell **(b)** and 12-16 cell **(c)** embryos, relative abundance and localization of Aurora B and -C was similar. Embryos were arrested in prometaphase after treatment with colcemid. DNA was counterstained with DAPI. Square boxes are blowups of each corresponding smaller box. Scale bars are 5 µm.

Discussion

In this study, we analyzed localization of the different members of the CPC in human oocytes and preimplantation embryos, with a focus on the kinase subunits Aurora B and Aurora C. Contrary to INCENP, Survivin and Borealin that were detected in all stages investigated, Aurora B and C showed dynamic expression patterns at both the *in situ* protein and transcript level. We hypothesized a role for Aurora C during mitosis in the early stages of embryo development. Here we show that Aurora C is indeed the more prominent Aurora kinase present in cleavage stage embryos, based on fluorescent staining intensity at prometaphase in zygotes, 2 cell and 4 cell embryos (Figure 4.4d,e; Figure 4.5a). Aurora B was either not detected, or expressed at significantly lower levels than found from the 8 cell stage onwards (Figure 4.4d-h). Although we did detect Aurora B transcripts, we did not observe significant amounts of protein on prometaphase chromosomes in zygotes, 2 cell and 4 cell stage embryos. Similarly, in mouse oocytes, although *Aurkb* mRNA was present, western blot analysis failed to detect Aurora B, indicating regulation of expression at the translational level (Yang *et al.*, 2010).

Since Aurora C overexpression can completely restore cell cycle progression in Aurora B-deficient HeLa cells (Slattery *et al.*, 2009), Aurora C may be able to replace Aurora B and perform the same essential functions in cleavage stage embryos. Nevertheless, although Aurora B and Aurora C show high sequence similarities, structural differences exist (Brown *et al.*, 2004). The N- and C-terminal domains of Aurora C exhibit unique sequences and Aurora C lacks the so-called KEN-box and A-box sequences which target Aurora B for degradation via the anaphase-promoting complex/cyclosome (APC/C) after mitosis (Nguyen *et al.*, 2005). This implies that Aurora C is less susceptible to degradation, thus more stable throughout the cell cycle. In line with this, Aurora C protein levels were observed to peak after Aurora B during the later part of the M-phase (Sasai *et al.*, 2004). Thus, as the first embryonic cell cycles lack active transcription, there might be a need for an Aurora kinase that is independent of degradation at the end of M-phase, but with otherwise overlapping functions. However, our data also suggests a possible complementary role of Aurora B and C during the first embryonic cell divisions. Aurora C was observed to cover a larger area on zygotic prometaphase chromosomes, whereas Aurora B was more restricted to the centromeric regions. Although we can not exclude the possibility that Aurora B is present on the chromosome arms at amounts that fall below detection levels, similar observations regarding differences in Aurora B and C localization have been made in mouse MI oocytes (Shuda *et al.*, 2009; Sharif *et al.*, 2010). This suggests that Aurora C may have a role during the first embryonic cell cycle that does not overlap with Aurora B. The observed association of Aurora C with pericentric heterochromatin in spermatocytes leads us to hypothesize that this function could be related to pericentric heterochromatin organization, an hypothesis that awaits further investigation.

We expected that the expression pattern of *AURKC* would be similar to *ZP3*, with maternal transcripts gradually disappearing during embryo development, to be replaced by *AURKB* after activation of the embryonic genome. However, we observed a brief upregulation of *AURKC* mRNA after embryonic gene activation, reaching similar levels as *AURKB*. The presence of both kinases was also detected at the protein level but with a variation in staining intensity between day 3 and 4 (Figure 4.4f, g). Our data indicate that a switch in the Aurora B

to Aurora C ratio is gradually made on day 4 of embryo development, and that Aurora B is the chromosomal passenger of choice only after cavitation.

Owing to ethical limitations, we used embryos developed from tripronuclear zygotes as a model for embryo development up to the 4 cell stage. In human embryos, the first cleavage divisions are maternally directed until activation of the embryonic genome (Braude *et al.*, 1988) and it is therefore unlikely that the extra set of chromosomes present has an impact on expression and localization of CPC proteins in those early stages. Further in support of our findings on the presence of Aurora C during preimplantation embryo development, a recent study on mouse embryos carrying a targeted disruption of the *Aurkb* gene showed that these embryos were able to develop up to the blastocyst stage (Fernandez-Miranda *et al.*, personal communication). This is in contrast to mouse embryos that lack other components of the CPC, which are unable to progress beyond the cleavage stages (Cutts *et al.*, 1999; Uren *et al.*, 2000b; Yamanaka *et al.*, 2008b). Fernandez-Miranda *et al.* (personal communication) demonstrated that Aurora C was responsible for compensating loss of Aurora B function.

Although findings in most embryos investigated by us are consistent with an increase in the Aurora B/ Aurora C signal ratio from day 3 to day 4, we observed one prometaphase cell in a day 3 embryo showing clear Aurora B staining around the centromeres with no Aurora C, and the opposite in a day 4 (12-16 cell) embryo (data not shown). These variations are also visible at the mRNA level (Figure 4.4c) and may be related to a variation in timing and extent of activation of the embryonic genome. In human embryos, this is reported to start between the 4- and 8 cell stages of development (Braude *et al.*, 1988), with a major burst of transcription occurring at the 8 cell stage (Tesarik *et al.*, 1986). However, human IVF embryos demonstrate a lack of synchronicity in making the switch from maternal to embryonic gene activity (Tesarik, 1989). It was further observed in human and bovine embryos that development to the morula stage is possible without activation of the embryonic genome (Tesarik, 1989; Pavlok *et al.*, 1993; Schramm *et al.*, 2003) but these embryos lack the ability to form a blastocyst (Tesarik, 1989; Pavlok *et al.*, 1993).

In a previous study by us on the incidence of chromosomal abnormalities in human IVF embryos, we reported the proportion of aneuploid cells within an embryo to decline after cavitation (Santos *et al.*, 2010b). This coincides with the time of disappearance of Aurora C mRNA and protein at the inner centromere that we observe here. It is tempting to speculate that the presence of Aurora C may contribute to the observed high incidence of chromosome segregation errors during embryo development before compaction. However, Aurora C could fully compensate for the absence of Aurora B in mediating SAC function, as measured by cell cycle progression in HeLa cells (Sasai *et al.*, 2004; Slattery *et al.*, 2009) and mouse preimplantation embryos (Fernandez-Miranda *et al.*, personal communication). A possible cause for the high error rate may be found in the variation in the Aurora B to Aurora C ratio between embryos, as observed in this study. Ectopic overexpression of Aurora B in cell lines results in polyploidy (Tatsuka *et al.*, 1998) and over-expression of Aurora B is associated with cancer cell lines and primary tumors (Tatsuka *et al.*, 1998; Katayama *et al.*, 2003), indicating that tight regulation of Aurora B expression levels is crucial for accurate chromosome segregation. Moreover, overexpression of an Aurora C kinase-deficient mutant disrupts the

Aurora B-INCENP complex and induces polyploidy (Chen *et al.*, 2005), and overexpression of Aurora C has been reported in several cancer cell lines (Kimura *et al.*, 1999). So the question arises whether not maintaining the correct balance in expression of both kinases, before and after activation of the embryonic genome, is the underlying problem causing chromosome segregation errors.

Aurora C has already been described as an important kinase during human male meiosis (Dieterich *et al.*, 2007), where lack of functional Aurora C severely disrupts the meiotic process. In the current study, we describe for the first time localization of Aurora C to the region surrounding the centromeres in human spermatocytes. Aurora C associates with pericentric heterochromatin during pachytene, then spreads onto the chromosome arms at diakinesis and condenses at the centromeres again at metaphase. In mouse spermatocytes, Aurora C has been described to appear at the diplotene stage (an extremely short stage in the human male), following a similar pattern (Tang *et al.*, 2006). Our observations are also consistent with the phenotype observed in male patients carrying the Aurora C kinase c.144delC mutation (Dieterich *et al.*, 2009) and with a functional role for Aurora C during human male meiosis.

In agreement with the high mRNA expression of Aurora C reported in human oocytes (Assou *et al.*, 2006), we also observed Aurora C on the region surrounding the centromeres in MI and MII oocytes. Although timing of fixation in these human oocytes can not be performed optimally due to ethical reasons, our observations are similar to those recently described in mouse oocytes (Yang *et al.*, 2010), pointing to a conserved role for Aurora C in female mammalian meiosis. However, this function may be partly redundant in human oocytes, as the two female homozygous *AURKC* mutants are apparently fertile and without further phenotypes (Dieterich *et al.*, 2009). We also detected Aurora B transcripts in human IVF oocytes, although at a lower level than Aurora C. On (pro)metaphase chromosomes in MI oocytes, signal intensity of Aurora C was greater than Aurora B (Figure 4.2c), whereas in MII oocytes this signal ratio was more variable. Data in mouse as to the relative importance of Aurora B and C during meiosis I and II are contradictory (Shuda *et al.*, 2009; Vogt *et al.*, 2009; Yang *et al.*, 2010). The interpretation of these studies is complicated by the lack of specific inhibitors for Aurora B and C, as well as the possibility of the two kinases binding to each other *in vivo* (Li *et al.*, 2004). Thus overexpression of a kinase dead form of one kinase may affect functioning of the other (Chen *et al.*, 2005). To complicate matters further, polymorphisms in the mouse *Aurkc* gene that result in an amino acid sequence change have been described in inbred strains (Hu *et al.*, 2000), underscoring the need for proper knockout models. However, taken together, the evidence indicates that there is room for plasticity in the balance of Aurora B and C in the female germline, with the two proteins able to compensate for each other. The fact that human male patients with a mutation in *AURKC* are sterile may indicate differences in this plasticity between the male and the female germline.

Interestingly, in rhesus macaque oocytes, Aurora B mRNA expression was observed to decrease significantly when IVM oocytes were compared to *in vivo* matured oocytes (Mtango and Latham, 2008). A similar observation was made in human oocytes (Jones *et al.*, 2008b), indicating that oocyte maturation conditions may contribute to regulation of Aurora B mRNA expression. In future studies it will be interesting to explore whether the variability in the levels

of AURKB and AURKC we observe in the current study can be related to oocyte or embryo quality and patient characteristics, such as maternal age.

We set out to characterize the CPC in human preimplantation development, in order to identify causes for the observed high post-zygotic chromosome segregation error rate. Although known as a meiotic kinase, we present evidence that Aurora C is the main enzymatic subunit of the CPC during preimplantation embryo development up to the 8 cell stage, and continues to be present next to Aurora B during the compaction stage. This is in contrast to the constitution of the CPC in somatic mitotic cells, and indicates a role for Aurora C during preimplantation embryo development. Even though it is unlikely that the presence of Aurora C alone explains the high chromosome segregation error rate, the data presented here provide novel information regarding possible mechanisms. Further investigation of differences between Aurora B and C substrates and binding partners, as well as regulation of expression of these kinases before and after activation of the embryonic genome in relation to oocyte quality, may help in identifying crucial factors.

Acknowledgements

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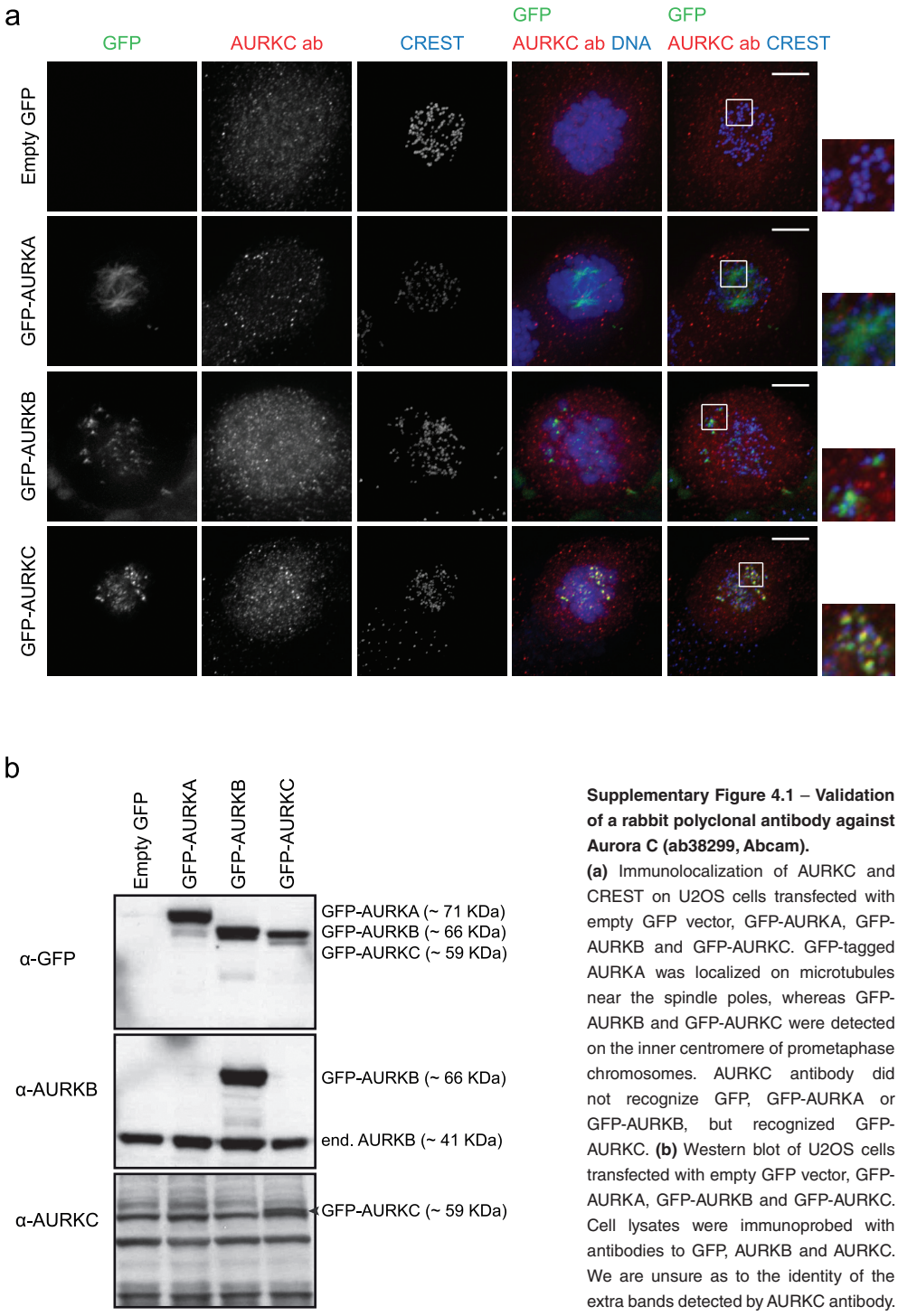
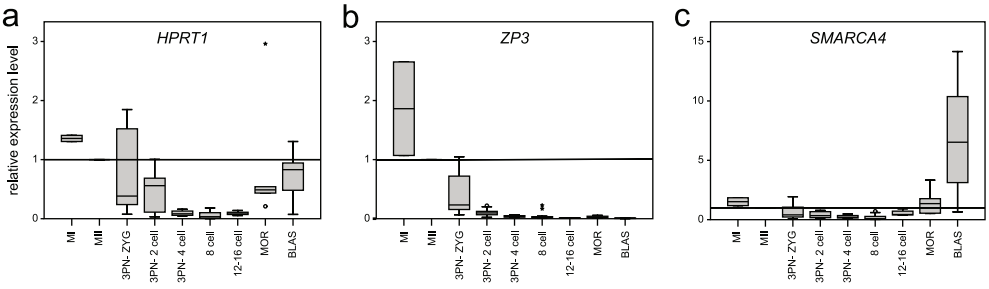
Supplementary tables and figures

Supplementary Table 4.1 – Average Ct-values after pre-amplification and RT-qPCR for indicated Taqman assays in single oocytes and preimplantation embryos at nine developmental stages: oocytes at MI (n=2) and MII (n=5), zygotes (3PN, n=13), 2 cell embryos (3PN- 2 cell, n=10), 4 cell embryos (3PN- 4 cell, n=4), 8 cell embryos (n=12), 12-16 cell embryos (n=6), morulas (MOR, n=5), and blastocysts (BLAS, n=10). Data are expressed as mean ±SD.

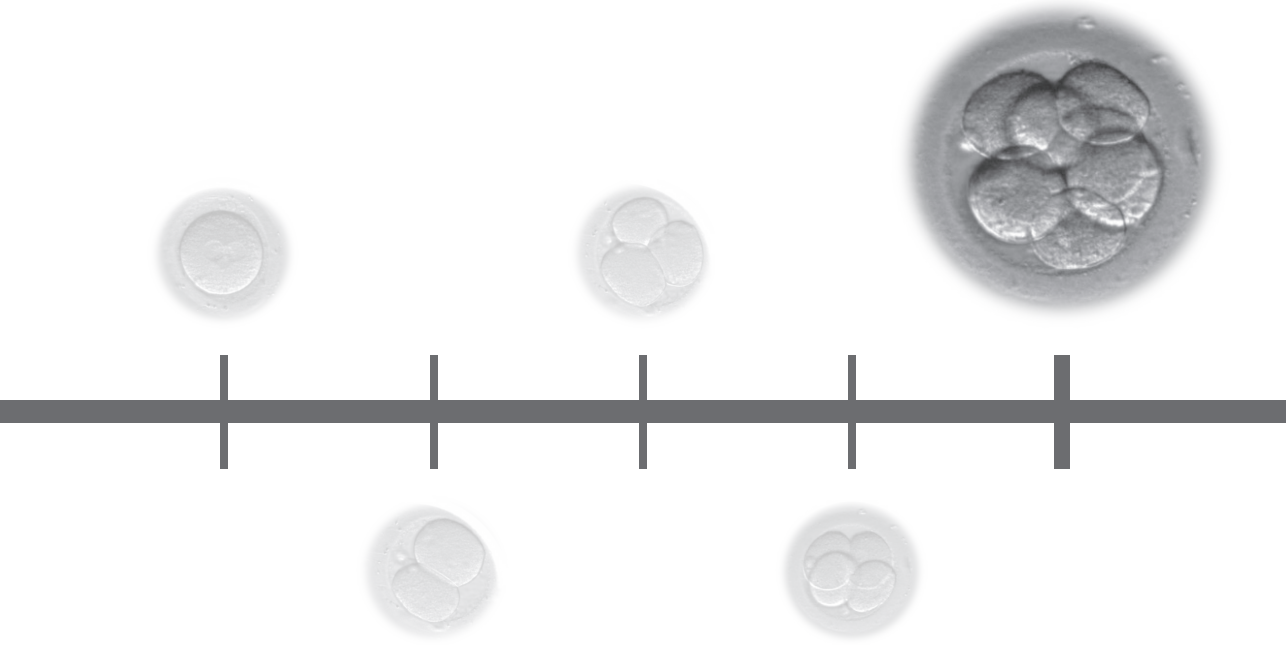
	MI	MII	3PN	3PN-2C	3PN-4C	8C	12-16C	MOR	BLAS
AURKB	26.67 ±0.41	26.87 ±1.18	27.95 ±1.67	28.22 ±1.09	29.09 ±1.41	29.53 ±1.44	27.06 ±0.54	25.85 ±1.36	25.75 ±1.51
AURKC	24.93 ±0.26	25.50 ±1.37	26.50 ±1.56	27.41 ±1.33	29.18 ±0.40	28.22 ±1.72	27.17 ±0.83	26.51 ±1.09	29.87 ±1.65
BIRC5	25.54 ±0.38	25.89 ±0.79	26.68 ±1.84	27.27 ±0.94	28.15 ±0.77	27.93 ±1.31	26.20 ±0.42	25.32 ±0.94	24.22 ±1.24
CDCA8	23.64 ±0.48	24.69 ±1.57	25.38 ±2.28	25.53 ±1.59	26.26 ±0.70	27.77 ±2.17	25.92 ±0.36	25.09 ±0.93	25.11 ±1.55
HPRT1	23.39 ±0.06	23.83 ±1.42	24.96 ±1.50	25.47 ±1.55	27.39 ±0.65	29.07 ±2.21	27.31 ±0.45	24.59 ±1.26	24.57 ±1.19
INCENP	28.55 ±0.69	28.06 ±0.41	29.00 ±1.54	29.47 ±0.92	29.70 ±0.87	29.93 ±1.13	27.36 ±0.41	26.62 ±0.84	24.94 ±1.19
SMARCA4	25.64 ±0.32	26.20 ±1.07	27.51 ±1.58	28.09 ±1.31	28.46 ±0.80	29.41 ±1.56	27.15 ±0.48	25.97 ±1.02	24.05 ±1.56
ZP3	18.88 ±0.66	19.63 ±0.99	21.42 ±1.27	23.17 ±1.01	24.42 ±0.58	25.01 ±1.55	26.04 ±0.28	24.97 ±0.98	27.43 ±1.46



Supplementary Figure 4.1 – Validation of a rabbit polyclonal antibody against Aurora C (ab38299, Abcam).
(a) Immunolocalization of AURKC and CREST on U2OS cells transfected with empty GFP vector, GFP-AURKA, GFP-AURKB and GFP-AURKC. GFP-tagged AURKA was localized on microtubules near the spindle poles, whereas GFP-AURKB and GFP-AURKC were detected on the inner centromere of prometaphase chromosomes. AURKC antibody did not recognize GFP, GFP-AURKA or GFP-AURKB, but recognized GFP-AURKC. **(b)** Western blot of U2OS cells transfected with empty GFP vector, GFP-AURKA, GFP-AURKB and GFP-AURKC. Cell lysates were immunoprobed with antibodies to GFP, AURKB and AURKC. We are unsure as to the identity of the extra bands detected by AURKC antibody.



Supplementary Figure 4.2 – Relative expression levels after RT-qPCR of **(a)** *HPRT1*, **(b)** *ZP3* and **(c)** *SMARCA4* in single oocytes and preimplantation embryos at nine developmental stages: oocytes at MI (n=2) and MII (n=5), zygotes (3PN, n=13), 2 cell embryos (3PN- 2 cell, n=10), 4 cell embryos (3PN- 4 cell, n=4), 8 cell embryos (n=12), 12-16 cell embryos (n=6), morulas (MOR, n=5), and blastocysts (BLAS, n=10). The mean expression level at the oocyte MII stage was taken as a reference to calculate the relative levels of the other stages. Note the scale of the y-axis differs.



Chapter 5

Histone modification dynamics and centromeric targeting of the chromosomal passenger complex in human preimplantation embryos

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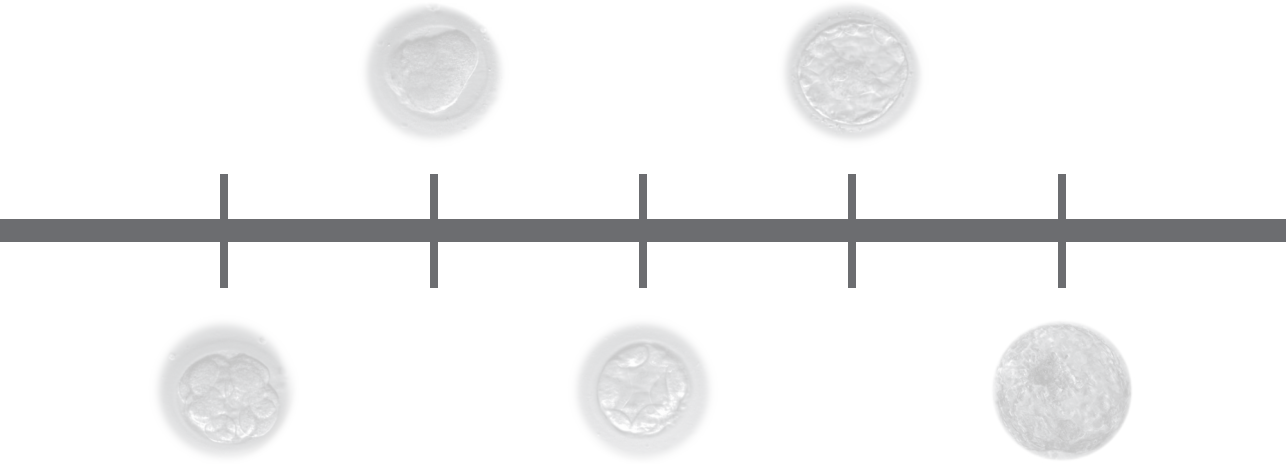
In preparation



Abstract

The great majority of human preimplantation embryos contain aneuploid cells. The bulk of such aneuploidies result from chromosome segregation errors occurring during the first mitotic division, but the molecular mechanisms are poorly understood. The chromosomal passenger complex (CPC) regulates chromosome segregation, and in order to do so it must be precisely localized at the inner centromere. In somatic cells, centromeric localization of the CPC depends on two histone modifications at the centromeres: phosphorylation of histone H3 at Thr3 (H3pT3) and H2A at Thr120 (H2ApT120) by Haspin and Bub1 kinases, respectively. We investigated the dynamics of these phosphorylations in human preimplantation embryos in the context of the known epigenetic asymmetry between the chromatin derived from sperm and oocyte for trimethylation of histone H3 at Lys 9 (H3K9me3).

We observed H2ApT120 enriched at centromeres and co-localization with the CPC in all stages of embryonic development investigated. However, H3pT3 was ubiquitously distributed on chromosomes of human zygotes and only became enriched at centromeres from the 2 cell stage onwards. Inhibition of Aurora kinase activity in zygotes resulted in decreased H3T3 phosphorylation and altered CPC localization, as described in somatic cells. However, maternal and paternal chromosomes were affected differently and CPC subunits co-localized with H3K9me3-positive regions.

Our results suggest that in human zygotes the mechanism regulating targeting of the CPC to the inner centromeres partially differs from that described in somatic cells. The differences found may compensate for the epigenetic asymmetry between parental chromosomes, assuring correct CPC localization to centromeres for all chromosomes, independent of their parental origin.

Introduction

Chromosome aneuploidies are frequently observed in human preimplantation IVF embryos (Mantzouratou and Delhanty, 2011; Voet *et al.*, 2011) and believed to be the leading cause of fetal wastage and low IVF success rates. Some of these aneuploidies are of meiotic origin, but we and others have shown that most of them occur post-meiotically, as a result of errors during the cleavage divisions (Baart *et al.*, 2006; Vanneste *et al.*, 2009; Santos *et al.*, 2010b). The proportion of aneuploid cells within an embryo subsequently declines towards the blastocyst stage (Ruangvutilert *et al.*, 2000; Bielanska *et al.*, 2002a; Santos *et al.*, 2010b), suggesting that chromosome segregation mechanisms are more error-prone during the cleavage divisions than in the blastocyst. The underlying molecular mechanisms contributing to these errors during the cleavage divisions of human preimplantation embryos remain largely unknown.

Mitotic errors are rare in adult somatic cells, as they are equipped with elaborate mechanisms that regulate and monitor chromosome segregation. The pericentric heterochromatin region of the chromosome has been shown to play a crucial role in chromosome segregation. This region folds between the centromeres and is characterized by the presence of DNA repeat sequences, together with a distinct epigenetic signature. It shows enrichment for DNA methylation, the histone variant H2A.Z and histone modifications such as trimethylation of lysine residue 9 on histone H3 (H3K9me3) and trimethylation of lysine residue 20 on histone H4 (H4K20me3; for review see Boyarchuk *et al.*, 2011). Targeted disruption of this signature leads to chromatid cohesion defects and chromosome segregation errors (Peters *et al.*, 2001; Rangasamy *et al.*, 2004). Moreover, a cancer-associated reduction of H3K9me3 levels at the pericentric heterochromatin was recently reported to be associated with chromosomal instability (Slee *et al.*, 2012). H3K9me3 is known to recruit the three isoforms of heterochromatin protein 1 (HP1α, β, and γ) (Bannister *et al.*, 2001) that are important for heterochromatin structure and function. In addition, pericentric heterochromatin is known to be important to limit incorporation of the histone H3 variant CENP-A to the centromeres (Lam *et al.*, 2006). CENP-A is the epigenetic mark that specifies the centromere and serves as the basis for kinetochore formation, creating the primary site for microtubule attachment (Black and Bassett, 2008; Verdaasdonk and Bloom, 2011). HP1 also provides a direct link between pericentric heterochromatin and the kinetochore, by recruiting one of the components of the kinetochore to the centromeric region, an interaction important for establishment of the inner centromere (Kiyomitsu *et al.*, 2010).

Accurate chromosome segregation is also dependent on kinetochore capture by the mitotic spindle and subsequent formation of bipolar attachment. This is achieved through a stochastic trial-and-error process, whereby active destabilization of erroneous attachments is necessary during prometaphase to provide a new opportunity to attach in a bi-oriented fashion. Aurora kinase B is a key player in this mechanism of attachment error-correction (Vader *et al.*, 2008). Aurora B belongs to a family of serine-threonine kinases that is conserved from yeast to humans. This kinase is a member of the Chromosomal Passenger Complex (CPC), together with the Inner Centromere Protein (INCENP), Borealin and Survivin (Carmena and Earnshaw, 2003). We previously showed that in human cleavage stage embryos the main kinase subunit of the CPC is an alternative subunit called Aurora kinase C (Avo Santos *et al.*, 2011). *In vitro*

experiments have shown that Aurora C shares Aurora B substrates such as CENP-A, histone H3 at Ser 10, INCENP, Survivin and Borealin (Slattery *et al.*, 2008). Aurora C is also capable to fully support mitotic progression in the absence of Aurora B in human somatic cells (Slattery *et al.*, 2009) and mouse embryo development (Fernandez-Miranda *et al.*, 2011). For Aurora B, and in analogy Aurora C, to be able to destabilize erroneous attachments, it must be targeted and anchored precisely at the inner centromere. Disturbance of CPC localization results in chromosome misalignment, cytokinesis failure and SAC override (Bassett *et al.*, 2010; Becker *et al.*, 2010).

A model for the mechanism by which the CPC is targeted to the inner centromere has recently been proposed (Kawashima *et al.*, 2010; Kelly *et al.*, 2010b; Tsukahara *et al.*, 2010; Wang *et al.*, 2010; Yamagishi *et al.*, 2010). According to this model, CPC targeting depends on two distinct histone modifications: phosphorylation of histone H3 at Thr 3 (H3pT3) by the kinase Haspin and phosphorylation of histone H2A at Thr 120 (H2ApT120) by Bub1 kinase (Figure 5.1a). In fission yeast, Haspin interacts with cohesion-associating protein Pds5 and the homologue of HP1, Swi6 (Yamagishi *et al.*, 2010). In human cells Haspin may localize to heterochromatin at cohesion-sites along the chromosome (Dai *et al.*, 2005), phosphorylating histone H3 and creating binding sites for Survivin (Kelly *et al.*, 2010b; Wang *et al.*, 2010). Parallel to this, Bub1, a mitotic kinase that localizes to the kinetochores, phosphorylates histone H2A on centromeric heterochromatin, creating a binding site for shugoshin 1 (Sgo1). Shugoshin acts as a centromeric adaptor that binds the CPC via Borealin and protects cohesins from premature degradation (Kawashima *et al.*, 2010; Tsukahara *et al.*, 2010).

The intersection of H3pT3 and H2ApT120 marks are suggested to define CPC localization to the inner centromeres (Yamagishi *et al.*, 2010) and a collaboration between the two pathways has been proposed to enhance enrichment at the inner centromere (Wang *et al.*, 2011). Bub1-H2ApT120-Sgo1-CPC pathway is believed to contribute to the centromeric concentration of H3pT3 by promoting CPC accumulation in the inner centromeres. Moreover, phosphorylation of Haspin by Aurora B promotes full phosphorylation of H3pT3, which in turn contributes to further CPC accumulation at the inner centromeres, creating a positive feedback loop between Aurora B and Haspin (Wang *et al.*, 2011) (Figure 5.1a).

Ectopically expressed Haspin has been observed along the chromosome arms in prophase, followed by centromeric enrichment in prometaphase through anaphase. Likewise, phosphorylation of H3T3 starts at late G2/ early prophase and by late prophase it spreads along the chromosome arms. However, at late prometaphase/metaphase it gets enriched at centromeres (Dai *et al.*, 2005; Yamagishi *et al.*, 2010). A recent publication by Qian *et al.* has shown that dephosphorylation of H3 at T3 is specifically regulated by the phosphatase activity of protein phosphatase PP1γ together with its regulatory subunit Repo-Man (Qian *et al.*, 2011). PP1γ/Repo-Man complex is therefore responsible for opposing the Haspin-mediated H3T3 phosphorylation on the chromosome arms, allowing enrichment of H3pT3 at centromeric regions (Qian *et al.*, 2011). This way, the collaboration of Bub1-H2ApT120-Sgo1-CPC and Haspin-H3pT3-CPC phosphorylation pathways, in combination with the counteracting dephosphorylation activity of PP1γ/Repo-Man, ensures a robust mechanism of CPC localization to the inner centromeres.

In summary, the interplay between histone modifications associated with pericentric heterochromatin and its recruitment of HP1, as well as mitosis specific phosphorylation marks, contributes to kinetochore formation and establishment of the inner centromeric region in somatic cells. In this context, an interesting feature of mammalian embryos is the epigenetic asymmetry between the paternal and maternal chromosomes after fertilization (Santos *et al.*, 2005; Puschendorf *et al.*, 2008). In spermatozoa, the DNA is associated with protamines instead of histone-containing nucleosomes, facilitating high-density packaging in the sperm head. After fertilization, dramatic changes occur on the paternal chromatin, as the protamines are replaced by maternally provided nucleosomes and functional chromatin domains, such as the pericentric heterochromatin, have to be established *de novo* (Albert and Peters, 2009; Probst and Almouzni, 2011). In mouse and human early embryos this results in different maternal and paternal chromatin states at the pericentric heterochromatin, which is exemplified by H3K9me3. While the maternal chromatin is enriched for this mark, the paternal chromatin is initially largely devoid of this modification (Puschendorf *et al.*, 2008; van der Heijden *et al.*, 2008). Considering the importance of H3K9me3 for accurate chromosome segregation (Peters *et al.*, 2001), the impact of the H3K9me3-parental asymmetry on regulation of chromosome segregation remains unknown.

Our previous observations on CPC localization in human preimplantation embryos suggested that the CPC was less constricted to the inner centromeric region on zygotic prometaphase chromosomes, when compared to the blastocyst stage (Avo Santos *et al.*, 2011). This lead us to question the mechanism regulating CPC targeting to the inner centromere in human zygotes, and if this is affected by the aforementioned epigenetic asymmetry. Therefore, we aimed to further characterize the distribution of the CPC in relation to phosphorylation dynamics of H2AT120 and H3T3 during mitosis in early human embryos from the zygote to the blastocyst stage, as well as regulatory proteins involved in the Bub1-H2ApT120-Sgo1-CPC and Haspin-H3pT3-CPC pathways.

Materials and Methods

Culture, collection and treatment of human preimplantation embryos

Triploid embryos (3PN) resulting from abnormal fertilization, i.e. oocytes fertilized by two spermatozoa simultaneously or where the second polar body failed to extrude, were used to study the first and second cleavage divisions (days 1 and 2 post-fertilization). Surplus fresh or cryopreserved embryos donated for research were used to study embryo developmental stages from day 3 to day 5 (Figure 5.1b). Tripronuclear zygotes and surplus embryos were donated with written informed consent by couples undergoing routine IVF at the Erasmus MC University Medical Center. The use of both types of surplus embryos was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO NL38053.000.11) and the local institutional ethics committee.

Embryo culture and assessment of embryo morphology were performed as described previously (Hohmann *et al.*, 2003). Embryos were cultured in G1 or G2 v5 PLUS medium (Vitrolife) where applicable, according to the manufacturers' instructions. Cryopreservation

was performed in straws using a slow freezing standard protocol and subsequent thawing was performed as described previously (Santos *et al.*, 2010b).

Time-lapse imaging and treatment of zygotes and embryos

Tripronuclear human zygotes were incubated in a time-lapse embryo monitoring system (EmbryoScope™, Unisense Fertilitech) at approximately 20h post-insemination (hpi) (Figure 5.1b). This allowed monitoring of nuclear envelope breakdown (NEB), which marks entry into prometaphase. In mouse embryos metaphase plate formation occurs about 30min after NEB and the first mitotic division is completed after 2h (Ciemerych *et al.*, 1999; Sikora-Polaczek *et al.*, 2006). In humans these timings have not been described yet. Our own time-lapse observations on human IVF embryos have shown the time interval between disappearance of pronuclei and first cleavage was 2.7±0.5h. We estimated that incubation of human 3PN zygotes for 2h after NEB in G1 medium with colcemid (1,5 µg/ml) would allow arrest at late prometaphase, whereas an incubation of only 30min after NEB would correspond to early prometaphase, as confirmed by the observed lower degree of chromosome condensation. For the experiments where the effect of Aurora kinase inhibition was investigated, 3PN zygotes were incubated for 2h in colcemid (1,5 µg/ml) in combination with ZM447439 (2 or 20µM). All embryos were then processed for chromosome spreads, unless stated otherwise. To investigate the effect of colcemid treatment on our results, some zygotes were fixed 30min or 2h after NEB in the absence of any inhibitor. To arrest zygotes at the metaphase stage, these were incubated in G1 medium with MG132 for 2h after NEB.

After thawing of cryopreserved embryos, day 3 (8 cell stage) embryos were first incubated for 1-2h in G2 medium and then incubated for 5-8h in G2 medium containing colcemid (1.5µg/ml, Invitrogen) for cell cycle arrest at prometaphase by destabilization of the mitotic spindle. Thawed day 4 (morula stage) embryos were cultured for 24h in G2 medium and embryos that developed into blastocysts were then incubated for 5-8h in G2 medium containing colcemid (1.5µg/ml) to induce prometaphase arrest.

Single embryo RT-qPCR

Quantification of mRNA levels was performed in individual single tripronuclear zygotes and blastocysts obtained after culture of day 3 or day 4 cryopreserved embryos in G2 medium, without addiction of any of the cell cycle inhibitors mentioned above. Zygotes and blastocysts were incubated in EmbryoMax® Acidic Tyrode's Solution (Millipore) for 1-2 min for removal of the zona pellucida and rinsed in G-MOPS Plus medium (Vitrolife) before transfer to the lysis buffer solution provided in the Taqman® PreAmp Cells-to-Ct Kit (Applied Biosystems). Lysis, preamplification and RT-qPCR were performed according to the manufacturer's protocol with minor adjustments, as described elsewhere (Avo Santos *et al.*, 2011). The following Taqman Gene Expression Assays were used: HPRT1 (Assay ID: Hs99999909_m1; amplicon size 100bp); BUB1 (Hs00177821_m1; amplicon size 61bp); GSG2, Haspin (Hs01072471_s1; amplicon size 89bp); CDCA2, Repo-Man (Hs00299250_m1; amplicon size 93bp), PPP1CC, PP1γ (Hs01566021_m1; amplicon size 83bp).

Results were analyzed using Sequence Detection Software version 1.2.3 (Applied

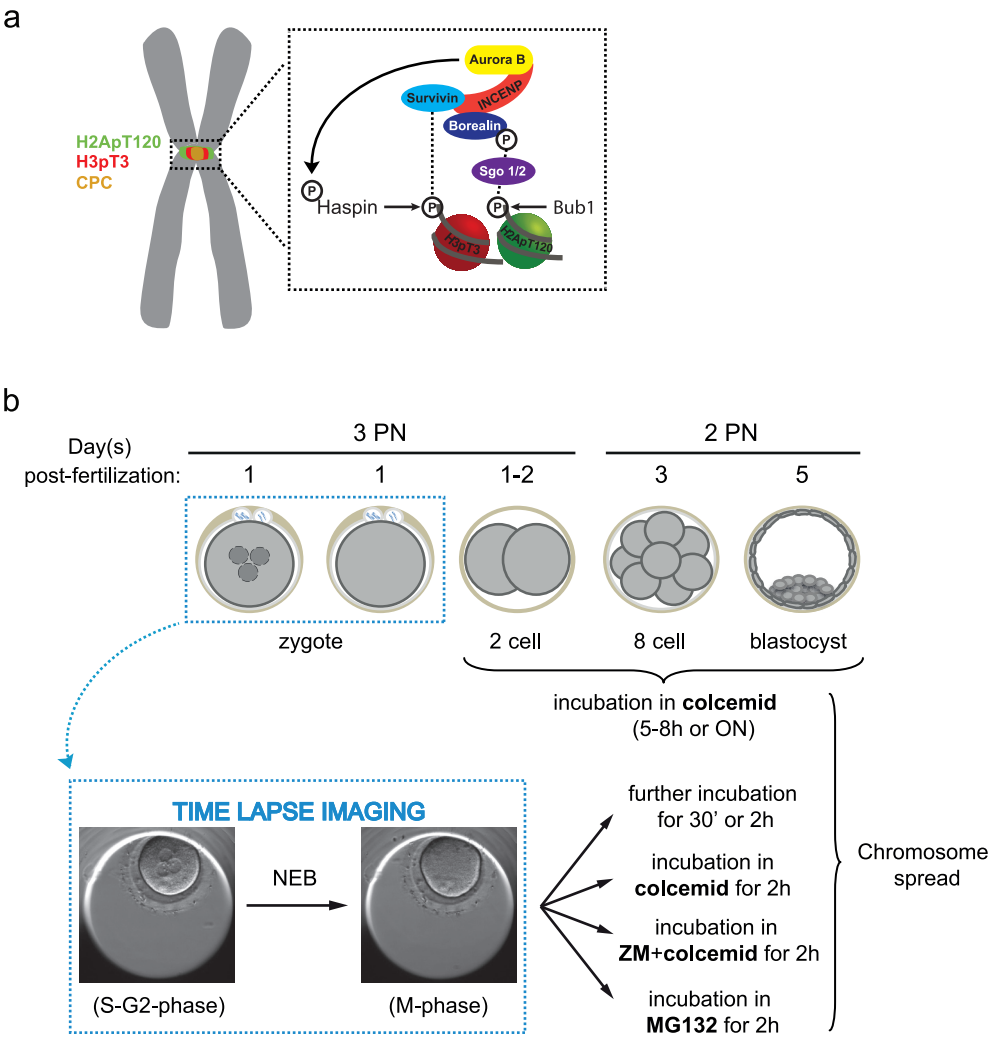


Figure 5.1 – Schematic representation of the CPC recruitment model and experimental setup.
(a) Depiction of the model that explains recruitment of the CPC to the inner centromere. Phosphorylation of H3T3 by Haspin acts to bring the CPC to chromosomes. The Survivin subunit of the CPC binds specifically to H3pT3. Furthermore, Bub1 phosphorylation of histone H2AT120 recruits shugoshin. Shugoshin acts as a centromeric adaptor that binds the CPC via Borealin. Intersection of H3pT3 and H2ApT120 marks defines CPC localization to the inner centromeres.
(b) Fresh tripronucleate (3PN) embryos were used to study the zygote and 2 cell stages, whereas surplus fresh or cryopreserved (2PN) embryos were used to study the 8 cell and blastocyst stages. NEB was monitored by time-lapse imaging to allow immediate incubation of prometaphase zygotes in medium containing colcemid, ZM+colcemid or MG132. Embryos at the 2 cell stage and later were incubated in medium containing colcemid in a standard incubator. All embryos were fixed with 1% PFA to obtain chromosome spreads on glass slides, unless stated otherwise.

Biosystems) and expressed as cycle threshold (Ct) values. Gene expression levels were normalized over HPRT1 gene expression, according to the 2-ΔCT method (Livak and Schmittgen, 2001). Differences in BUB1, GSG2, CDC2A and PPP1CC expression between zygotes and blastocysts were analyzed using the Mann-Whitney test performed with GraphPad Prism software. A p-value ≤ 0.05 was considered statistically significant.

Antibodies for immunostaining

The following antibodies were used in this study: mouse monoclonal against INCENP (1:1000; Upstate) and SgoL1 (1:100, Abnova); rabbit polyclonal against H2ApT120 (1:2500; Active motif), H3pT3 (1:1,000, Upstate), Repo-Man (CDCA2) (1:200, Abcam), H3pS10 (1:100, Cell Signaling), H3K9me3 (1:200, Abcam) and HP1α (1:100, Bethyl Labs); sheep polyclonal against Bub1 (1:100, a kind gift from G. Kops, The Netherlands); and human centromere autoantigen (CREST, HCT-0100, 1:1000, Cortex Biochem). Primary antibodies were detected by labeling with the appropriate secondary antibodies conjugated with Alexa fluor 488, 594 or 633 (1:200, Molecular Probes).

Chromosome spreads and fixation of whole embryos

Before submitted to chromosome spreads or whole mount fixation, all embryos were subjected to removal of the zona pellucida with acidic Tyrode's solution. Chromosome spreads of human embryos were prepared as described previously (Avo Santos *et al.*, 2011) and used for all the stainings shown, with the following exceptions. Zygotes at the PN stage stained for HP1α, H2ApT120 and H3pT3 were fixed whole mount in 4% PFA as described (Avo Santos *et al.*, 2011) and zygotes and 8 cell embryos stained for Repo-Man were fixed for 15 min at room temperature (RT) using pre-extraction medium (PEM) containing 100 mM PIPES [pH 7.2], 5 mM $MgCl_2$, 2.5 mM EGTA, 2% formaldehyde and 0.1% Triton X-100. Chromosome spread preparations were stored at -20oC, while PEM-fixed embryos were stored in PBS/ 0.05% Na-azide for a maximum of 3 months until used for immunostaining.

Injection of H3pT3

Injection of an antibody against H3pT3 (Upstate) diluted 1:10 in PVP medium (ICSI™ sperm handling solution, Vitrolife) in live 3PN zygotes was performed right after NEB on a heated stage of a Nikon IX-70 microscope, equipped with micromanipulation tools. Approximately 10 pl of antibody/PVP-mixture was injected in the cytoplasm of the zygotes. After injection embryos were incubated for 15 min in G-MOPS medium at 37°C (Vitrolife) before fixation in PEM buffer.

Immunofluorescence and antibody stripping

For immunofluorescence stainings, chromosome spreads and whole embryos were rinsed in PBS-T (PBS, 0.01% v/v Tween-20) and blocked with blocking solution (PBS-T, 2% w/v bovine serum albumin, 5% normal goat serum) for 30 min and incubated with primary antibodies at 4°C overnight. After washing with PBS-T, chromosome spreads or whole embryos were incubated with the appropriate secondary antibodies for 1h, washed with PBS-T and mounted with vectashield mounting solution (Vector Laboratories) containing Hoechst 34580 (5 µg/ml, Invitrogen) for DNA counterstaining. Images were obtained using a Zeiss Axio Imager M2 confocal laser scanning microscope, equipped with four diode lasers (405, 488, 555, 639), an Axiocam camera, and Zen 2009 software (all Carl Zeiss). Images were processed with Image J and Adobe Photoshop CS3 software.

For immunofluorescence antibody stripping of chromosome spreads, a western blot

stripping buffer (Pierce Biotechnology) was used as described (van de Werken *et al.*, in preparation). After incubation of chromosome spreads with the stripping buffer for 3 min at RT, slides were washed twice in PBS-T before undergoing a second round of immunofluorescence.

Quantification of immunofluorescence and statistical analysis

Quantification of immunofluoresence in chromosome spreads of human embryos from the zygote to the blastocyst stage was carried out using ImageJ (NIH) and using images obtained at identical illumination settings. For assessment of the localization of INCENP relative to CREST, a vertical line was drawn following the chromosome arms and perpendicular to the axis connecting the two sister centromeres. The length of the INCENP and CREST signals was measured in pixels along this line. The ratio of INCENP/CREST signal length was calculated for all chromosomes that were spread in a way that allowed accurate immunofluoresence quantification.

For assessment of the localization and distribution of H2ApT120 and H3pT3 relative to INCENP, the average pixel intensity of H2ApT120, H3pT3 and INCENP staining was determined along the whole length of representative chromosomes and values plotted in graphs using Excel software (Microsoft).

For quantification of the mean immunofluorescence intensity in maternal *versus* paternal chromosomes of zygotes treated with ZM+colcemid, the area of H3K9me3-positive (maternal) chromosomes was selected out and the mean H3pT3 fluorescence intensity was calculated. The mean H3pT3 fluorescence intensity on paternal chromosomes was calculated by subtracting the mean H3pT3 intensity on maternal chromosomes to the total mean H3pT3 intensity (all chromosomes).

Statistical analyses were carried out using GraphPad Prism software. One-way ANOVA followed by Bonferroni's multiple comparison test allowed comparison of INCENP/CREST signal length at the zygote, 8 cell and blastocyst stages. A Mann-Whitney test was used to test differences in the mean H3pT3 fluorescence intensity between maternal and paternal chromosomes after ZM+colcemid treatment. A p-value ≤ 0.05 was considered statistically significant.

Results

Localization of the CPC during the first mitosis compared to subsequent cell divisions

Our previous work studying CPC constitution in human preimplantation embryos indicated a difference in localization of the CPC in zygotes compared to blastocysts during prometaphase, with the CPC being less constricted to the inner centromere at the zygote stage (Avo Santos *et al.*, 2011). We aimed to explore and quantify this difference further. During the previous study, we confirmed co-localization of INCENP with Aurora B and C in the human zygote and blastocyst, respectively. We therefore used a mouse monoclonal anti-INCENP antibody to evaluate CPC localization relative to the inner centromeric region as delimited by the centromeres. This was done by immunolocalization of INCENP relative to localization of a human autoantibody against the centromere (CREST) on chromosome spreads of human

embryos arrested at prometaphase with colcemid (Figure 5.2a), determining the spreading of the INCENP signal relative to CREST.

In zygotic prometaphases, INCENP was not confined to the center of paired CREST dots, i.e. the inner centromere, but was also present in the adjacent areas corresponding to the pericentric regions. At the blastocyst stage, however, INCENP was mostly restricted to the inner centromere. These differences in INCENP localization were translated into a significantly higher mean ratio between the length of INCENP and CREST signals in the zygote compared to the blastocyst stage (Figure 5.2b). The mean INCENP/CREST length ratio in 8 cell embryos, although lower than in zygotes, was also significantly higher than in blastocysts. Together these results suggest that CPC localization at prometaphase changes from the zygote to the blastocyst stage. As the embryo developed, CPC enrichment became increasingly confined to the inner centromeres. This prompted us to investigate the mechanisms that determine CPC localization at the inner centromere in human zygotes and test the model proposed by Yamagishi and co-workers (Yamagishi *et al.*, 2010), suggesting that targeting of the CPC to the inner centromeres is determined by the intersection of H3pT3 mark with H2ApT120.

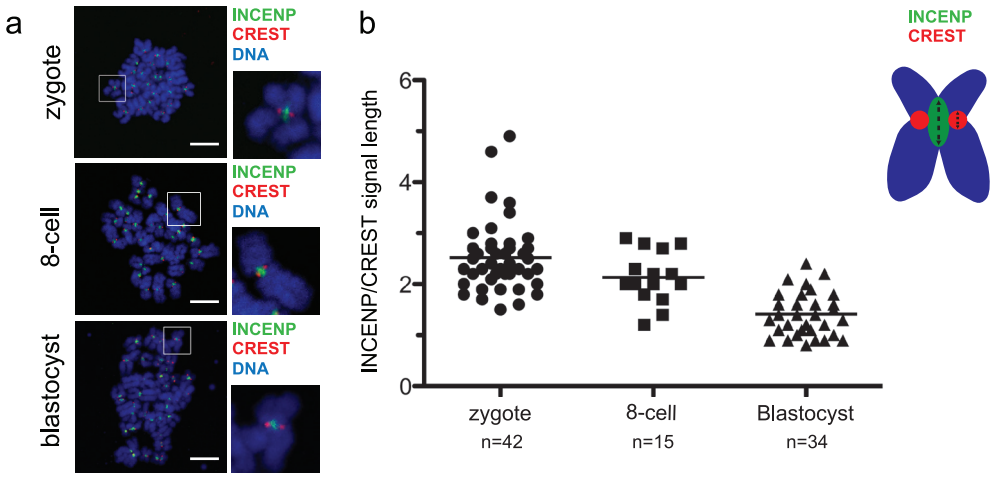


Figure 5.2 – Localization of the CPC relative to the centromere changes throughout preimplantation development of human embryos. (a) Chromosome spreads of prometaphase-arrested embryos showing immunolocalization of INCENP relative to the centromere (CREST antigen). DNA was counterstained with Hoechst. Square boxes are blow-ups of each corresponding smaller box. Scale bars represent 10 µm. **(b)** Localization of INCENP relative to centromere (CREST) represented as the ratio of pixel length of these two marks. The graphic represents the values measured in three zygotes, two 8 cell embryos and three blastocysts (n= number of chromosomes analyzed). Horizontal bars represent mean values. Significant differences between groups of chromosomes analyzed ***$p < 0.05$.

Functional Bub1-H2ApT120-Sgo1-CPC pathway in human preimplantation embryos

Immunolocalization of H2ApT120 in zygotes, 8 cell and blastocyst stage human embryos revealed that, similar to somatic cells, phosphorylation of H2AT120 was strongly enriched at centromeric regions, along the interkinetochore axis (Figure 5.3a). Quantification of H2ApT120 staining intensity along the whole length of representative chromosomes showed

that distribution of H2ApT120 was similar to INCENP distribution (Figure 5.3a). To exclude an effect of colcemid or timing of fixation on these results, H2ApT120 dynamics were further investigated in zygotes at the late G2-phase (zygotes at 20hpi with pronuclei still visible), early prometaphase (30 min after NEB), late prometaphase (2h after NEB) and at metaphase (2h after NEB in the presence of MG132). Immunolocalization data showed the presence of H2AT120 phosphorylation from late G2-phase until metaphase, with centromeric enrichment from early prometaphase to metaphase (Supplementary Figure 5.1). Gene expression analysis of BUB1, the kinase responsible for phosphorylation of H2AT120, showed similar levels of relative mRNA expression in zygotes and blastocysts (Figure 5.3c). Consistent with these results and to what has been published before (Kawashima *et al.*, 2010), we detected Bub1 at the kinetochores and Sgo1 at inner centromeres in human zygotes (Figure 5.3b). Together, these data suggest a functional Bub1-H2ApT120-Sgo1-CPC pathway in human zygotes and later stages investigated, with Bub1 phosphorylating histone H2AT120, which recruits Sgo1 to the centromeres (Kawashima *et al.*, 2010; Yamagishi *et al.*, 2010).

Human zygotes fail to show centromeric enrichment of H3pT3 on (pro)metaphase chromosomes

In HeLa cells, phosphorylation of H3T3 becomes enriched at the inner centromere during prometaphase (Dai *et al.*, 2005; Yamagishi *et al.*, 2010). However, in arrested zygotes at late prometaphase, immunolocalization analysis of H3pT3 revealed ubiquitous phosphorylation on the whole length of the chromosome, without enrichment at the centromere (Figure 5.4a). To exclude an effect of colcemid or timing of fixation on this finding, H3pT3 dynamics were further investigated in zygotes at late G2-phase, early prometaphase (30 min after NEB), late prometaphase (2h after NEB) and at metaphase (2h after NEB in the presence of MG132) (Supplementary Figure 5.2). The absence of H3pT3 staining in late G2-phase zygotes indicates that H3T3 phosphorylation is probably initiated at the onset of mitosis. Subsequently, ubiquitous phosphorylation of H3T3 was consistently observed on chromosomes at prometaphase and metaphase, without centromeric enrichment. However, at the 2 cell, 8 cell and blastocyst stage H3T3 phosphorylation was observed to be mainly restricted to the inner centromeric regions at late prometaphase (Figure 5.4a). Meanwhile, simultaneous detection of INCENP revealed centromeric enrichment for this CPC subunit in all stages investigated, including in the zygote, when H3pT3 fails to enrich at centromeric regions.

The joint action of PP1γ together with the PP1 interacting protein Repo-Man was shown in U2OS cells to dephosphorylate H3T3 during prometaphase, ensuring its centromeric enrichment (Qian *et al.*, 2011; Vagnarelli *et al.*, 2011). The persistent phosphorylation of H3T3 on the chromosome arms at prometaphase and metaphase in human zygotes is reminiscent of the phenotype of Haspin overexpression or Repo-Man knockdown (Qian *et al.*, 2011). As the balance between PP1γ/Repo-Man and Haspin was demonstrated to be important for correct localization of the CPC, we analyzed their expression in human zygotes and blastocysts. Contrary to expectations, quantification of Haspin mRNA (*GSG2*) levels revealed low relative expression of Haspin in human zygotes, whereas Repo-Man mRNA (*CDCA2*) levels between zygotes and blastocysts were not significantly different (Figure 5.4c). However, this does not

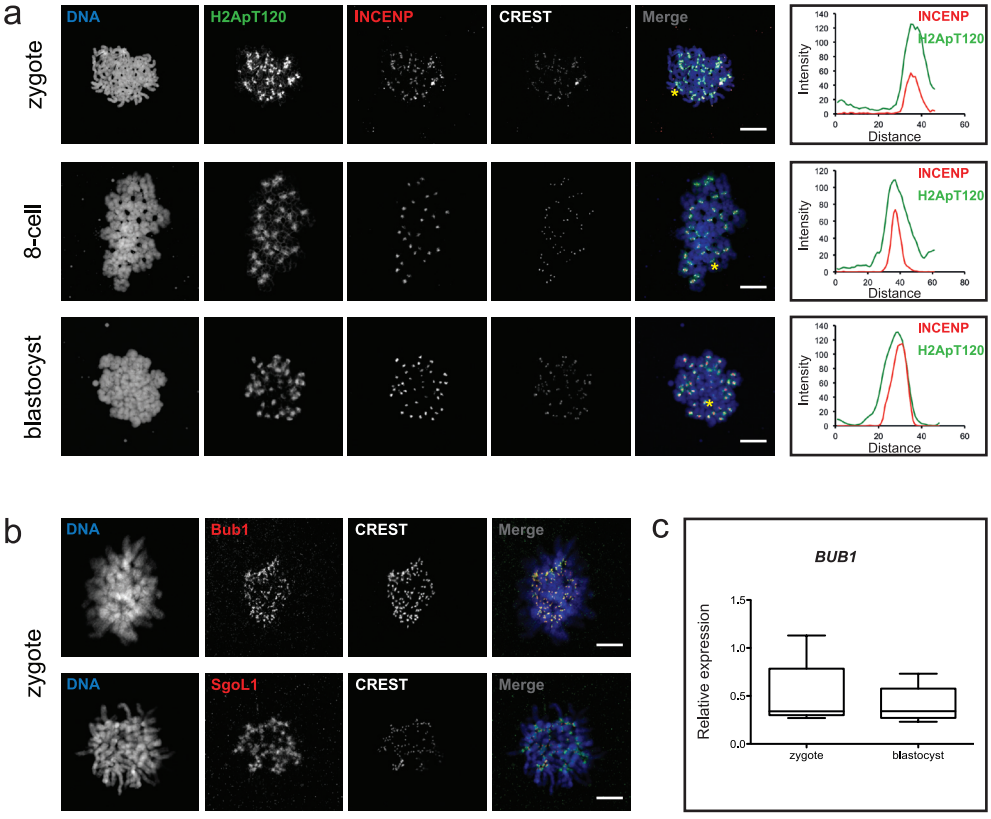


Figure 5.3 – A functional Bub1-H2ApT120-Sgo1-CPC pathway in human preimplantation embryos. (a) Immunolocalization of H2ApT120 and INCENP relative to centromeres (CREST) in prometaphase-arrested human embryos at the zygote (n=11), 8 cell (n=7) and blastocyst (n=6) stage. For each stage of development, a graph was plotted representing the distribution of H2ApT120 relative to INCENP along a representative chromosome (*). Intensity was measured in pixels and distance as arbitrary units. **(b)** Immunolocalization of Bub1 (n=4) and SgoL1 (n=4) relative to centromeres (CREST) in prometaphase-arrested human embryos at the zygote stage. **(c)** Relative expression of *BUB1* over *HPRT1* in human zygotes (n=5) and blastocysts (n=5). DNA was counterstained with Hoechst. All scale bars represent 10 µm.

exclude the possibility of Haspin protein accumulation in the cytoplasm of human oocytes, as evidenced by the presence of H3pT3 on the chromatin. Immunodetection of Repo-Man in human zygotes compared to embryos of later developmental stages, shows that Repo-Man can be detected in similar levels on the chromosome arms of prometaphase-arrested zygotes, as well as at later embryonic stages, when H3pT3 is absent from the chromosome arms (Figure 5.4b). Thus, the observed phosphorylation of H3T3 on the chromosome arms of zygotes and lack of enrichment at the centromeres is not likely to be explained by lack of Repo-Man. Levels of PP1γ mRNA (*PPP1CC*) were significantly lower in the zygote compared to the blastocyst stage (Figure 5.4c). As this phosphatase is also implicated in removal of the CPC from the chromatin at anaphase, a process that is apparently normal in the zygote (Avo Santos *et al.*, 2011), the reason for the persistent H3T3 phosphorylation remains unknown.

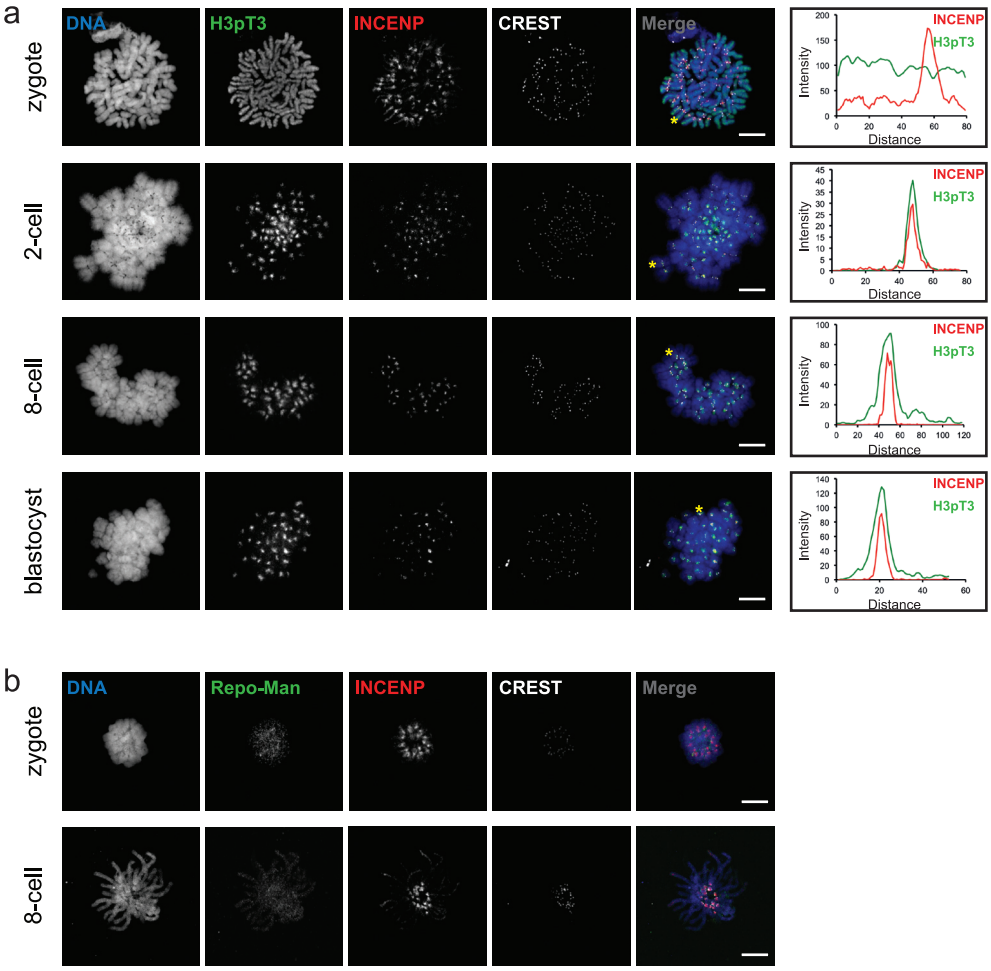


Figure 5.4 – Haspin persistent phosphorylation of H3T3 on chromosome arms and lack of centromeric enrichment in human zygotes at prometaphase does not affect centromeric accumulation of INCENP. (a) Immunolocalization of H3pT3 and INCENP relative to centromeres (CREST) in prometaphase-arrested human embryos at the zygote (n=12), 2 cell (n=5), 8 cell (n=6) and blastocyst (n=5) stage. For each stage of development, a graph was plotted representing the distribution of H3pT3 relative to INCENP along a representative chromosome (*). Intensity was measured in pixels and distance as arbitrary units. **(b)** Immunolocalization of Repo-Man and INCENP relative to centromeres (CREST) in prometaphase-arrested human embryos at the zygote (n=2) and 8 cell (n=3) stage. **(c)** Relative expression of *GSG2* (n=5), *CDCA2* (n=4) and *PPP1CC* (n=4) over *HPRT1* in human zygotes and blastocysts. *p<0.05 compared to the zygote stage. DNA was counterstained with Hoechst. All scale bars represent 10 µm.

However, our findings indicate that centromeric enrichment of H3pT3 is not necessary for inner centromeric localization of INCENP in the zygote.

Feedback loop between Haspin and Aurora B/C

In somatic cells it has been shown that Haspin phosphorylates H3T3, providing a chromatin-binding site for the CPC at the centromeres, and in return Aurora B phosphorylates Haspin to promote further phosphorylation of H3T3 (Wang *et al.*, 2011). The same authors showed that this positive feedback loop is disturbed when cells are treated with the Aurora kinase inhibitor ZM447439 (ZM), which leads to reduced phosphorylation of endogenous Haspin and concomitant reduction of H3pT3. We aimed at investigating whether a similar feedback loop is also active in human zygotes, where Aurora C is likely to be involved in the establishment of the ubiquitous H3pT3 on (pro)metaphase chromosomes.

As ZM treatment inhibits all Aurora kinase activity (Ditchfield *et al.*, 2003), we are not able to distinguish Aurora kinase B from Aurora kinase C activity. To test the effect of ZM treatment on Aurora kinase activity, we first determined the levels of phosphorylation of histone H3 on Ser10 (H3pS10) on zygotes at late prometaphase. Using a concentration that is commonly used in somatic cells [2 μM ZM; (Ditchfield *et al.*, 2003)], H3pS10 signal was high and well defined (Figure 5.5a), similar to what was observed in non-treated embryos (Avo Santos *et al.*, 2011). Using a concentration of 20 μM ZM, which had been previously shown to efficiently abolish H3pS10 in mouse zygotes (Teperek-Tkacz *et al.*, 2010), we observed complete loss of H3pS10 in human zygotic prometaphases. Both concentrations of ZM in combination with colcemid were subsequently used to evaluate the effect of inhibition of Aurora B and C kinase activity on the levels of H3T3 phosphorylation and CPC (INCENP) localization (Figure 5.5b). At 2 μM ZM, reduced H3pT3 levels were observed on most chromosomes. Centromeric enrichment of INCENP did not seem disturbed, although levels appeared lower than in controls (compare Figures 5.4b and 5.5b). At 20 μM ZM, we detected not only a clear reduction of H3T3 phosphorylation on human zygotes, but also a disruption of INCENP enrichment at the inner centromere. INCENP was spread along the chromosome arms or detected at low levels in some chromosomes.

These data suggest that, similar to what has been described in somatic cells, in human zygotes the positive feedback loop between Aurora B/C and Haspin is functional. It also shows that centromeric enrichment of the CPC is, at least in part dependent on H3T3 phosphorylation, although centromeric enrichment of H3pT3 seems dispensable. To investigate the role of H3pT3 in CPC recruitment more directly, we injected the antibody to pT3 in zygotes at the prometaphase stage in the presence of colcemid. Injection of an H3pT3 specific antibody was previously shown to block the docking site recognized by Survivin, resulting in a failure of the CPC to enrich at the inner centromere (Wang *et al.*, 2010). We detected the injected antibody along the chromosome arms as expected and observed INCENP to localize along the arms with some enrichment at the inner centromere (Figure 5.5c). Although these results may indicate that blocking the recruiting mark for the CPC disturbs centromeric enrichment, we are not able to prove that the antibody we used is indeed blocking Survivin recruitment. Therefore the results may simply reflect timing of fixation at an earlier stage of prometaphase, with INCENP still present on the arms.

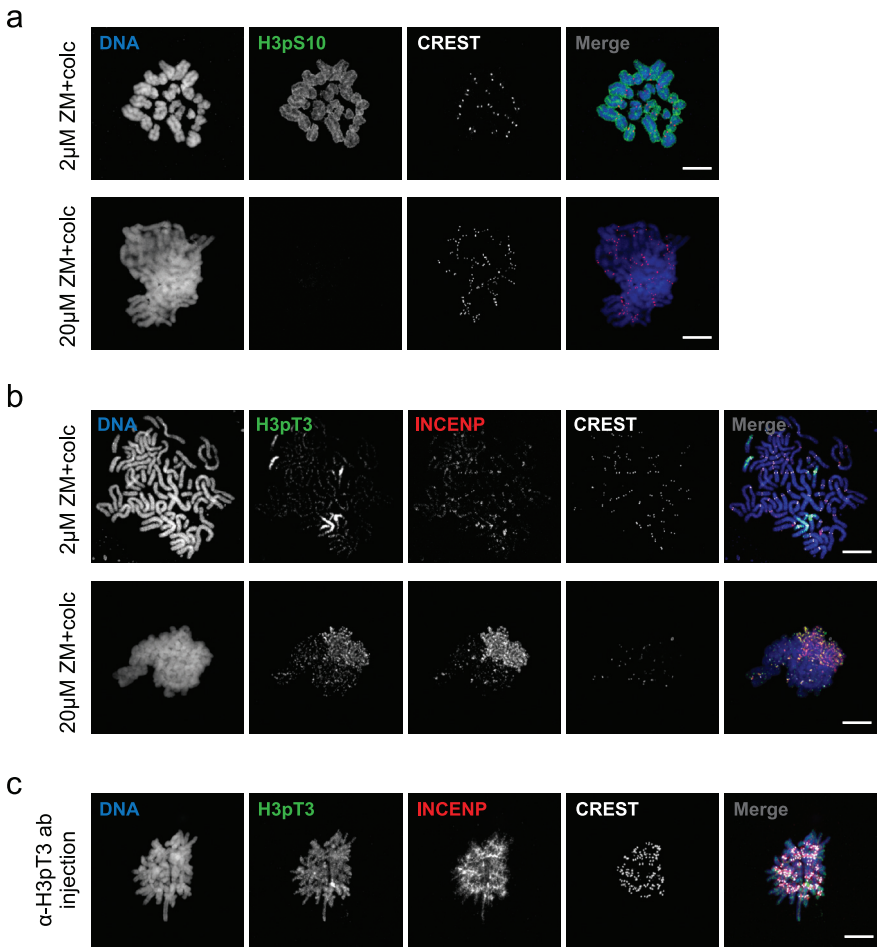


Figure 5.5 – **Phosphorylation of H3T3 and localization of CPC are dependent on Aurora B/C kinase activity.** After NEB, human zygotes were treated with 2 and 20 μM ZM447439 and colcemid (ZM+colc) for 2h followed by detection of **(a)** H3pS10 and CREST; **(b)** H3pT3, INCENP and CREST. **(c)** Injection of anti-H3pT3 antibody in zygotes immediately after NEB, followed by fixation and immunolocalization of the injected antibody together with INCENP and CREST.

Different susceptibility of maternal and paternal chromatin to Aurora B/C inhibition

To our surprise, when treating human zygotes with the high concentration of Aurora kinase inhibitor ZM, we observed a clear difference in the levels of reduction of H3T3 phosphorylation on different chromosomes. Consistent with this, INCENP localization was also affected differently, as in some chromosomes it was observed to locate along the chromosome arms, whereas in other chromosomes INCENP signal was reduced to such low levels that it became difficult to evaluate its localization. We hypothesized that the different reaction to the ZM treatment on chromosomes within one zygote was related to the parental origin of the chromosomes. Therefore, after detection of H3pT3 and INCENP and image analysis, we performed antibody stripping followed by a new round of immunostaining to detect trimethylation of H3K9. This allowed us to distinguish maternal (enriched for H3K9me3) from paternal (low levels of H3K9me3) chromosomes (van der Heijden *et al.*, 2009). Our results clearly showed

that the effect of Aurora C inhibition by ZM was more pronounced on the paternal compared to the maternal chromatin (Figure 5.6a), which resulted in a significantly lower mean H3pT3 fluorescence intensity on the paternal chromatin (Figure 5.6a'). On the paternal chromosomes, foci of accumulated INCENP co-localized to H3K9me3-positive regions (arrows, Figure 5.6a-b). It has previously been shown that inhibition of Aurora B, and the concomitant reduction in H3pS10, results in retention of HP1 proteins on mitotic chromosomes (Fischle *et al.*, 2005; Hirota *et al.*, 2005). It has also recently been shown that both INCENP and Sgo1 bind HP1α during mitosis (Kang *et al.*, 2011). As in the absence of H3pS10, HP1 is not evacuated from the chromatin and remains bound to H3K9me3, we asked whether the observed asymmetric distribution of INCENP in ZM-treated zygotes was due to its association to H3K9me3-anchored HP1α. Immunodetection of HP1α in ZM-treated zygotes showed an asymmetric distribution on the parental chromosomes (Figure 5.6d), following the pattern of H3K9me3 localization, as observed at the G2-phase in non-treated zygotes (Figure 5.6e). In zygotes arrested in late prometaphase with colcemid, HP1α was symmetrically distributed at centromeric regions, co-localizing with INCENP (Figure 5.6f).

Recruitment of Sgo1 to centromeres requires phosphorylation of histone H2A on T120 by Bub1 (Yamagishi *et al.*, 2010). ZM treatment was previously shown to abolish phosphorylation of H2AT120 at the centromeres in U2OS cells (van der Waal *et al.*, 2012). However, in 7 out of 10 zygotes, ZM treatment did not abolish centromeric H2ApT120, as evidenced by immunofluorescent analysis as a "first round" (not shown) and after stripping (Figure 5.6c). Still, Sgo1 did not show centromeric enrichment after ZM treatment, but also did not co-localize with HP1α (Figure 5.6d). The consistent absence of centromeric Sgo1 and INCENP in ZM treated zygotes may indicate that H2ApT120 alone may not be sufficient for their recruitment after Aurora B/C inhibition.

Discussion

Our study demonstrates that during the first mitotic division in human zygotes the CPC is less constricted to the inner centromere than at later developmental stages. When investigating the pathways involved in targeting the CPC to the inner centromere in human preimplantaion embryos, H2AT120 phosphorylation dynamics were found to be similar to what has been described for somatic cells. Likewise, we found localization of Bub1 and Sgo1 to be normal, suggesting a functional Bub1-H2ApT120-Sgo1-CPC pathway. Conversely, immunolocalization of H3pT3 in human zygotes revealed that, contrary to what has been shown in somatic cells, there is no centromeric enrichment of this mark in the zygote. We persistently observed strong staining on chromosome arms as prometaphase progressed with and without spindle poisons and even at the metaphase stage. Centromeric enrichment of INCENP, however, was already observed at early prometaphase. In contrast, from the 2 cell stage onwards, H3T3 phosphorylation was observed to be enriched at the centromeres, following the pattern of distribution of INCENP, as described in somatic cells. Inhibition of Aurora B/C kinase activity resulted in decreased H3pT3 phosphorylation and localization of INCENP to the chromosomal arms, instead of accumulation at the inner centromere. Furthermore, maternal and paternal

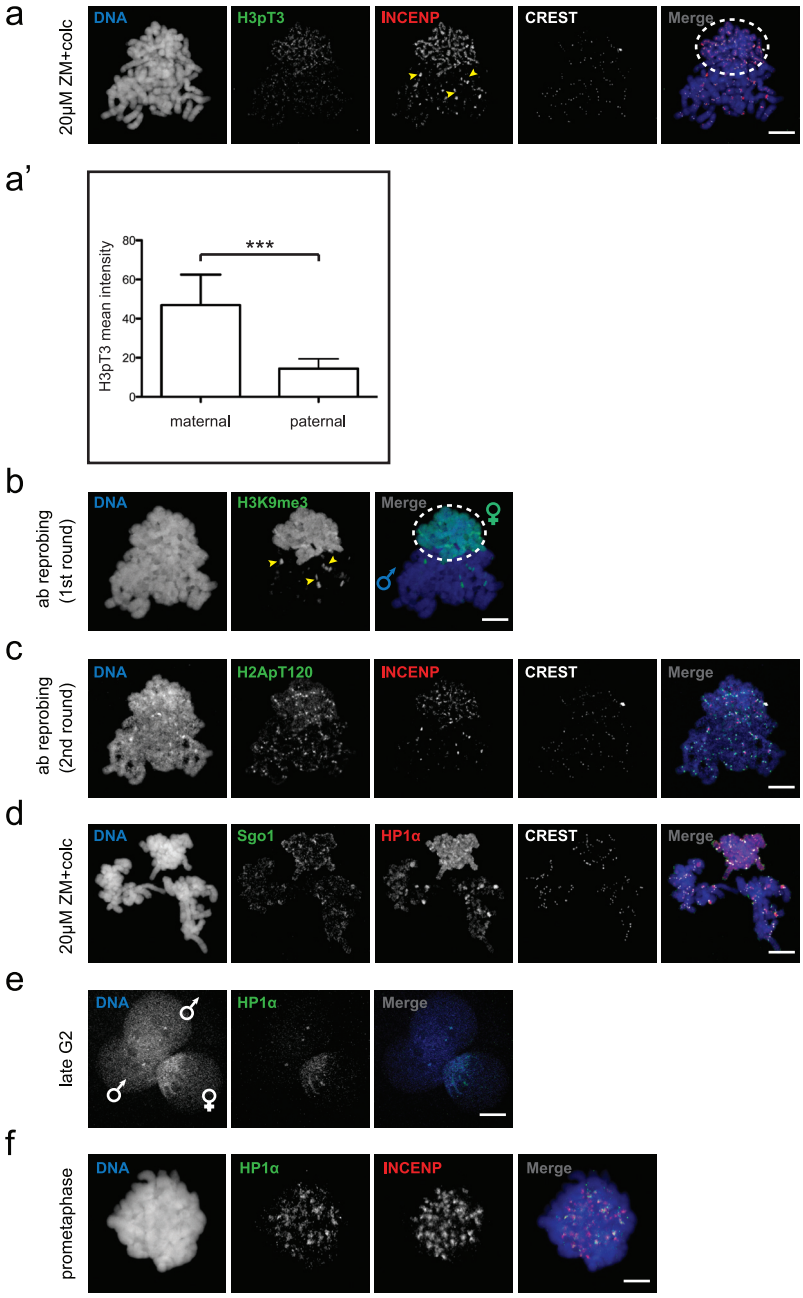


Figure 5.6 – ZM treatment reveals parental asymmetry in H3pT3 and INCENP localization. After NEB, human zygotes were treated with 20 μM ZM447439 and colcemid (ZM+colc) for 2h followed by detection of **(a)** H3pT3, INCENP and CREST. **(b)** After a first round of antibody stripping, the same zygotes were stained with H3K9me3 to allow identification of maternal (H3K9me3-enriched) and paternal chromatin (low levels of H3K9me3). **(c)** A second round of antibody stripping allowed staining of the same zygotes with H2ApT120, INCENP and CREST. **(d)** Zygotes treated with 20 μM ZM+colc for 2h followed by detection of Sgo1, HP1α and CREST. **(e)** Zygote at late G2 stained for HP1α. **(f)** Zygote at late prometaphase stained for HP1α, INCENP and CREST. DNA was counterstained with Hoechst. Scale bars represent 10 μm. **(a')** Quantification of H3pT3 mean fluorescence intensity on maternal and paternal chromatin (n=4). Mean ± SD values are shown. Significant differences between maternal and paternal chromosomes ***p<0.05.

chromosomes showed different susceptibility to Aurora kinase inhibitions, suggesting a role for the epigenetic asymmetry between sperm- and oocyte-derived chromatin on the mechanisms regulating CPC localization.

For Aurora B, and in analogy Aurora C, to be able to correct erroneous attachments of chromosomes to the mitotic spindle, the CPC must be targeted and anchored precisely at the inner centromeres. Induced displacement of CPC from centromeres onto chromosome arms of HeLa cells by actinomycin D induces chromosome misalignment, cytokinesis failure and spindle assembly checkpoint override (Becker *et al.*, 2010). But even more subtle alterations of CPC localization compromise its error-correction function. Altered localization of Aurora B to the neocentromere of a mutated chromosome 4 of a patient-derived cell line resulted in diminished capacity to correct misattachments and align at the metaphase plate (Bassett *et al.*, 2010). The error-correction function of the CPC is therefore severely compromised when the complex is not precisely targeted to the inner centromere.

We investigated CPC localization to the inner centromere in zygotes compared to 8 cell and blastocyst stage in an attempt to understand whether the high rates of aneuploidy reported in cleavage stage embryos (Ruangvutilert *et al.*, 2000; Bielanska *et al.*, 2002a; Santos *et al.*, 2010b) could be related to a less accurate localization of the CPC in early embryos compared to blastocyst. We found that in zygotes CPC localization is not solely confined to the inner centromere but also slightly present at the pericentric regions, confirming our previous observations (Avo Santos *et al.*, 2011). As human embryos develop to the 8 cell and blastocyst stages, INCENP localization becomes increasingly more restricted to the inner centromere.

We further investigated the mechanisms regulating CPC localization to the inner centromere in zygotes compared to later embryonic stages. Similar to somatic cells, in human preimplantation embryos phosphorylation of H2AT120 is enriched at the centromeres in all stages of embryo development studied. This, together with identification of Bub1 and Sgo1 suggests a conserved Bub1-H2ApT120-Sgo1-CPC, where Bub1 phosphorylates histone H2AT120, which in turn recruits Sgo1 to centromeres. Interaction of Sgo1 with the CPC subunit Borealin then allows centromeric localization of the CPC at prometaphase (Kawashima *et al.*, 2010; Yamagishi *et al.*, 2010). Strikingly different from somatic cells, phosphorylation of H3T3 was persistently ubiquitously distributed on (pro)metaphase chromosomes in human zygotes. At this stage of development centromeric enrichment of H3pT3 seems unnecessary for centromeric CPC localization, which may depend solely on phosphorylation of H2AT120 by Bub1 and/or other so far unknown mechanisms. How H3T3 phosphorylation is generalized to whole chromosome arms in zygotes remains unexplained, as we were able to detect PP1γ/Repo-Man in zygotes.

In somatic cells, the accumulation of CPC at centromeres is further promoted by a positive feedback loop between Aurora B and Haspin (Wang *et al.*, 2011). We therefore tested whether the Haspin-Aurora B/C feedback loop is active during prometaphase of human zygotes. Our experiments using ZM for inhibition of Aurora kinase activity resulted in residual phosphorylation of H3T3 and disturbed CPC localization, suggesting that in zygotes, similarly to somatic cells, Aurora B/C kinase activity is necessary for full Haspin-mediated H3T3 phosphorylation and contributes to centromeric CPC localization. Moreover, we observed that

the effect of ZM treatment on H3pT3 phosphorylation levels and CPC localization differed between the maternal and paternal chromosomes, in relation to their parental epigenetic asymmetry. Maternal H3K9me3-rich chromosomes were able to attract higher levels of residual H3pT3 and INCENP on the chromosome arms, whereas on paternal chromatin, both H3pT3 and INCENP co-localized with the few H3K9me3-positive regions present.

During late G2 to prophase in human zygotes H3K9me3, and consequently HP1, have an asymmetrical distribution resulting from the epigenetic asymmetry of parental chromosomes after fertilization (van der Heijden *et al.*, 2009; van de Werken *et al.*, 2012). In somatic cells, phosphorylation of H3S10 by Aurora B in prophase displaces HP1 from H3K9me3 heterochromatin sites (Fischle *et al.*, 2005; Hirota *et al.*, 2005). In prometaphase, HP1α associates with centromeres in a non-H3K9me3 binding-dependent manner through binding to INCENP and Sgo1 (Kang *et al.*, 2011). Consistent with this, in human zygotes arrested at prometaphase we observed HP1α co-localized with INCENP at the inner centromere on both maternal and paternal chromatin. After Aurora B/C inhibition and in the absence of H3S10 phosphorylation, HP1α still localizes to H3K9me3 sites on the chromosomes. Under high ZM conditions, there is also striking distribution of H3pT3 and INCENP, as they co-localize with H3K9me3 and HP1α. However, in zygotes treated with low concentrations of ZM, INCENP still localizes (symmetrically) to centromeres in all chromosomes. These results suggest that phosphorylation of H3S10, possibly together with the ubiquitous phosphorylation of H3T3, may be important to “cloak” the epigenetic asymmetry between the parental chromosomes, ensuring an equal distribution of the CPC to all chromosomes. In the absence of H3S10 phosphorylation, CPC localization to the chromatin may occur by direct binding of INCENP to H3K9me3-bound HP1α. Furthermore, our results also suggest Haspin localization to heterochromatic regions via HP1α.

Hence, we propose that in human zygotes, the Haspin-H3pT3-CPC pathway for targeting of the CPC to the inner centromere is altered to compensate for the inherited epigenetic asymmetry. Aurora C kinase activity may have a major role in this, promoting ubiquitous phosphorylation of H3T3. Combined with this, the Bub1-H2ApT120-Sgo1-CPC pathway may be more important for centromeric enrichment and localization of the CPC in the zygote. Although it is not clear how the two pathways interact in order to define CPC centromeric localization in human zygotes, it seems that the Bub1-H2ApT120-Sgo1-CPC pathway on its own is not sufficient to maintain centromeric CPC localization, as disturbance of the Aurora B/C-Haspin feedback loop resulted in loss of CPC and Sgo1 centromeric localization, but not of H2ApT120.

Together these findings suggest that the mechanisms regulating CPC function, localization and activity during the first cell division in human embryos are distinct from those described in somatic cell divisions. In somatic cells, overexpression of Haspin results in persistent H3pT3 on the chromosome arms and concomitant dislocation of the CPC to the arms (Qian *et al.*, 2011). This may indicate that centromeric enrichment of the CPC in zygotes depends solely on phosphorylation of H2AT120 by Bub1 and/or other mechanisms yet to be identified and may offer an explanation for our observation that CPC localization was less confined to the inner centromere in zygotes. If centromeric targeting of the CPC in zygotes relies on only one

recruitment pathway, this process may be more susceptible to disturbances. As even subtle disturbances in CPC localization seem to predispose a chromosome to misalignment (Bassett *et al.*, 2010), this may result in an increased rate of missegregation. However, at present it remains unclear to what extent the differences we observed affect the capacity of CPC to correct erroneous attachments. In future studies it will be interesting to assess if the error correcting ability of the CPC is different in the zygote and if differences exist on maternal and paternal chromosomes.

Acknowledgments

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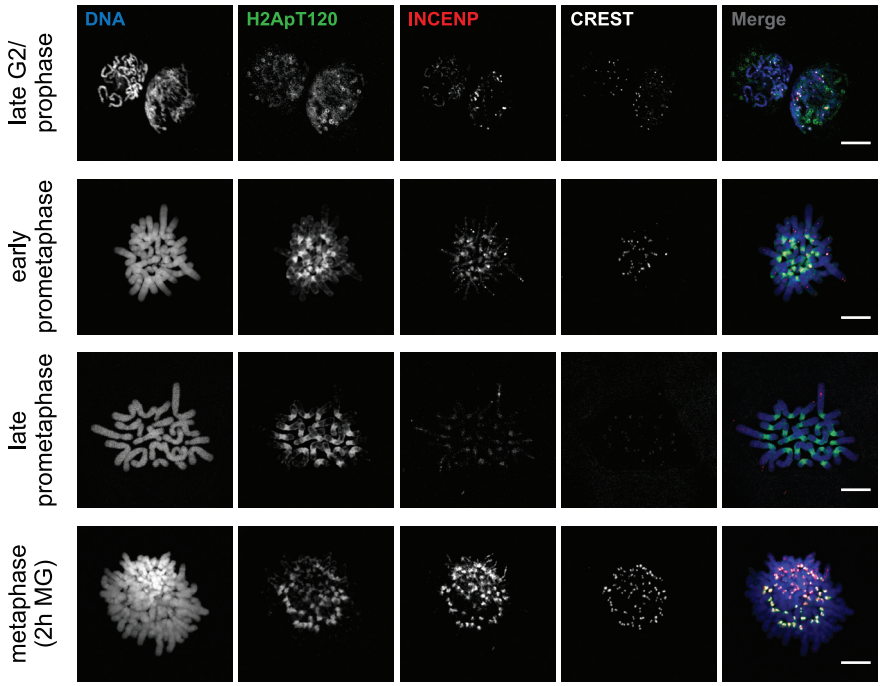
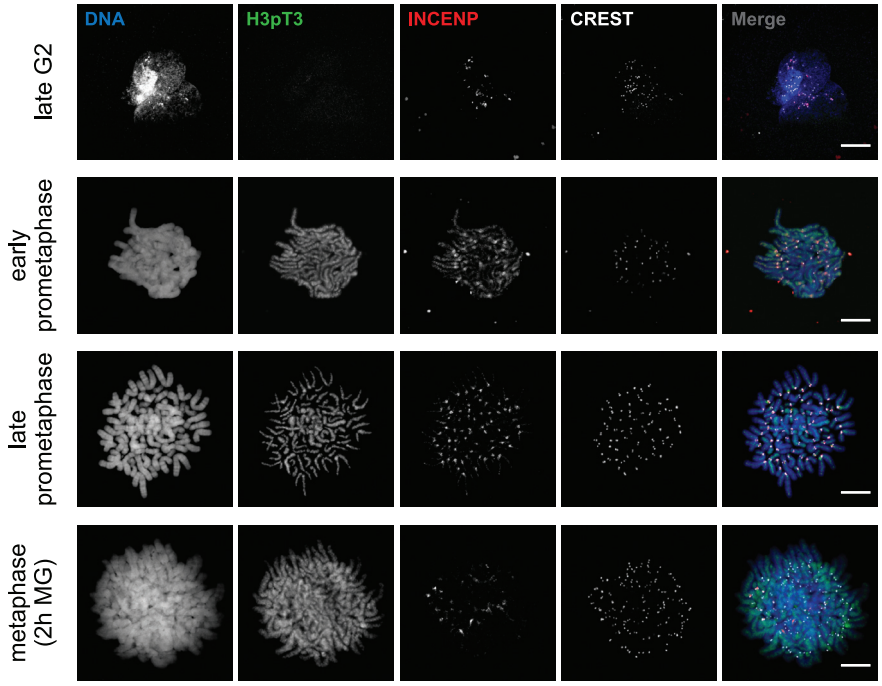
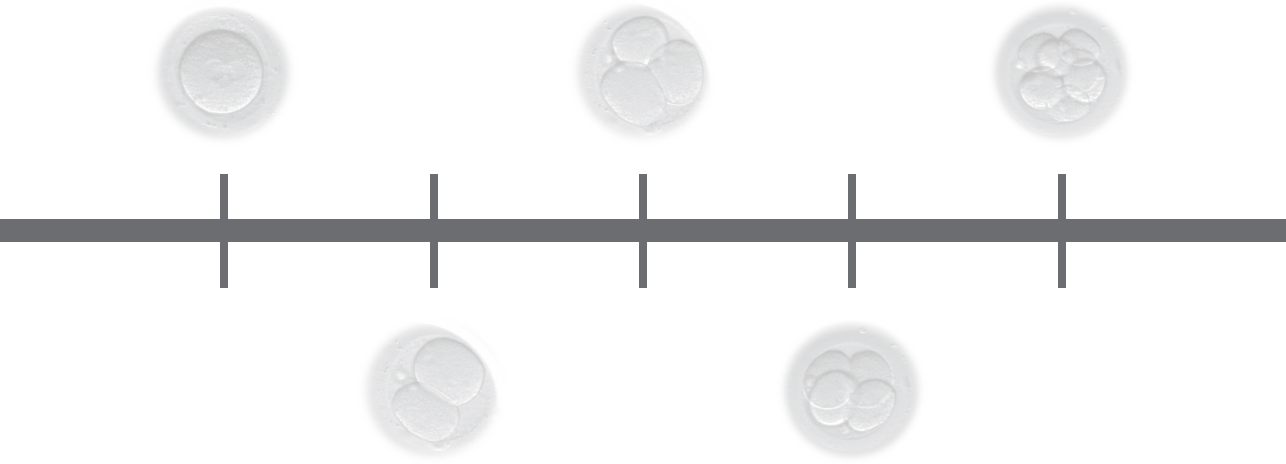
Supplementary figures



Supplementary Figure 5.1 – Phosphorylation of H2AT120 at different stages of the first mitosis in human embryos. Immunolocalization of H2ApT120 and INCENP relative to centromeres (CREST) in human zygotes at late G2/prophase, early prometaphase, late prometaphase and metaphase stage. Embryos at early and late prometaphase zygotes were fixed 30 min and 2h after NEB, respectively, in the absence of cell cycle inhibitors. Embryos at metaphase were treated with MG132 for 2h after NEB. DNA was counterstained with Hoechst. Scale bars represent 10 µm.



Supplementary Figure 5.2 – **Phosphorylation of H3pT3 at different stages of the first mitosis in human embryos.** Immunolocalization of H3pT3 and INCENP relative to centromeres (CREST) in human zygotes at late G2, early prometaphase, late prometaphase and metaphase stage. Embryos at early and late prometaphase zygotes were fixed 30 min and 2h after NEB, respectively, in the absence of cell cycle inhibitors. Embryos at metaphase were treated with MG132 for 2h after NEB. DNA was counterstained with H Hoechst. Scale bars represent 10 µm.



Chapter 6

The first embryonic cleavage division in humans: molecular mechanisms influencing chromosome segregation

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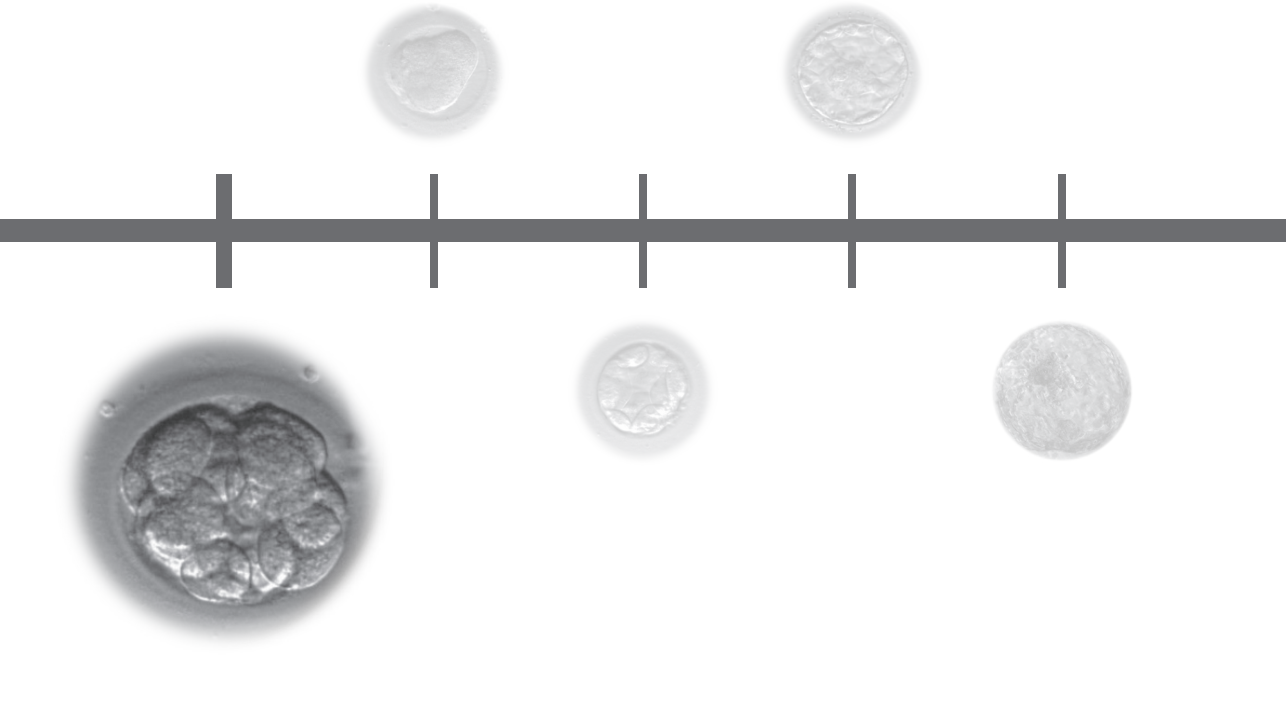
In preparation



Abstract

Aneuploidy and chromosomal mosaicism are commonly identified in human preimplantation *in vitro* fertilized embryos, and believed to contribute to the relatively low IVF success rates. Most of these chromosomal imbalances originate from defective chromosome segregation during the first mitotic divisions. In adult somatic cells, chromosome segregation errors are rare, as cells are equipped with elaborate checkpoint mechanisms that prevent errors.

The first mitotic division of human embryos is clearly distinct from somatic cell divisions as it is accompanied by cytoplasmic volume reduction and it relies on the reserves of mRNA and proteins present in the oocyte. However, a number of other notable differences exist, which so far may have been disregarded and could help understanding the peculiar increased incidence of chromosome segregation errors in human preimplantation embryos. Here we review the current literature with the aim of cataloguing mechanisms that take place exclusively during the first embryonic division and how they differ from those described in somatic mitosis and mouse embryonic divisions. One of the most striking differences is the pronounced parental epigenetic asymmetry present in human zygotes. We conclude that the unique features of the first cleavage division allow us to set it apart from somatic mitotic cell division. We furthermore hypothesize an important paternal contribution to the incidence of segregation errors. Investigating this hypothesis will open up new research strategies to elucidate the molecular origin of embryo aneuploidy.

Contents

1. Introduction

Chromosomal abnormalities are the leading cause of fetal wastage and congenital defects, being identified in 50-60% of first trimester spontaneous abortions (reviewed in Lebedev, 2011). The introduction of fluorescence *in situ* hybridization (FISH) for preimplantation genetic diagnosis (PGD) after *in vitro* fertilization (IVF) allowed an insight on the chromosomal constitution of human embryos as early as 3 days after fertilization, when embryos have about 8 cells. PGD-FISH studies reported over the past two decades show that on average 60% of IVF human embryos have at least one aneuploid cell (Mantzouratou and Delhanty, 2011). More recently, comparative genomic hybridization (CGH) and array CGH analysis for PGD confirmed the widespread incidence of chromosomal abnormalities, revealing that in fact, when the copy number of all chromosomes is analyzed, aneuploid cells can be identified in 80% of all preimplantation embryos (Voullaire *et al.*, 2000; Wells and Delhanty, 2000). Although some of these chromosomal abnormalities can be traced back to a meiotic origin, the majority have originated during the first mitotic divisions of early preimplantation development (Marquez *et al.*, 2000; Bielanska *et al.*, 2002a; Coonen *et al.*, 2004; Daphnis *et al.*, 2005; Baart *et al.*, 2006; Santos *et al.*, 2010b; van Echten-Arends *et al.*, 2011). This results in chromosomally mosaic embryos, i.e., embryos whose cells have different chromosomal constitution (Delhanty *et al.*, 1993). Embryonic mosaicism is estimated to affect 73% of all human preimplantation embryos (reviewed by van Echten-Arends *et al.*, 2011). Interestingly, in a study using an array-based method allowing genome-wide screening of the chromosome copy number in single embryonic cells from morula stage embryos, the reported high frequency of chromosome instability was comparable to observations made on aneuploid human cancers (Vanneste *et al.*, 2009; Voet *et al.*, 2011). The intriguing phenomenon of chromosomal mosaicism is not exclusive of human embryos. It has also been described in bovine, equine, porcine and non human primate embryos (Viuff *et al.*, 2000; Rambags *et al.*, 2005; Dupont *et al.*, 2010) both in *in vitro* and *in vivo* produced embryos (Viuff *et al.*, 2000; Zijlstra *et al.*, 2008). From this it is clear that chromosome segregation in cells of the preimplantation embryo of these species is more error prone than in dividing adult somatic cells. In contrast, mouse preimplantation embryos are apparently devoid of chromosomal mosaicism, as they show very low rates of aneuploidy (1-2%) (Tateno *et al.*, 2011).

According to the classic definition, the first embryonic cleavage division is a mitotic division, as opposed to preceding meiotic divisions. During this first mitotic division, sister chromatids of a diploid set of chromosomes are separated to two daughter cells. However, intriguing differences exist between a zygote and a normal mitotic cell. This review aims to systematically analyze mechanisms underlying the events leading to chromosome segregation during the zygotic cleavage division and compare these to what is known to occur during somatic mitosis and mouse embryonic divisions. In doing so, we will identify possible causes for the observed high rate of chromosome segregation errors and open up new research strategies. It will also become clear that the first embryonic cleavage division shows unique features that set it apart from both meiosis and mitosis.

2. Fertilization and the transition from maternal to embryonic control

Mitosis produces two daughter cells with the same number of chromosomes as in the mother cell. Meiosis however, is a specialized type of cell division where two sequential cell divisions take place after a single round of DNA duplication, generating gametes with a haploid set of chromosomes. During the first division (meiosis I) two homologous chromosomes segregate from each other, and in the second division (meiosis II) sister chromatids of each chromosome segregate to opposite poles.

In most vertebrates, mature oocytes are arrested at metaphase of the second meiotic division (MII). Upon fertilization by a sperm cell, the oocyte completes the second meiotic division resulting in formation of the second polar body and the haploid maternal pronucleus. At the same time, the highly condensed chromatin of the sperm nucleus decondenses, resulting in the formation of the haploid paternal pronucleus. The parental pronuclei are maintained physically separate in the ooplasm during the subsequent G1-, S- and G2-phase. Upon entry in the first embryonic mitosis, maternal and paternal chromatin are condensed into chromosomes that align at a common metaphase plate (Ciemerych *et al.*, 1999) (Figure 1.2, chapter 1).

To facilitate the transition from maternal to embryonic control, during oogenesis mammalian oocytes store an abundance of mRNAs, proteins and macromolecular structures in the ooplasm. These oocyte-stored molecules play an active regulatory role in promoting the first cell cycles following fertilization, when embryos are transcriptionally inactive (Matzuk *et al.*, 2002; Bettegowda and Smith, 2007). During transcription quiescence, post-transcriptional and post-translational mechanisms enable activation of the embryonic genome, the subsequent cleavage stages of embryogenesis and the initial establishment of embryonic cell lineages. Embryonic genome activation (EGA) occurs in a stepwise manner, with some genes being transcribed well in advance of the major genome activation event. In the mouse, the maternal to embryonic transition occurs at the 2 cell stage (Moore, 1975; Bensaude *et al.*, 1983). In humans, EGA was believed to occur at the 4- to 8 cell stage (Braude *et al.*, 1988), but recent reports suggest that it starts as early as at the 2 cell stage for a select number of genes followed by a major wave of transcription between the 6- and the 8 cell stage (Vassena *et al.*, 2011). In somatic cells, transcription of most of the genes important for faithful chromosome segregation are tightly cell cycle regulated. It is unknown how early embryos compensate for this.

3. Epigenetic signature of centromeric and pericentric heterochromatin in somatic cells

In somatic cells, DNA is wrapped around nucleosomes, which consist of a histone H3-H4 tetramer and two histone H2A-H2B dimers, together forming the chromatin. The N-terminal tails of these core histones can undergo a range of post-translational modifications, including acetylation, methylation, phosphorylation and ubiquitination (Jenuwein and Allis, 2001; Fischle *et al.*, 2003). Such post-translational "marks", together with variants of the canonical histones and chromatin-associated proteins, determine chromatin structure and thereby functional

chromatin domains. Heterochromatin domains, as opposed to euchromatin, remain condensed and transcriptionally silent throughout the cell cycle.

In the context of chromosome segregation, the constitutive heterochromatin domains at the centromeric and pericentric regions are of particular importance. In humans these regions consist of DNA sequences of α-satellite repeats for centromeric, and satellite II and III repeats for pericentromeric heterochromatin. However, centromeric and pericentromeric regions are not purely specified by DNA sequences, but epigenetic mechanisms play a dominant role. The centromere is determined by incorporation of a variant of histone H3.1, named CENP-A (Black and Bassett, 2008), and forms the basis for attachment of the chromosome to the spindle, as described below (section 7).

Pericentromeric heterochromatin can be found at the regions flanking the centromeres and has a specific epigenetic signature. Trimethylation of lysine 9 on histone H3 (H3K9me3) is the classic example of a repressive post-translation modification involved in the establishment of pericentric heterochromatin. This repressive mark is established by histone methyltransferases Suv39h1 and 2 (Peters *et al.*, 2001). H3K9me3 is bound by the three isoforms of heterochromatin protein 1 (HP1α, β, γ), which in turn recruit the histone H4 lysine 20 (H4K20) histone methyltransferases and DNA methyltransferases that all together establish a transcriptionally repressed chromatin state (reviewed in Probst and Almouzni, 2011). Similarly to H3K9me3, trimethylation of histone 64 on histone H3 (H3K64me3) has also been shown to accumulate at pericentric heterochromatin regions in a Suv39h pathway-dependent manner (Daujat *et al.*, 2009). Maintenance of these epigenetic marks is required for proper centromere function and chromosome segregation in mice, where lack in Suv39h methyltransferases results in loss of H3K9me3 and HP1 at pericentric regions and consequent chromosome missegregation (Peters *et al.*, 2001). Furthermore, cancer associated alteraration of H3K9me3 distribution at the pericentric regions is believed to be involved in chromosomal instability (Slee *et al.*, 2012).

Another epigenetic mark essential for formation of pericentric heterochromatin is DNA methylation of cytosines at CpG dinucleotides (5-methylcytosine; 5mC). Global genome hypomethylation is observed in a variety of tumors, where chromosomal instability is considered a driving force for tumorogenesis (Costello and Plass, 2001). This, together with the reported increased chromosome instability observed in DNMT-deficient human cell lines, suggests that proper DNA methylation at pericentromeric regions is necessary to ensure correct chromosome segregation (Karpf and Matsui, 2005).

The pericentric regions are in addition characterized by the presence of two histone variants, H2A.Z and H3.3. Depletion of histone H2A.Z results in chromosome segregation defects (Greaves *et al.*, 2007). Histone 3.3 is incorporated into the chromatin by the protein ATRX. Mutations or knockdown of ATRX expression results in various problems, including premature separation of sister chromatids and chromosome missegregation (Ritchie *et al.*, 2008).

Taken together, centric and pericentric heterochromatin have a distinct epigenetic signature and disturbances in this signature are clearly associated with an increased incidence of chromosome segregation errors, although the underlying molecular mechanisms are still

the subject of much research.

4. Different epigenetic signatures on paternal and maternal chromatin in zygotes and early embryos

4.1 Histone modifications establishing pericentric heterochromatin

A crucial epigenetic process after fertilization is the re-establishment of the paternal chromatin structure in order to form a functional embryonic genome. At the time of fertilization, the maternal genome is marked by histone modifications inherited from the oocyte, whereas the paternal genome is packaged with protamines and thus initially largely devoid of such modifications.

In mouse oocytes, the majority of somatic epigenetic heterochromatin marks are retained on the meiotic chromosomes and transferred to the zygote: pericentric chromatin is enriched in H3K9me3, H3K64me3 and HP1β, but lacks HP1α (Meglicki *et al.*, 2008; Puschendorf *et al.*, 2008; Daujat *et al.*, 2009). In sperm however, most heterochromatin marks are lost, as histones are replaced by protamines during spermiogenesis. After fertilization, dramatic changes occur in the paternal chromatin organization, as the protamines are replaced by maternally provided nucleosomes. To compensate for the lack of the typical pericentric hetrochromatin modifications found in somatic cells (and maternal chromatin), paternal pericentric heterochromatin regions are enriched in maternally provided Polycomb Repressive Complex 1 (PRC1), an alternative repressive chromatin factor that transiently re-establishes parental pericentric heterochromatin (Puschendorf *et al.*, 2008). Paternal pericentric heterochromatin of mouse zygotes is also enriched in Polycomb Repressive Complex 2 (PRC2), responsible for trimethylation of lysine 27 of histone H3 (H3K27me3) (Puschendorf *et al.*, 2008). Thus, in mouse zygotes the Polycomb pathway compensates for the absence of a classic pericentric heterochromatin signature on paternal chromatin and thereby functions as a transient backup mechanism for pericentric heterochromatin formation. This remarkable asymmetry in the parental pericentric heterochromatin signatures can be observed until the 8 cell stage, when pericentric domains become indistinguishable (Merico *et al.*, 2007; Puschendorf *et al.*, 2008).

In human embryos, paternal heterochromatin was recently shown not to be transiently formatted by PRC proteins (van de Werken *et al.*, 2012). In late human zygotes, no PRC-proteins or H3K27me3 were detected on paternal chromatin by immunofluorescent analysis. However, H3K9me3 was observed at heterochromatic regions. This pattern of broad maternal and heterochromatic paternal localization of H3K9me3 is maintained until the 8 cell stage, after which it adopts a symmetric pattern. These observations are in sharp contrast to the lack of this modification on paternal chromatin in mouse embryos. H3K9me3 is also observed at heterochromatic regions in human spermatozoa (van de Werken *et al.*, 2012). To test possible transmission to the embryo, human sperm was injected into mouse oocytes. In these heterologous zygotes, enrichment for H3K9me3 was consistently observed in heterochromatic regions on human paternal chromatin, indicating a paternal origin of the modified histones. This indicates a human paternal epigenetic contribution to constitutive heterochromatin formation and an absence of a maternally provided backup mechanism. Thus, human and mouse

zygotes have evolved different mechanisms for the re-establishment of paternal pericentric heterochromatin.

4.2 DNA methylation

DNA methylation also shows pronounced parental asymmetry. At fertilization, both the paternal and the maternal genome are highly methylated in mouse zygotes, but shortly thereafter, before DNA replication takes place, the paternal genome is actively demethylated (Rougier *et al.*, 1998; Mayer *et al.*, 2000; Oswald *et al.*, 2000; Santos *et al.*, 2002). Nevertheless, pericentric heterochromatic regions remain methylated (Salvaing *et al.*, 2012). Loss of 5mC in the paternal pronucleus has been correlated with the appearance of a recently discovered cytosine modification, 5-hydroxymethylcytosine (5hmC) (Inoue and Zhang, 2011; Iqbal *et al.*, 2011; Ruzov *et al.*, 2011; Wossidlo *et al.*, 2011). The maternal genome, on the other hand, is passively demethylated, a result from subsequent rounds of DNA replication in the absence of maintenance DNA methyltransferase Dnmt1 in morula stage embryos (Rougier *et al.*, 1998; Mayer *et al.*, 2000; Reik *et al.*, 2001). Parental asymmetry in 5mC and 5hmC is therefore detected until the 8 cell stage (Iqbal *et al.*, 2011) and at the blastocyst stage, a wave of global *de novo* methylation restores DNA methylation patterns, which will be maintained thereafter in somatic lineages (Santos *et al.*, 2002). Interestingly, the pattern of paternal genome demethylation is not conserved in mammalian species. Active demethylation of the paternal genome has been reported also in the rat and cattle, but not in sheep, rabbit and goat, whereas results in the pig are still contradictory (Beaujean *et al.*, 2004a; Beaujean *et al.*, 2004b; Fulka *et al.*, 2004; Shi *et al.*, 2004; Wossidlo *et al.*, 2011). Little is known about DNA demethylation in human preimplantation embryos, and some of the data available is contradictory. According to Fulka *et al.*, in about 50% of human zygotes the maternal genome is enriched for 5mC, similar to mouse zygotes, whereas in the other 50% of the zygotes both the paternal and the maternal genome are enriched for 5mC (Fulka *et al.*, 2004). Until the 8 cell stage, declining levels of methylation suggest passive demethylation (Fulka *et al.*, 2004; Santos *et al.*, 2010a), but it is unknown what happens at paternal pericentric heterochromatin.

4.3 Histone variants incorporated after fertilization

During repackaging of the protamine-stripped paternal genome with nucleosomal chromatin there is replication-independent preferential incorporation of histone variant H3.3 into the paternal genome, concomitant with formation of the paternal pronucleus. Incorporation of histone H3.3 in the maternal genome does not occur until nearly an hour after its deposition on the paternal genome (Torres-Padilla *et al.*, 2006). Deposition of histone H3.3 on the paternal chromatin of mouse zygotes is involved in the establishment of pericentric heterochromatin in the paternally-derived chromosomes (Santenard *et al.*, 2010). Inhibition of histone H3.3 deposition in mouse oocytes by loss of maternal ATRX (one of the histone chaperones involved in deposition of histone H3.3) causes centromere instability and chromosome missegregation in both MII oocytes and preimplantation embryos. This suggests that incorporation of H3.3 is required for centromere stability and the epigenetic control of heterochromatin function during meiosis and the transition to the first mitosis (Baumann *et al.*, 2010).

After fertilization, histone H2A.Z is lost from the zygotic genome as a result of its minimal incorporation and its accumulation is only detected again at the blastocyst stage (Nashun *et al.*, 2010). Nashun *et al.* further demonstrated that induced incorporation of H2A.Z in the chromatin of preimplantation embryos is detrimental to embryo development to the blastocyst stage, suggesting that removal of H2A.Z from the zygotic chromatin is required for normal preimplantation development in the mouse. In human zygotes however, it remains to be investigated whether histone variants H3.3 and H2AZ contribute to the establishment of pericentric heterochromatin.

In summary, early mouse and human embryos show parental asymmetry at the pericentric regions for most of the epigenetic marks that have shown to be crucial in somatic cells. So far, little is known about how preimplantation embryos compensate for this asymmetry during mitosis. Interestingly, the asymmetry persists until the 8 cell stage (Puschendorf *et al.*, 2008; van de Werken *et al.*, 2012), coinciding with the stages where most of the aneuploidies arise in human embryos.

5. A paternal epigenetic contribution to chromosome segregation errors?

Research into the origin of embryo aneuploidy has so far focussed on the contribution of the oocyte, and a link between maternal age and an increased risk for meiotic errors has been well established. However, chromosomal mosaicism in human embryos can occur independently of meiotic segregation errors (Baart *et al.*, 2006; Vanneste *et al.*, 2009; Santos *et al.*, 2010b) and does not appear to correlate with maternal age (Marquez *et al.*, 2000; Katz-Jaffe *et al.*, 2005). Compromised spermatogenesis is associated with increased chromosome segregation errors, as evidenced by an increased incidence of mosaicism in embryos derived from non-obstructive azoospermia patients, compared with those from ejaculated sperm (Silber *et al.*, 2003; Magli *et al.*, 2009). Moreover, research into the parental origin of the extra chromosome in human autosomal trisomies shows the occurrence of post-zygotic errors involving the paternal chromosomes (Nicolaidis and Petersen, 1998; Conlin *et al.*, 2010). Thus, a paternal contribution to chromosome segregation errors in embryos should also be considered.

The paternal genome in mature sperm is associated with protamines, instead of nucleosomes, facilitating high-density packaging in the sperm head. About 4-30% of the DNA in human sperm remains associated with nucleosomes (Tanphaichitr *et al.*, 1978; Gusse *et al.*, 1986; Gatewood *et al.*, 1990; Bench *et al.*, 1996), in contrast to mouse sperm DNA, where only 1% of DNA is nucleosome associated (Balhorn *et al.*, 1977). Mounting evidence indicates that specific regions in sperm DNA retain a somatic-like structure (for review see Miller *et al.*, 2010). In particular, these regions can be differentially marked by modified histones (Hammoud *et al.*, 2009; Brykczynska *et al.*, 2010) or histone variants (Gatewood *et al.*, 1990). In humans, the nucleosome/protamine ratio has been shown to vary between individuals, and is reportedly higher in sperm from infertile patients than that of fertile men (Ramos *et al.*, 2008; Hammoud *et al.*, 2011). This appears to be associated with incomplete nucleosome to protamine remodeling during spermiogenesis (Ramos *et al.*, 2008). Genome-wide analysis also identified differences in histone retention, as well as histone modifications in sperm of infertile men (Hammoud *et*

al., 2011).

It was previously demonstrated that sperm-derived histones contribute to zygotic chromatin in humans (van der Heijden *et al.*, 2008), and this has also been established for mouse (van der Heijden *et al.*, 2006). Therefore, sperm derived histones could influence the order of repackaging genes to a nucleosomal state and/or expression following fertilization (Gatewood *et al.*, 1990; Hammoud *et al.*, 2009). As described above, it was recently shown that in humans, the mark specifying pericentric heterochromatin is inherited from the sperm cell, by means of transfer of H3K9me3 to the zygote (van de Werken *et al.*, 2012). Since the histone content and modification level in human sperm is more variable than in mouse, aberrant levels of sperm-retained histones passed on to the zygote may thus directly interfere with correct heterochromatin formation. As a number of the mechanisms we describe below are dependent on a correct pericentric heterochromatin signature, we hypothesize that this may in turn impact on chromosome segregation during the first and consecutive cleavage divisions. In this context, an interesting observation was made in cancer cells, where a reduction in the level of H3K9me3 at the pericentric region was observed to result in an increased incidence of chromosome missegregation (Slee *et al.*, 2012).

6. Cell cycle control in preimplantation embryos

6.1 Cell cycle control during the first cleavage division

The duration of mitosis in mammalian somatic cells takes on average approximately 1h, with a relatively long prometaphase and a short metaphase (~25 min and ~8 min, respectively, in HeLa cells) (Meraldi *et al.*, 2004). Nevertheless, theses timings can be altered when, in the presence of unattached or non-bioriented chromosomes, cells cannot satisfy the spindle assembly checkpoint (SAC), a complex survaillance mechanism that delays anaphase until biorientiion of all chromosomes at the metaphase plate (Musacchio and Salmon, 2007) (Figure 1.3, chapter 1). Under such circumstances, cells are delayed (arrested) in mitosis for many hours (up to 4h in rodents and 22h in humans) (Rieder, 2011). The SAC is composed of Mad1, Mad2 and Bub3 proteins and BubR1, Bub1 and Mps1 kinases, and when activated it generates an inhibitory signal that blocks the anaphase promoting complex (APC/C) (Musacchio and Salmon, 2007). The APC/C is an E3 ubiquitin ligase complex responsible for the onset of anaphase, as it mediates proteolytic degradation of key mitotic substrates via the ubiquitin proteasome pathway (Schmidt *et al.*, 2006). Once all pairs of sister kinetochores are properly captured by the spindle microtubules and are under tension, the SAC is silenced and the APC/C becomes active, ubiquitinating securin and cyclin B, which are then degraded by the 26S proteasome (Peters, 2006). This results in the activation of separase, removal of sister chromatid cohesion, and sister chromatid separation, so that cells enter anaphase and exit mitosis.

The duration of the stages of mitosis can be determined by the observation of chromosome movements and/or localization of SAC markers. Disapearance of Mad2 and Bub1 from kinetochrores delineates the inactivation of SAC and marks the end of prometaphase (Hoyt *et al.*, 1991; Li and Benezra, 1996). In mouse embryos, it has been shown that the first mitotic

division takes almost double the time than the second mitosis (119 *vs* 70 min, respectively). This difference results from a prolonged metaphase in the first mitosis (Ciemerych *et al.*, 1999). The duration of prometaphase however, does not differ among the first two divisions, as demonstrated by Mad2 localization at the kinetochores for a similar period of time (Sikora-Polaczek *et al.*, 2006). This indicates that prolongation of the first embryonic mitosis occurs due to a transient metaphase arrest, which resembles the cytostactic factor (CSF)-mediated metaphase II arrest in oocytes. The CSF, whose identity is still not fully uncovered, inhibits the APC/C, but this inhibition is believed to be independent of the SAC (Tsurumi *et al.*, 2004). Similarly, in *Xenopus* embryos the first embryonic mitosis is also estimated to take longer than the second mitosis (25 *vs* 15 min respectively) (Chesnel *et al.*, 2005). It is therefore believed that the prolonged duration of the first mitotic division may occur by a mechanism similar to the arrest at the second meiotic division.

The similarities between MII arrest in oocytes and the prolonged metaphase observed in mouse zygotes, together with the notion that EGA only occurs at the 2 cell stage, suggest that some maternally provided meiotic pathways may remain active during the first mitosis of mouse embryos (Kubiak *et al.*, 2008). It is however unclear which mechanisms are involved in the metaphase arrest in zygotes, as the Mos/MAPK pathway, necessary for CSF establishment in MII oocytes, does not seem to be active during the first two mitosis of mouse embryo development (Verlhac *et al.*, 1994; Kalab *et al.*, 1996).

Similarly to mouse, the first mitosis also appears prolonged in human embryos. Time-lapse observations on human IVF embryos have shown the time interval between disappearance of pronuclei and first cleavage to take almost 1 hour longer than the second mitosis (2.7±0.5 h *vs* 1.9±0.3 h, respectively) (E. B. Baart, personal communication). It is unknown, however, whether prolongation in the duration of the first mitosis is caused by a prolongued metaphase as in mouse and *Xenopus* zygotes.

6.2 Activity of the SAC in early embryos

The SAC improves chances of a successful mitosis by delaying anaphase to provide the cell an opportunity to correct attachment errors. However, it is not necessary for the completion of mitosis (Rieder and Maiato, 2004). The high incidence of chromosome aneuploidies and mosaicism during the first cleavage divisions in human embryos has been proposed to result from a dysfunctional SAC, allowing embryonic cells to divide while not all chromosomes have achieved proper attachment (Los *et al.*, 2004). However, our work suggests otherwise, as we were able to arrest human embryos at all stages of preimplantation development at prometaphase of the cell cycle using spindle poisons such as colcemid and nocodazole (Avo Santos *et al.*, 2011), an indication of a functional SAC that is capable of APC/C inhibition in the presence of unattached kinetochores. Furthermore, using RNAi-based silencing of Bub3, BubR1 and Mad2 in morula stage mouse embryos, Wei *et al.* have shown that SAC is essential for correct chromosome segregation during the first embryonic divisions (Wei *et al.*, 2011).

In vertrebrate cells, a single unattached kinetochore can delay anaphase for hours (Rieder *et al.*, 1994). However, the biochemical signal produced by each unattached kinetochore is not an “all-or-none” event, but can in fact be weakened. The kinetochore-derived inhibitor that

sends the wait-anaphase signal can be quantitatively reduced in concentrations of signal-producing components (Kops *et al.*, 2005). This creates a situation in which more than one unattached kinetochore is needed to produce enough signal to inhibit anaphase onset, and in which chromosome separation can occur with unaligned chromosomes. It may thus be that the relatively large cytoplasmic volume present in cells of the early embryo may dilute kinetochore signalling to the SAC. Only when all kinetochores are unattached due to the addition of spindle poisons, the amount of signal generated is enough to activate the SAC. Alternatively, a weakened signal may also be caused by reduced expression of SAC proteins, as reported for Bub1 in immature rhesus macaque oocytes (Dupont *et al.*, 2012).

6.3 Distinct constitution of the CPC during the cleavage divisions

Kinetochore capture by the mitotic spindle is a stochastic trial-and-error process. While unattached chromosomes activate the SAC to delay anaphase until biorientation is achieved, erroneous attachments cannot induce SAC response. Therefore, during prometaphase, the activity of Aurora kinase B allows destabilization of non-bipolar microtubule-kinetochore interactions, providing a new opportunity to attach in a bi-oriented fashion (Vader *et al.*, 2008). Aurora B belongs to a family of serine-threonine kinases that is conserved from yeast to humans. This kinase is a member of the Chromosomal Passenger Complex (CPC), together with the Inner Centromere Protein (INCENP), Borealin and Survivin (reviewed in Carmena and Earnshaw, 2003). These three non-enzymatic subunits of the CPC are essential for the correct localization of Aurora B to the inner centromere (reviewed in Ruchaud *et al.*, 2007).

Aurora kinase C, first identified in the testis (Bernard *et al.*, 1998), has been coined the meiotic counterpart of Aurora B. Aurora C is known to be important during human male meiosis, where lack of functional kinase disrupts meiotic progression, leading to infertility (Dieterich *et al.*, 2007). In human IVF oocytes, transcript and protein levels of Aurora C are considerably higher than Aurora B levels, suggesting that the main kinase involved in meiotic maturation is Aurora C (Assou *et al.*, 2006; Avo Santos *et al.*, 2011). However, contrary to men, the lack of Aurora C kinase activity in women does not impair completion of meiosis, as two women with homozygous *AURKC* mutation are reported to be fertile (Dieterich *et al.*, 2009). This suggests redundancy in the function of Aurora B and C in the female, with the two kinases having similar functions and compensating for (the lack of) each other. Experiments in somatic cell lines support this, as induced expression of Aurora C is able to fully compensate for the absence of Aurora B and allow mitotic progression (Sasai *et al.*, 2004; Slattery *et al.*, 2009).

In human embryos, Aurora C is the most prominent kinase in the CPC during the first three embryonic mitosis, when Aurora B levels are considerably lower (Avo Santos *et al.*, 2011). Immunodetection of both Aurora kinases on prometaphase chromosomes revealed differences in localization. Aurora B was confined to the interkinetochore region of the centromere, while Aurora C covered a larger area, beyond the interkinetochore limit, possibly at the pericentromeric heterochromatin. Similar observations regarding differences in Aurora B and C localization have been made in mouse MI oocytes (Shuda *et al.*, 2009; Sharif *et al.*, 2010). This points to non-overlapping roles for Aurora B and C during early human embryo development. However, this does not exclude some degree of redundancy between the two

kinases. Mouse embryos carrying a targeted disruption of the *Aurkb* gene are able to develop up to the blastocyst stage (Fernandez-Miranda *et al.*, 2011). This was shown to be due to the endogenous presence of Aurora C, which compensates for the loss of Aurora B. In contrast, mouse embryos lacking other subunits of the CPC, are unable to progress beyond the cleavage stages (Cutts *et al.*, 1999; Uren *et al.*, 2000b; Yamanaka *et al.*, 2008b). It is unknown to which extent the distinct CPC constitution in human zygotes, where Aurora C co-exists with low levels of Aurora B, may influence CPC localization and/ or function.

It is generally accepted that Aurora B contributes to the SAC. However there is open discussion on whether Aurora B communicates to the SAC directly or indirectly, by creating unattached kinetochores that in turn stimulate production of the SAC wait-anaphase signal. In a recent communication, Fuller and Stukenber propose a model where Aurora B can contribute both directly and indirectly to the SAC, depending on the levels of the kinase at the centromeres. According to their hypothesis, small levels of Aurora B are sufficient to generate an occupancy signal (involved in SAC regulation); while higher levels of the kinase are required for tension signaling (involved in attachment error-correction) (Fuller and Stukenberg, 2009). The question is then if the high levels of Aurora C present in the oocyte and zygote can fully take over the attachment error-correction function of Aurora B. If not, according to Fuller and Stukenbers's model, low levels of Aurora B in human zygotes could explain the apparent reduced stringency of SAC during the first mitotic divisions in human embryos compared to somatic mitotic divisions.

6.4 Differences in mechanisms regulating CPC localization and function in zygotes

Correct localization of the CPC is crucial for its ability to destabilize misattachments. Pericentric heterochromatin appears to play an important role in CPC localization. The N-terminal end of HP1 binds to H3K9me3 (Lachner *et al.*, 2001), while the C-terminal end interacts with other proteins (Hayakawa *et al.*, 2003), among which is the CPC subunit INCENP (Ainsztein *et al.*, 1998; Kang *et al.*, 2011). A role for pericentric heterochromatin in CPC locaization is further demonstrated by an experimental model consisting of a human cell line incorporating a mutated copy of chromosome 4 (Bassett *et al.*, 2010). This chromosome 4 contains an epigenetically silenced centromere and a neocentromere has formed, meaning that the CENP-A containing nucleosomes (see section 7) have translocated to a different location, devoid of centromeric and pericentric DNA repeat sequences (Amor *et al.*, 2004). This results in an altered distribution of Aurora kinase B and impairment of its error-correction ability. As a result, the chromosome containing the neocentromere was more frequently involved in misattachment and failed alignment at the metaphase plate.

In somatic cells, targeted localization of the CPC to the inner centromere was shown to depend on two centromeric-specific histone modifications: phosphorylation of histone H3 at Thr3 (H3T3) and H2A at Thr120 (H2AT120), performed by Haspin and Bub1 kinases, respectively (Kawashima *et al.*, 2010; Kelly *et al.*, 2010a; Tsukahara *et al.*, 2010; Wang *et al.*, 2010; Yamagishi *et al.*, 2010). According to the model proposed, the intersection of these two marks determines CPC localization to the inner centromere at prometaphase (Yamagishi *et al.*, 2010). Phosphorylation of H2AT120 allows recruitment of Shugosin to the centromeres

that in turn interacts with the CPC subunit Borealin (Kawashima *et al.*, 2010; Yamagishi *et al.*, 2010). Phosphorylation of H3T3 recruits the CPC subunit Survivin (Kelly *et al.*, 2010a; Wang *et al.*, 2010). In somatic cells, this mark starts to appear on the chromosome arms during prophase and then concentrates at the inner centromere during prometaphase (Wang *et al.*, 2010), by the counteracting dephosphorylation activity of the phosphatase complex PP1/Repo-Man (Qian *et al.*, 2011). We recently investigated the Haspin-H3pT3 pathway in human preimplantation embryos and observed persistent ubiquitous phosphorylation of H3T3 on (pro)metaphase chromosomes in human zygotes, with a complete lack of centromeric enrichment for H3pT3, but not INCENP (chapter 5). In somatic cells, overexpression of Haspin also results in persistent H3pT3 on the chromosome arms and concomitant dislocation of the CPC to the arms (Qian *et al.*, 2011). This may indicate that centromeric enrichment of the CPC in zygotes may depend either solely on phosphorylation of H2AT120 by Bub1 or in combination with another mechanism yet to be identified.

Kinase activity of Aurora B and C is often assessed by its capacity to phosphorylate serine 10 of histone H3 (H3pS10) during mitosis (Crosio *et al.*, 2002; Sasai *et al.*, 2004). In somatic cells, H3pS10 is first detected in G2 phase and reaches a maximum in metaphase chromosomes, being rapidly dephosphorylated with the onset of anaphase and following the pattern of Aurora B kinase activity (Crosio *et al.*, 2002). Interestingly, the phosphorylation/dephosphorylation cycle of H3S10 during the first two mouse embryonic mitosis is distinct from somatic divisions and resembles that of meiotic oocyte divisions, with H3pS10 persisting during anaphase and telophase (Teperek-Tkacz *et al.*, 2010). This suggests a different regulation of H3pS10 (de)phosphorylation, which indicates prolonged kinase activity of Aurora B/C in oocytes and early embryos (Wang *et al.*, 2006; Swain *et al.*, 2007; Teperek-Tkacz *et al.*, 2010). Furthermore, inhibition of Aurora kinases with ZM447439 also exerts an effect on the first embryonic mitosis that is distinct from the second mitosis, but comparable with the one observed during meiotic divisions (Teperek-Tkacz *et al.*, 2010).

In human zygotes, inhibition of Aurora kinase activity resulted in a differential effect on CPC localization between maternally- and paternally-derived chromosomes (chapter 5). Higher levels of INCENP, as well as higher residual H3pT3 levels were observed on the chromosome arms of maternal, H3K9me3-rich chromosomes. On paternal chromatin, some INCENP and residual H3pT3 co-localized with a few H3K9me3 positive regions. We hypothesize that in the zygote the activity of Aurora C and its phosphorylation of H3S10, possibly together with the persistent ubiquitous phosphorylation of H3T3, plays a key role in assuring that the epigenetic asymmetry between the parental chromosomes for H3K9me3 is “cloaked” and will not affect CPC localization. In future studies it will be interesting to assess if the error-correcting ability of the CPC differs on maternal and paternal chromosomes.

Together these findings suggest that the mechanisms regulating CPC localization and activity during the first cell division in mouse and human embryos are distinct from those described in somatic cell divisions. It remains unclear to what extent such differences affect the capacity of CPC to correct erroneous attachments.

7. The centromere and *de novo* assembly of the kinetochore on paternal chromatin

Incorporation of the H3 variant CENP-A forms the epigenetic mark that specifies the centromere (Black and Bassett, 2008). It serves as the site of DNA-protein and protein-protein interactions required for the formation of the kinetochore (Guse *et al.*, 2011). Centromeres are maintained across generations through epigenetic mechanisms (Black and Cleveland, 2011). During DNA replication in S-phase, CENP-A is equally partitioned to the newly formed sister centromeres, leaving each centromere with only half the amount of CENP-A. This situation is remedied only after mitosis, when at the onset of G1 phase newly formed CENP-A containing nucleosomes are incorporated (Jansen *et al.*, 2007). Characterisation of the histones present in mature human sperm identified CENP-A (Gatewood *et al.*, 1990; Zalensky *et al.*, 1993), indicating that the "identity" of the centromeres is preserved when the paternal genome is transferred to the zygote. However, it is not known when CENP-A loading to re-establish the full complement occurs on the sperm chromatin: after the second meiotic division and during spermiogenesis or after fertilization and before the onset of the first embryonic S-phase. Interestingly, CENP-A dosage is important for centromere function and CENP-A synthesis is normally restricted to S- and G2-phase of the cell cycle. When CENP-A expression is artificially increased, the centromeric region expands (Van Hooser *et al.*, 2001), causing aneuploidy in human cells (Amato *et al.*, 2009). This expansion can be limited by the presence of H3K9me3 on the surrounding heterochromatin (Lam *et al.*, 2006; Ohzeki *et al.*, 2012). Therefore, it has been hypothesized that the overall ratio of CENP-A containing chromatin and heterochromatin is important for the capability of the centromere to form a functional kinetochore (Lam *et al.*, 2006; Okamoto *et al.*, 2007; Gonzalez-Barrios *et al.*, 2012).

The kinetochore consists of more than 80 proteins in humans and forms the primary site for microtubule attachment (Maiato *et al.*, 2004; Cheeseman and Desai, 2008), as well as being required for establishment of the inner centromere and proper function of the SAC (Vagnarelli and Earnshaw, 2004; Chan *et al.*, 2005). The kinetochore consists of an inner kinetochore that forms directly on the CENP-A containing nucleosomes and is also called the constitutive centromere-associated network (CCAN). Most of its subunits are present at the centromeres throughout the cell cycle (Takeuchi and Fukagawa, 2012). The outer kinetochore, also called the KMN network, is assembled on top of this and comprises three different protein complexes (KNL1, Mis12 and Ndc80 complexes). The outer kinetochore starts to form around the G2 phase and disassociates again at the end of mitosis (Hori and Fukagawa, 2012). So far, no reports exist on the presence of CCAN proteins in sperm, indicating that the inner kinetochore needs to be formed *de novo* on the paternal chromatin after fertilization.

It has recently been shown that HP1 provides a direct link between pericentric heterochromatin and kinetochore formation, by recruiting the Mis12 complex to the centromeric region during interphase (Kiyomitsu *et al.*, 2010). It is unclear if and how the asymmetry on maternal and paternal chromosomes for H3K9me3 and HP1 impacts on centromere formation and kinetochore assembly in the zygote.

8. The human sperm cell contributes the centrosome to zygotic spindle formation

The formation of the bipolar spindle is a crucial event for cell division, which ensures correct chromosome segregation to daughter cells. The centrosome, which comprises a pair of centrioles surrounded by the pericentriolar material, is the organizing center of the mitotic spindle in somatic cells. However, mammalian oocytes, except in the mouse, are deprived of centrosomes. During fertilization in humans, the sperm-derived centrosome is responsible for spindle assembly in the zygote (Sathananthan *et al.*, 1991). Sperm contribution to centrosomes and spindle formation in human zygotes is therefore directly related with the ability to correctly segregate chromosomes.

The increased incidence of mosaicism in dispermic human zygotes compared to monospermic or digynic embryos can be considered evidence of a sperm contribution to abnormal spindle organization (Palermo *et al.*, 1994). However, this may simply reflect the presence of an extra centrosome delivered by the second sperm cell, as extra centrosomes have been shown to induce transient multipolar spindles. Although these poles can fuse to reform a bipolar spindle (Quintyne *et al.*, 2005), this can result in chromosomes where one sister kinetochore attaches to both spindle poles (merotelic attachment) with subsequent missegregation (Ganem *et al.*, 2009). Nevertheless, the transfer of a defective centrosome from the sperm to the oocyte has been proposed to contribute to chromosome segregation errors in the embryo (Delhanty, 2005; Mantikou *et al.*, 2012). Attempts have been made to assess functionality of the sperm centrosome by insemination of rabbit or bovine oocytes with human sperm and evaluation of its ability to organize microtubules (Terada, 2007). However, so far no clear relationship between sperm quality, centrosomal dysfunction and spindle formation in the zygote has been established in human IVF (Schatten and Sun, 2011).

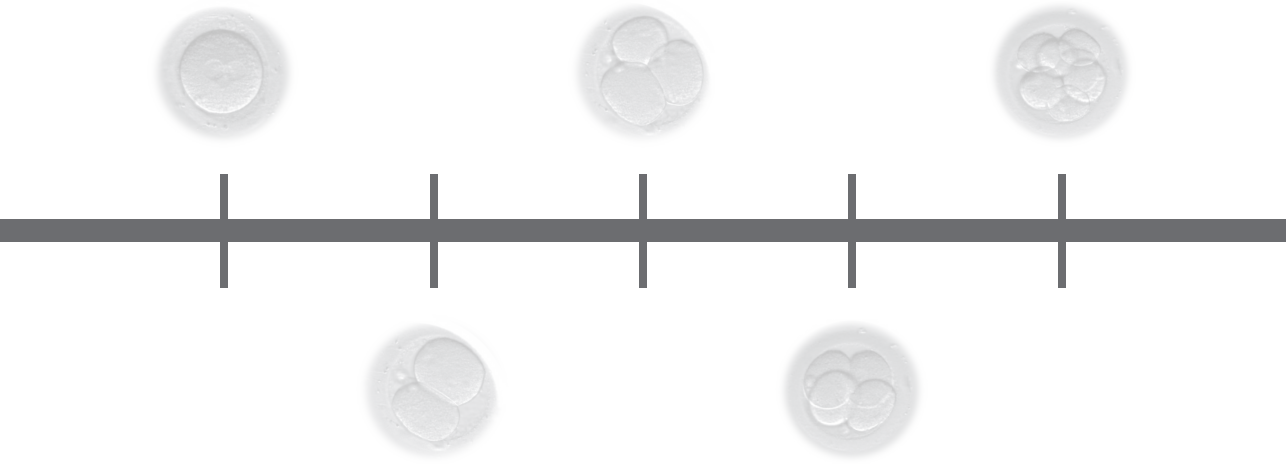
9. Conclusions and future perspectives

Research into the origin of embryo aneuploidy has so far focussed on the contribution of the oocyte, and a link between maternal age and the increased risk for meiotic errors has been well established. Meiotic chromosome segregation errors in oogenesis are currently explained by a “two-hit” hypothesis (Lamb and Hassold, 2004). Homologous chromosomes can form more susceptible crossover configurations during the meiotic pairing process (first hit), which later results in segregation errors due to age related perturbations in the oocytes’ spindle and checkpoint function (second hit) (Nagaoka *et al.*, 2012). This model, however, does not explain the high rate of chromosomal mosaicism in human embryos, which can occur independently of meiotic segregation errors (Baart *et al.*, 2006; Vanneste *et al.*, 2009; Santos *et al.*, 2010b) and does not seem to correlate with maternal age (Marquez *et al.*, 2000).

Here we reviewed a number of mechanisms involved in the events leading to chromosome segregation during human preimplantation development, with a special emphasis on the first embryonic division. By establishing a parallel with the mechanisms governing cell division in somatic cells and mouse embryos we were able to identify unique features of the first embryonic division in humans that help to identify possible origins of the high incidence of chromosome

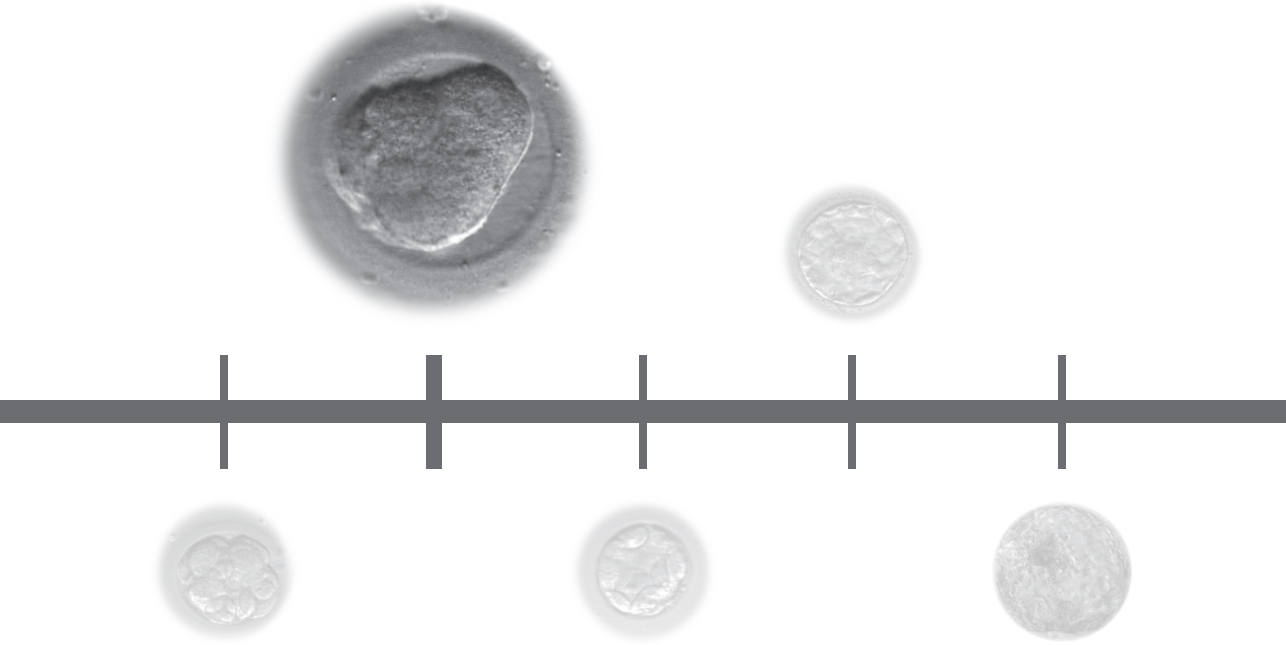
segregation errors. Most of the differences in the cell cycle control between somatic cells and early embryos are present both in human and mouse. Both species also share a clear epigenetic asymmetry between maternal and paternal chromatin established right after fertilization. Nevertheless, mouse preimplantation embryos are known to show very low rates of aneuploidy compared to humans (1-2% *vs* 80%, respectively) (Voullaire *et al.*, 2000; Wells and Delhanty, 2000; Tateno *et al.*, 2011). Our knowledge is still too limited to explain this striking difference and more research is necessary. However, from the literature revised in this review, it has become clear that the epigenetic signature passed on by the sperm chromatin may contribute to the poor performance of human embryos regarding chromosome segregation. The histone content of human mature sperm varies significantly between individuals and higher levels of histone retention seem to be associated with subfertility. In the mouse, on the other hand, sperm histone content is not only much lower, but also consistent between specimens, and high content of histones retained on sperm has been linked to an increased incidence of chromosome missegregation. Therefore, we propose that the particular asymmetric chromatin constitution of human zygotes, greatly influenced by the inherited sperm histone content, may constitute a "first hit" on the mechanisms leading to chromosome missegregation in human preimplantation embryos. In addition to this, other factors such as oocyte quality and age may determine how well the zygote's cell cycle machinery can compensate for defects and ensure correct segregation of all chromosomes. In fact, the reported differences in cell cycle duration and checkpoint mechanisms may not be causing the missegregations, but rather represent an adaptation that allows correct chromosome segregation under such special circumstances as in the case of epigenetic asymmetry on parental chromatin.

Further research on the mechanisms leading to the increased incidence of chromosome missegregations in human embryos may help provide a better understanding of human infertility and ultimately allow the development of novel diagnostic tools to improve gamete and embryo selection and increase pregnancy rates after IVF.



Chapter 7

General discussion



Ovarian stimulation: to what extent does it affect preimplantation development?

Patients in need of IVF techniques undergo ovarian stimulation to allow the growth and maturation of multiple follicles and therefore compensate for inefficiencies in IVF procedures. However, as ovarian stimulation implies the administration of high doses of exogenous gonadotropins that overrule the natural selection of a single follicle, it is important to evaluate its impact on embryo development and other IVF outcomes. In **chapter 2** of this thesis the literature relevant to this topic was reviewed. Several studies have attempted to establish a link between ovarian stimulation and IVF outcomes such as epigenetic competence of oocytes and embryos, embryonic implantation and perinatal outcomes. However, no consensus has been reached, as some studies point towards a link between the use of ovarian stimulation and poor IVF outcomes, while others do not find such correlation. The difficulty to eliminate confounding risks factors such as maternal age and subfertility of ART patients, which on its own may contribute to poor outcomes, makes it hard to find irrefutable evidence for a link between ovarian stimulation and poor IVF outcomes. Nevertheless, it has become generally accepted that ovarian stimulation affects the occurrence of chromosomal abnormalities in IVF-derived embryos (Munne *et al.*, 1997; Troncoso *et al.*, 2003; Katz-Jaffe *et al.*, 2005; Baart *et al.*, 2007a; Haaf *et al.*, 2009). Most chromosomal abnormalities identified result from post-zygotic mitotic segregation errors, leading to chromosomal mosaicism (Baart *et al.*, 2006; Vanneste *et al.*, 2009; Santos *et al.*, 2010b), and there is some direct evidence that the use of a conventional high dose gonadotropin stimulation protocol increases the incidence in mitotic segregation errors (Baart *et al.*, 2007a).

Mounting evidence suggests that the exposure of developing oocytes to supraphysiological concentrations of gonadotropins may disturb oocyte maturation (Elbling and Colot, 1985; Vogel and Spielmann, 1992; Roberts *et al.*, 2005). During maturation, the oocyte accumulates mRNAs and proteins that will sustain embryo development during the first cleavage divisions, before activation of the embryonic genome. High levels of exogenous gonadotropins may interfere with this process, altering the levels of maternally stored mRNAs and proteins important for correct chromosome segregation. This may contribute to the increased incidence of aneuploidies and chromosomal mosaicism in preimplantation embryos from IVF patients. Indeed, several studies performed in human and animal models illustrate the link between oocyte maturity and mRNA expression of genes related to chromosome segregation and cell cycle regulation (Jones *et al.*, 2008b; Mtango and Latham, 2008; Dupont *et al.*, 2010). Future studies evaluating the levels of mRNA and proteins involved in chromosome segregation and the mitotic checkpoint found in mature oocytes in relation to ovarian stimulation protocols using high *versus* low doses of gonadotropins will be important to confirm this.

The impact of chromosomal mosaicism on embryonic development

In **chapter 3** the impact chromosomal mosaicism may have on the developmental capacity of embryos from the morula to the blastocyst and peri-implantation stages was investigated. The great incidence of chromosomal mosaicism on cleavage stage day 3 embryos results mainly from post-zygotic chromosomal errors (Mantzouratou and Delhanty, 2011). Such high

frequency of aneuploidy has been hypothesized to result from the inefficiency of the cell cycle checkpoints during cleavage divisions, before initiation of embryonic genome activation (Los *et al.*, 2004). According to this idea, once embryonic genome activation is initiated at around the morula stage (day 4), the establishment of functional cell cycle checkpoints prevents new errors. Indeed, a number of studies reported a decline in the percentage of aneuploidy towards the blastocyst stage (Ruangvutilert *et al.*, 2000; Bielanska *et al.*, 2002a). In **chapter 3** the results of FISH analysis for 10 chromosomes in human embryos at the morula (day 4), blastocyst (day 5) and peri-implantation (day 8) stage further confirmed the previous findings, showing a decreasing incidence of mosaicism over time, with a significant reduction of the percentage of embryos classified as chromosomally mosaic on day 5 and day 8. In our study a co-culture system was used to allow development of day 5 blastocysts up to day 8 peri-implantation stage, in contact with a monolayer of endometrial stromal cells. This is the first and so far the only study evaluating the chromosomal constitution of human embryos as late as day 8, a stage at which *in vivo* implantation is taking place. It is therefore remarkable to notice that although the incidence of mosaicism at day 8 is lower than at earlier stages, an estimate of 42% of embryos are still chromosomally mosaic. This indicates that in a good proportion of human embryos the presence of aneuploid cells may not be detrimental for embryonic development. However, the ratio of chromosomally normal and abnormal cells may need to be above a certain threshold level in order to allow successful embryo development (Evsikov and Verlinsky, 1998). Furthermore, we found a negative correlation between the percentage of chromosomally abnormal cells and the total number of cells in day 5 blastocysts and day 8 peri-implantation embryos, but not in day 4 or day 5 arrested embryos. We also found the highest incidence of mosaic embryos on day 4 compared to day 5 and day 8. This suggests that cavitation (i.e, blastocyst formation), which is accompanied by cell differentiation, may trigger the onset of selection against aneuploid cells and/or establishment of a growth advantage for chromosomally normal cells. This hypothesis is further supported by the work of Mantel *et al.*, where induction of differentiation in human embryonic stem cells is shown to induce apoptosis of chromosomally abnormal cells (Mantel *et al.*, 2007).

Thus, the work presented in **chapter 3** suggests that chromosomal mosaicism does not necessarily undermine normal human development. This indicates that we may currently underestimate the incidence of chromosomal mosaicism during and beyond preimplantation development. Future studies will be important to assess the impact of chromosomal mosaicism on loss of conception, fetal development and adult health.

How is chromosome segregation regulated in preimplantation human embryos?

In **chapter 4** constitution and localization of the CPC was described in an attempt to better understand the origins of aneuploidy in preimplantation human embryos. During prometaphase, active destabilization of erroneous attachments promoted by the activity of Aurora kinase B is necessary for the mechanism of attachment error-correction (Vader *et al.*, 2008). We showed that Aurora C kinase, until recently known as the meiotic counterpart of Aurora B, is the main

enzymatic subunit of the CPC from the zygote (day 1) up to the 8-16 cell stage (days 3/4) in human embryos. At around the morula stage (day 4), Aurora C levels decrease and after cavitation (day 5, blastocyst stage) Aurora B becomes the CPC kinase subunit of choice. The timing of the switch from Aurora C to Aurora B coincides with the moment in preimplantation development when the proportion of aneuploid cells declines significantly, as assessed in **chapter 3**. It is therefore tempting to hypothesize that the presence of Aurora C as the main CPC kinase up to the morula stage is related to the increased incidence of chromosome segregation errors identified during that same period of development. However, Aurora C can fully compensate for the absence of Aurora B in both HeLa cells (Sasai *et al.*, 2004; Slattery *et al.*, 2009) and mouse preimplantation embryos (Fernandez-Miranda *et al.*, 2011), allowing apparently normal cell cycle progression. Although these cells have not been investigated for low-level aneuploidy, there is so far no reason to assume that Aurora C has reduced error-correction activity. Nevertheless, our findings indicate other factors that may be involved in the higher error rate found in early human preimplantation embryos: 1) the variation in the Aurora B to Aurora C ratio in protein detected at the inner centromere between zygotes, and 2) differential localization of Aurora B compared with localization of Aurora C on prometaphase chromosomes of human zygotes.

Overexpression of Aurora B and Aurora C has been reported in human cancer cell lines (Tatsuka *et al.*, 1998; Kimura *et al.*, 1999), and ectopic overexpression of a kinase-deficient Aurora C was shown to induce polyploidy, as it caused disruption of the Aurora B-INCENP complex (Chen *et al.*, 2005). It seems therefore that maintenance of the correct balance between the amount of Aurora B and C is important for proper CPC function and prevention of chromosome segregation errors in human cell lines. Immunolocalization on metaphase chromosomes of mature human oocytes (MII) revealed variation in the amount of Aurora B compared to Aurora C (**chapter 4**). Although most oocytes showed a more intense Aurora C signal, in a subset of MII oocytes Aurora B signal was more intense than Aurora C. Since human embryos rely on the reserves of mRNA and proteins present in the oocyte during the first cleavage divisions, we would expect to observe such variation at the zygote, 2 cell and 4 cell stage as well. However, we observed Aurora C signal consistently stronger than Aurora B at all those stages. This suggests that the balance between the levels of Aurora B and C in oocytes may be related to oocyte maturation. The contradictory findings on the relative importance of Aurora B and C during mouse meiosis may also be explained by this, as findings may depend on whether hormonal stimulation was used prior to oocyte collection for *in vitro* maturation contradictory (Shuda *et al.*, 2009; Vogt *et al.*, 2009; Yang *et al.*, 2010). In the future it will be interesting to further test this hypothesis and evaluate whether the variability in the levels of Aurora B and C in MII oocytes can be related to oocyte and embryo quality, ovarian stimulation and maternal age.

Immunolocalization of Aurora B and C on chromosome spreads of zygotic prometaphases revealed not only higher levels of Aurora C compared to Aurora B, as well as differences in localization of these two kinases. Aurora B (present in very low levels) appeared to be more confined to the inner centromere, the area between the two sister kinetochores. In contrast, Aurora C accumulation was detected both at the inner centromere and pericentromeric

regions. This is consistent with observations in mouse MI oocytes (Shuda *et al.*, 2009; Sharif *et al.*, 2010) and our own observations during human spermatogenesis, where Aurora C was observed on pericentric regions during the pachytene stage of meiotic prophase. This may indicate differential targeting systems of Aurora B *versus* Aurora C. However, how such distinct localization can be achieved is unknown, since both Aurora B and C bind INCENP, an interaction that is important for correct localization of the CPC (Sasai *et al.*, 2004; Tang *et al.*, 2006).

For Aurora B/C to be able to take on the role of attachment error-correction, the CPC must be targeted and anchored precisely at the inner centromeres, as disturbance of its localization results in chromosome misalignment, cytokinesis failure and SAC override (Bassett *et al.*, 2010; Becker *et al.*, 2010). We investigated whether the extended localization of Aurora C to the pericentric regions in zygotes reflects a less confined CPC localization at that stage compared to later stages of development, where Aurora C is no longer the main kinase involved in the CPC. For this purpose, INCENP localization was investigated in zygotes, 8 cell and blastocyst stage as an indicator of CPC localization. The results presented in **chapter 5** confirmed our previous observations. In zygotes, INCENP localization is not confined to the inner centromeric regions of prometaphase chromosomes, but slightly extended to the pericentric regions. However, at the 8 cell and blastocyst stage INCENP becomes increasingly confined to the inner centromere. Further investigation of the mechanisms regulating CPC targeting to the inner centromere revealed that similar to somatic cells, in all stages of preimplantation embryo development there is centromeric-specific phosphorylation of H2AT120 by Bub1 kinase. This phosphorylation allows recruitment of Sgo1 to the centromeres that in turn interacts with the CPC subunit Borealin, allowing centromeric targeting of the complex at prometaphase (Kawashima *et al.*, 2010; Yamagishi *et al.*, 2010). Nevertheless, in somatic cells the Bub1-H2ApT120-Sgo1-CPC pathway does not act alone to direct CPC localization. The other histone modification known to contribute to targeting of the CPC is phosphorylation of histone H3T3 by Haspin kinase, a mark that recruits Survivin (Kelly *et al.*, 2010b; Wang *et al.*, 2010). In somatic cells, this mark starts to appear on the chromosome arms during prophase and then concentrates at the inner centromere during prometaphase (Wang *et al.*, 2010), by the counteracting dephosphorylation activity of the PP1/Repo-Man complex (Qian *et al.*, 2011). Surprisingly, when we investigated the Haspin-H3pT3-CPC pathway, we observed persistent ubiquitous phosphorylation of H3T3 on (pro)metaphase chromosomes in human zygotes, with a complete lack of centromeric enrichment. From the 2 cell stage onwards, however, phosphorylation dynamics resembled somatic cells.

In somatic cells, overexpression of Haspin also results in persistent H3pT3 on the chromosome arms and concomitant dislocation of the CPC to the arms (Qian *et al.*, 2011). This may indicate that centromeric enrichment of the CPC in zygotes may depend solely on phosphorylation of H2AT120 by Bub1 and/or other mechanisms yet to be identified. This may offer an explanation for our observation that CPC localization was less confined to the inner centromere in zygotes. If centromeric targeting of the CPC in zygotes relies on only one recruitment pathway, this process may be more susceptible to disturbances. As even subtle disturbances in CPC localization may predispose a chromosome to misalignment (Bassett *et*

al., 2010), this may result in an increased rate of missegregation.

Epigenetic asymmetry in human embryos and impact on chromosome segregation

Until now it has been suggested that the origins of chromosomal aneuploidy and mosaicism in preimplantation embryos are related to the lack of fully functional checkpoints resulting from the absence of embryonic transcription until around day 3 of development (Braude *et al.*, 1988; Los *et al.*, 2004). However, it has recently been shown that embryonic genome activation in humans is initiated as early as at the 2 cell stage (Vassena *et al.*, 2011). Furthermore, human embryos at all stages readily arrest when exposed to spindle poisons creating unattached kinetochores, indicating a functional SAC. We were also able to show mRNA expression of relevant SAC proteins (data not shown) and CPC activity in all stages of preimplantation development, including at the zygote stage. It seems thus unlikely that the lack of a functional mitotic checkpoint resulting from the absence of embryonic transcription fully explains the early embryo missegregations.

After fertilization, mammalian embryos show epigenetic asymmetry between the chromatin derived from the oocyte and sperm (Probst and Almouzni, 2011). While maternal chromatin maintains its somatic-like epigenetic marks, paternal chromatin needs to be re-established after the replacement of protamines by maternally provided nucleosomes (Albert and Peters, 2009). The most studied epigenetic mark involved in the establishment of constitutive pericentric heterochromatin is H3K9me3, which in human zygotes is broadly present on maternal chromatin, but restricted to a few heterochromatic regions on paternal chromatin (van der Heijden *et al.*, 2009; van de Werken *et al.*, 2012). This is an interesting feature of early embryos, especially considering the importance of the pericentric H3K9me3 heterochromatic signature for faithful chromosome segregation (Peters *et al.*, 2001; Slee *et al.*, 2012).

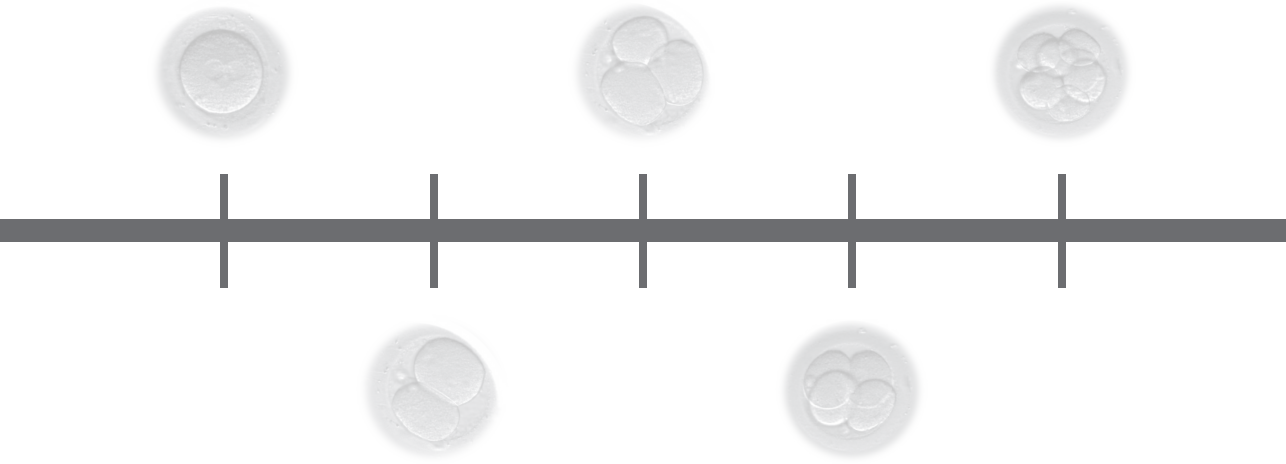
The work presented on **chapter 5** showed that inhibition of Aurora kinase activity in human zygotes resulted in a differential effect of CPC localization between maternally- and paternally-derived chromosomes, with maternal, H3K9me3-rich chromosomes able to attract higher levels of INCENP as well as higher residual H3pT3 levels on the chromosome arms. On paternal chromatin, both INCENP and residual H3pT3 co-localized with H3K9me3 positive regions. It seems thus possible that in the zygote the activity of Aurora C and its phosphorylation of H3S10, possibly together with the persistent ubiquitous phosphorylation of H3T3, plays a key role in assuring that the epigenetic asymmetry between the parental chromosomes for H3K9me3 is “cloaked” and will not affect CPC localization. In this light, it is interesting to notice that the end of epigenetic asymmetry and the switch from Aurora C to Aurora B as the main CPC kinase coincide around the morula stage (Avo Santos *et al.*, 2011; van de Werken *et al.*, 2012). It is only after that, at the blastocyst stage, that cell division regulatory mechanism may function optimally and embryos are able to select out aneuploid cells.

However, epigenetic asymmetry alone does not explain the increased incidence of chromosome segregation errors in human embryos. In mouse cleavage stage embryos

aneuploidy is rare, even though their epigenetic asymmetry for H3K9me3 is even more pronounced than in humans, with a complete absence of H3K9me3 at paternal pericentric regions (Puschendorf *et al.*, 2008). Remarkably, when mouse embryos are created by injection of round spermatids (100% histone-associated DNA) into oocytes, an increased incidence of chromosome missegregation is reported (Yamagata *et al.*, 2009). This, together with evidence that sperm derived modified histones are retained in the mouse embryo (van der Heijden *et al.*, 2006), leads us to hypothesize that the epigenetic signature passed on by the sperm chromatin may contribute to the erroneous chromosome segregation in embryos. Interestingly, higher levels of histone retention in sperm of infertile compared to fertile men have been described (Ramos *et al.*, 2008). It seems plausible that this variation in packaging of DNA with modified histones affects the proper transfer of epigenetic marks associated with pericentric heterochromatin. Furthermore, we observed that H3pT3 and CPC accumulation was significantly reduced on the paternal chromosomes when Aurora activity was compromised. This may indicate that paternal chromosomes are more readily predisposed for misalignment, a hypothesis that awaits further exploration. Future studies investigating the paternal contribution to chromosome aneuploidy and mosaicism may allow a better understanding on the origins of aneuploidy and mosaicism in human embryos, which so far has mainly focused on the contribution of the oocyte.

Concluding remarks

Chromosome segregation errors are a well known inherent feature of human preimplantation embryos. While most studies on the subject focused on finding the best methods to identify and quantify embryo aneuploidy and mosaicism and speculated about possible causes for post-zygotic mitotic errors, little has been done to unravel its molecular mechanism. The work described in this thesis provides evidence that chromosomal mosaicism is compatible with embryo implantation, with unknown consequences for further development and later health. We further uncovered several hitherto unknown differences in the mechanisms important for faithful chromosome segregation. Overall, our work provides the first insights into the regulation of chromosome segregation in human preimplantation embryos, while establishing a parallel to some of the molecular pathways described in somatic cells. Further systematic analysis of the consequences of the differences described in this thesis will contribute to a better understanding of the origins of aneuploidy in early human embryos. Ultimately, that knowledge may allow for optimization of the production and selection of IVF embryos and improve success rates after IVF.



Chapter 8

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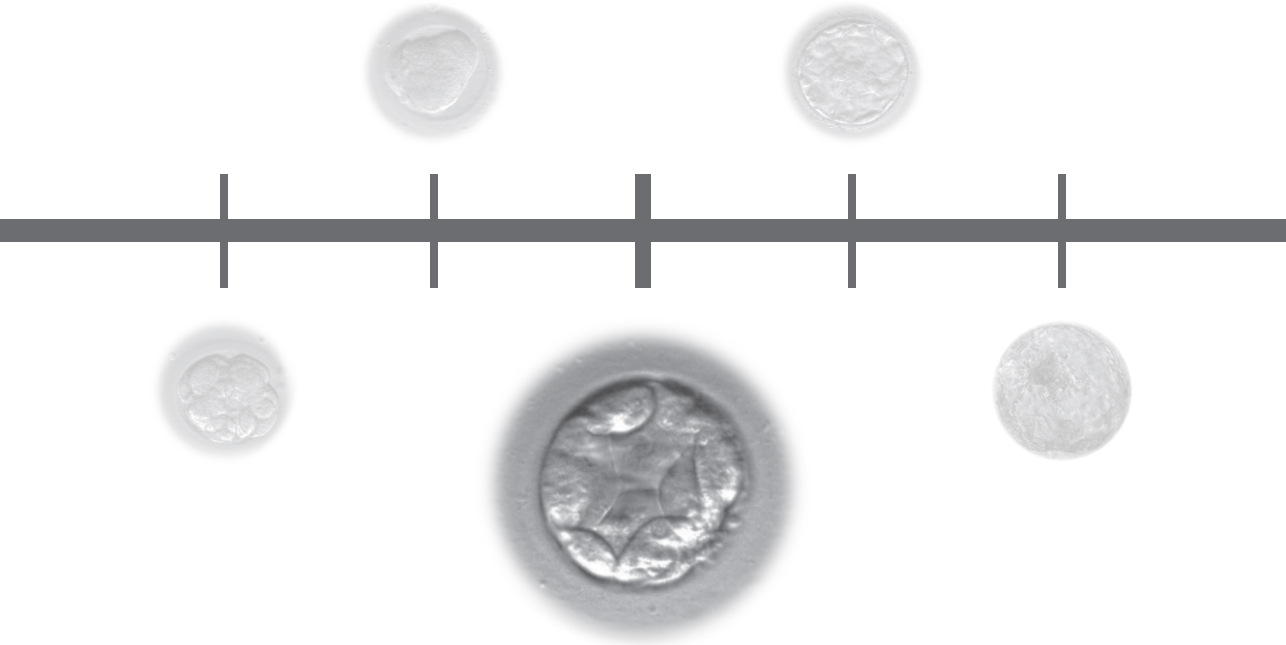

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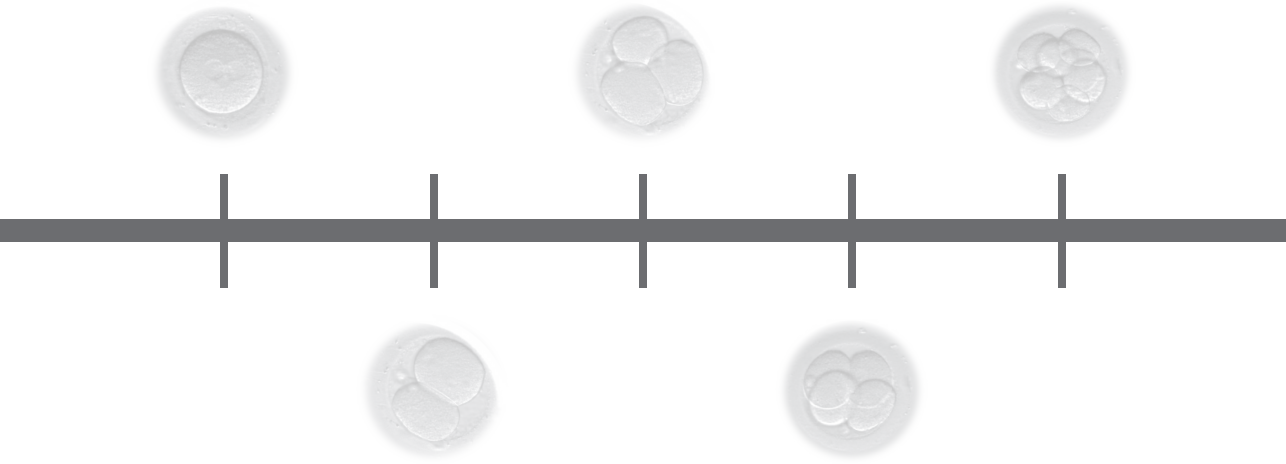
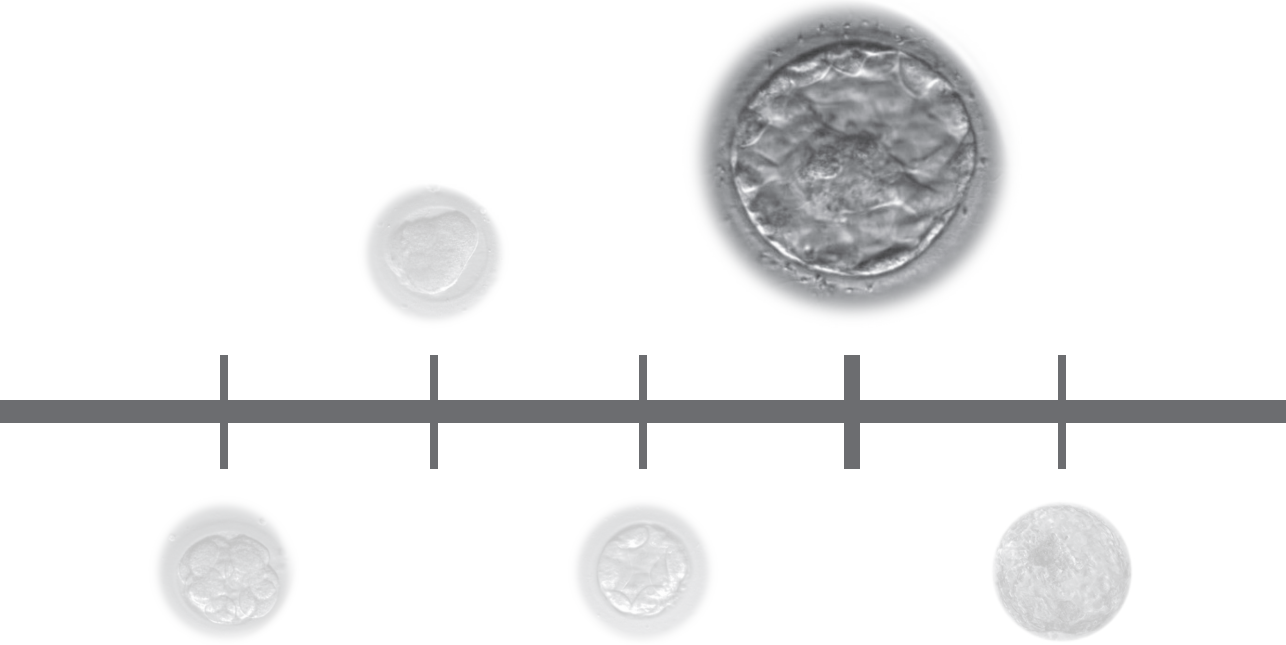


Chapter 9

Summary

Nederlandse samenvatting

Resumo em Português



Summary

Chapter 1

The introduction of this thesis gives an overview of the background information necessary to understand the rationale behind the studies conducted. We start by presenting chromosomal abnormalities as the main factor explaining the poor efficiency of human reproduction and chromosomal instability as an inherent feature of most IVF-produced preimplantation embryos. A brief summary on the endocrine mechanisms regulating follicle growth and oocyte maturation is given to allow the reader a better understanding on how ovarian stimulation used in IVF patients interferes with the natural selection of follicles and possibly affects the chromosomal competence of IVF-derived embryos. We then introduce the concept of chromosomal mosaicism in preimplantation embryos and review the mechanisms involved in the regulation of somatic cell divisions, i.e., the spindle assembly checkpoint and the chromosomal passenger complex. Finally we refer to the limited knowledge on the cell division regulatory mechanisms in mouse and human preimplantation embryos and acknowledge the need for a better understanding of the etiology of chromosome aneuploidy and mosaicism in early embryos.

Chapter 2

This chapter reviews the literature available in order to evaluate the impact of ovarian stimulation using high doses of exogenous gonadotropins on oocyte maturation, embryo development, epigenetic competence of oocytes and embryos, endometrial receptivity and perinatal outcomes. In this review we consider the evidence for these effects and address possible underlying mechanisms. We conclude that such mechanisms are still poorly understood and further knowledge is needed in order to increase the safety of ovarian stimulation and to reduce potential effects on embryo development and implantation, which will ultimately be translated into increased pregnancy rates and healthy offspring.

Chapter 3

Post-zygotic chromosome segregation errors are very common in human cleavage stage embryos after *in vitro* fertilization, resulting in chromosomally mosaic embryos. However, little is known regarding the significance of mosaicism for the developmental potential of preimplantation embryos. In this chapter we assessed chromosomal constitution and development of embryos from compaction to the peri-implantation stage at day 8 after fertilization.

From a pool of cryopreserved day 4 human embryos, a group was immediately fixed after thawing and all cells analyzed by fluorescent *in situ* hybridisation (FISH). The remaining group of thawed day 4 embryos was subjected to biopsy of one or two cells followed by FISH analysis. Detection of chromosomes 1, 7, 13, 15, 16, 18, 21, 22, X and Y was performed in two rounds of FISH. Biopsied embryos were kept in standard culture conditions for 24h. After that period, embryos arrested before cavitation were fixed and analyzed by FISH, whereas developing day 5 blastocysts were submitted to co-culture for a further 72h on a monolayer of

decidualized endometrial stromal cells, followed by fixation and FISH analysis. Cell numbers were counted and all nuclei analyzed by FISH. Data derived from a previous FISH analysis done by our group on cryopreserved good quality day 5 blastocysts were included in the present study.

Our results showed that an estimate 80% of day 4 embryos were mosaic and 11% showed a chaotic chromosomal constitution. FISH analysis of two blastomeres from morula stage embryos confirmed the high levels of mosaicism, showing that 54% of the biopsied embryos were mosaic, 40% normal, and 6% abnormal. FISH analysis of whole day 4, 5 and day 8 embryos showed a decrease in the incidence of mosaic embryos over time, from 83% on day 4 to 42% on day 8. Furthermore, we found a significant positive correlation between the total cell number and the percentage of normal cells in developing day 5 and day 8 embryos but not in day 4 morulas or embryos arrested before cavitation.

These data suggest that the developmental arrest of a significant proportion of mosaic embryos on day 4 together with the cell death or reduced proliferation of aneuploid cells within an embryo may be responsible for the observed decrease in the proportion of aneuploid cells from compaction to the peri-implantation stage.

Chapter 4

The work described in this chapter aims at identifying the molecular mechanisms that underlie the high incidence of chromosomal segregation errors observed in cleavage stage human embryos generated by IVF. For that purpose, we investigated the behavior of the chromosomal passenger complex (CPC) in human oocytes and embryos. This important mitotic regulatory complex consists of INCENP, Survivin, Borealin and Aurora B, with the meiotic kinase Aurora C as a possible alternative subunit.

We were able to detect all three non-catalytic subunits of the CPC (INCENP, Survivin and Borealin) at the inner centromere of prometaphase chromosomes in oocytes, and all stages of preimplantation development investigated (from zygote to blastocyst stage). However, we observed variations in the catalytic subunit involved in the CPC. Whereas Aurora B and C are both present in oocytes, Aurora C becomes the most prominent kinase in the CPC during the first three embryonic cell cycles. Moreover, we detected upregulation of Aurora C and Aurora B mRNA in early day 4 embryos and both proteins were detected at the morula stage. Subsequently, only Aurora B was detected in blastocysts. Therefore, in contrast to somatic cells, our results point to a specific role for Aurora C in the CPC during preimplantation embryo development up to the morula stage. Although the presence of Aurora C in itself may not explain the high chromosome segregation error rate, functional differences between Aurora B and C, as well as regulation of the balance in expression of these kinases up to the morula stage may help in identifying crucial factors.

Chapter 5

In this chapter we further investigated the molecular mechanisms that may be involved in the generation of chromosome missegregations during the first mitotic divisions. The CPC is an important component of the mitotic checkpoint, as it regulates chromosome segregation by

correcting erroneous attachments of the chromosomes to the mitotic spindle. In order to take on this role, the CPC must be precisely localized at the inner centromere. In somatic cells, centromeric localization of the CPC depends on two histone modifications at the centromeres: phosphorylation of histone H3 at Thr3 (H3pT3) and H2A at Thr120 (H2ApT120) by Haspin and Bub1 kinases, respectively. We therefore investigated the dynamics of these phosphorylations in human preimplantation embryos in the context of the epigenetic asymmetry between the chromatin derived from sperm and oocyte for trimethylation of histone H3 at Lys 9 (H3K9me3).

Our results showed that H2ApT120 is enriched at centromeres and co-localized with the CPC in all stages of embryonic development investigated. However, H3pT3 was ubiquitously distributed on chromosomes of human zygotes and only became enriched at centromeres from the 2 cell stage onwards. Inhibition of Aurora kinase activity in zygotes resulted in decreased H3T3 phosphorylation and altered CPC localization, as described in somatic cells. However, maternal and paternal chromosomes were affected differently and CPC subunits co-localized with H3K9me3-positive regions. These results suggest that in human zygotes the mechanism regulating targeting of the CPC to the inner centromeres partially differs from that described in somatic cells. We propose that the differences found may compensate for the epigenetic asymmetry between parental chromosomes, assuring correct CPC localization to centromeres for all chromosomes, independent of their parental origin.

Chapter 6

Aneuploidy and chromosomal mosaicism are commonly identified in human preimplantation IVF embryos, and believed to contribute to the relatively low IVF success rates. Most of these chromosomal imbalances originate from defective chromosome segregation during the first mitotic divisions. In adult somatic cells, chromosome segregation errors are rare, as cells are equipped with elaborate checkpoint mechanisms that prevent errors.

In this chapter we aimed at a better understanding of the cell cycle machinery involved in chromosome segregation during the first mitotic division of human embryos. We reviewed the current literature and catalogued mechanisms that take place exclusively during the first division in humans in comparison to those described in somatic mitosis and mouse embryonic divisions. One of the most striking differences is the pronounced parental epigenetic asymmetry present in human zygotes, which also differs from mouse. We conclude that the unique features of the first cleavage division allow us to set it apart from somatic mitotic cell division. We furthermore hypothesize a paternal epigenetic contribution to the incidence of segregation errors.

Chapter 7

In this chapter the most important conclusions from the conducted studies are summarized and implications for clinical practice and future research are discussed.

Nederlandse samenvatting

Reproductie is het fundamentele proces voor het voortplanten van alle soorten zoogdieren. Vergeleken met andere zoogdieren, is de humane reproductie bij mensen een relatief inefficiënt proces. Bij mensen is de kans op een spontane zwangerschap na tijdige geslachtsgemeenschap hoogstens 20-30%, een stuk lager dan in andere soorten zoals de resusaap (70%). De inefficiëntie van de humane voortplanting wordt vooral verklaard door het voorkomen van chromosomale afwijkingen die zijn geobserveerd in het merendeel van de onderzochte spontane abortusmonsters.

Chromosomen zijn georganiseerde structuren waarin menselijke cellen hun genetisch materiaal opslaan. Iedere cel bevat 23 paar chromosomen. Als een cel zich deelt om twee identieke dochtercellen te vormen, een proces dat mitose genoemd wordt, moet het genetische materiaal gelijk verdeeld worden om er voor te zorgen dat de genetische integriteit wordt bewaard. Foutieve chromosoomsegregatie kan leiden tot aneuploïdie, oftewel het vormen van dochtercellen met een abnormale hoeveelheid chromosomen.

Foutieve chromosoomsegregatie is een welbekend inherent kenmerk van humane pre-implantatie embryo's. Het onderliggende moleculaire mechanisme is echter nog niet goed bekend. Het in dit proefschrift beschreven werk bevat bewijs dat chromosomaal mosaïcisme verenigbaar is met de implantatie van het embryo, met onbekende consequenties voor de verdere ontwikkeling en de latere gezondheid. Ons werk bevat bovendien de eerste inzichten in de moleculaire mechanismen die chromosoomsegregatie reguleren in humane pre-implantatie embryo's.

Hoofdstuk 1

De inleiding van dit proefschrift geeft de achtergrondinformatie die nodig is om de gedachte achter de uitgevoerde studies te begrijpen. We beschrijven chromosomale afwijkingen als de belangrijkste factor om de lage efficiëntie van humane reproductie te verklaren en chromosomale instabiliteit als een eigenschap die inherent is aan de meeste embryo's die door IVF zijn ontstaan. We geven een korte samenvatting van de endocriene mechanismen die follikelgroei en eicelmaturatie reguleren. De lezer kan zo inzien hoe ovariële stimulatie, toegepast bij IVF patiënten, de natuurlijke selectie van follikels tegengaat en daardoor mogelijk de chromosomale integriteit van IVF-embryo's beïnvloedt. Daarna beschrijven we het concept van chromosomaal mosaïcisme in pre-implantatie embryo's en de mechanismen die betrokken zijn bij de regulatie van somatische celdelingen, zoals het spindle assembly checkpoint en het chromosomal passenger complex. Als laatste wijzen we op de beperkte kennis van deze mechanismen in muizen- en humane pre-implantatie embryo's en op het belang van een beter begrip van de etiologie van chromosomale aneuploïdie en mosaïcisme in embryo's.

Hoofdstuk 2

Dit hoofdstuk beschrijft een literatuurstudie naar de invloed van ovariële stimulatie met hoge doses exogene gonadotrofines op eicelmaturatie, embryo-ontwikkeling, epigenetische

eigenschappen van eicellen en embryo's, endometrium receptiviteit en perinatale uitkomsten. We beschrijven het bewijs voor deze effecten en bediscussiëren mogelijke onderliggende mechanismen. We concluderen dat er meer kennis van deze mechanismen nodig is om de veiligheid van ovariële stimulatie te verhogen en de mogelijke nadelige effecten op embryo-ontwikkeling en implantatie te reduceren, wat uiteindelijk zal leiden tot een grotere kans op zwangerschap en gezonde kinderen.

Hoofdstuk 3

Post-zygotische chromosoomsegregatie fouten komen zeer vaak voor in het klievingsstadium van humane embryo's die zijn ontstaan na IVF. Dit resulteert in chromosomaal mozaïeke embryo's. Er is echter weinig bekend over de invloed van mosaïcisme op de capaciteit van pre-implantatie embryo's om zich te ontwikkelen. In dit hoofdstuk bestudeerden wij de chromosomale samenstelling en de ontwikkeling van embryo's vanaf compactie tot het peri-implantatie stadium op dag 8 na bevruchting.

Van een groep op dag 4 gecryopreserveerde humane embryo's werd een deel direct na ontdooiing gefixeerd, om vervolgens alle cellen te analyseren met behulp van fluorescent *in situ* hybridisation (FISH). Van het andere deel van de embryo's werden 1 of 2 cellen weggehaald door middel van een biopsie en geanalyseerd met FISH. Detectie van chromosoom 1, 7, 13, 15, 16, 18, 21, 22, X en Y werd uitgevoerd in twee FISH rondes.

Gebiopteerde embryo's werden 24 uur onder standaard condities gekweekt. Daarna werden embryo's die niet verder ontwikkeld waren tot het cavitatie stadium gefixeerd en geanalyseerd met FISH. Embryo's die zich wel ontwikkelden tot een dag 5 blastocyst werden 72 uur gekweekt op een monolaag gedecidualiseerde stromale endometriumcellen en daarna gefixeerd. Het aantal cellen werd geteld en alle kernen werden geanalyseerd met FISH. Resultaten die onze groep eerder verkreeg in een FISH analyse van gecryopreserveerde dag 5 blastocysten werden meegenomen in deze studie.

Onze resultaten laten zien dat ongeveer 80% van de dag 4 embryo's mozaïek was en 11% een chaotische chromosomale samenstelling had. FISH analyse van twee blastomeren van embryo's in het morula stadium bevestigde de hoge aantallen van mosaïcisme; 54% van de gebiopteerde embryo's was mozaïek, 40% normaal en 6% abnormaal. FISH analyse van alle cellen van dag 4, dag 5 en dag 8 embryo's toonde aan dat de incidentie van mosaïcisme afneemt gedurende de ontwikkeling, van 83% op dag 4 tot 42% op dag 8. Daarnaast vonden we een significante positieve correlatie tussen het totaal aantal cellen en het percentage normale cellen in zich ontwikkelende dag 5 en dag 8 embryo's, maar niet in dag 4 morula's of embryo's die zich niet verder ontwikkeld hadden tot het cavitatie stadium.

Deze resultaten suggereren dat de afname van het percentage aneuploïde cellen van het compactie stadium tot het peri-implantatie stadium veroorzaakt zou kunnen worden door het niet verder ontwikkelen van mozaïeke embryo's vanaf dag 4 en door de celdood of verminderde proliferatie van aneuploïde cellen in een embryo.

Hoofdstuk 4

De studie die in dit hoofdstuk beschreven wordt, had als doel de moleculaire mechanismen

verantwoordelijk voor de hoge incidentie van chromosoomsegregatiefouten in humane IVF-embryo's te identificeren. Daarvoor bestudeerden wij het chromomal passenger complex (CPC) in humane eicellen en embryo's. Dit complex, dat belangrijk is voor de regulatie van mitose, bestaat uit INCENP, Survivin, Borealin en Aurora B, en heeft het meiotische kinase Aurora C als alternatieve subunit.

We detecteerden de drie niet-katalytische subunits van het CPC (INCENP, Survivin en Borealin) in het inner centromere gebied van prometafase chromosomen in eicellen en alle stadia pre-implantatie embryo's (van zygoot tot blastocyst). We detecteerden echter variatie in de katalytische subunit van het CPC; Aurora B en C waren beiden aanwezig in eicellen, maar gedurende de eerste drie embryonale celcycli was voornamelijk Aurora C aanwezig. Daarnaast vonden we ook een toename van Aurora B en C mRNA in dag 4 embryo's en beide eiwitten werden gedetecteerd in het morula stadium. Daarna, in het blastocyst stadium, werd alleen Aurora B gedetecteerd. Onze resultaten wijzen erop dat, anders dan in somatische cellen, Aurora C een specifieke functie heeft in het CPC gedurende pre-implantatie embryo ontwikkeling tot het morula stadium. Hoewel de aanwezigheid van Aurora C op zich geen verklaring is voor het hoge aantal chromosoomsegregatie fouten, zouden functionele verschillen tussen Aurora B en C en de regulatie van de balans in expressie van deze twee kinases tot het morula stadium wel mogelijkheden kunnen geven voor de identificatie van cruciale factoren.

Hoofdstuk 5

In dit hoofdstuk hebben we de moleculaire mechanismen bestudeerd die betrokken zouden kunnen zijn bij het ontstaan van chromosoomsegregatie fouten gedurende de eerste mitotische delingen. Het CPC is een belangrijk onderdeel van het mitotic checkpoint; het reguleert chromosoomsegregatie door verkeerde aanhechtingen tussen chromosomen en de spoelfiguur te corrigeren. Om die functie te kunnen uitvoeren, moet het CPC precies lokaliseren in het inner centromere gebied. In somatische cellen is deze lokalisatie afhankelijk van twee histon modificaties op de centromeren: fosforylatie van histon H3 op threonine 3 (H3pT3) en van histon H2A op threonine 120 (H2ApT120), die gekatalyseerd worden door respectievelijk Haspin en Bub1. We hebben daarom deze modificaties onderzocht in humane pre-implantatie embryo's in de context van de epigenetische asymmetrie van histon H3 trimethylatie op Lysine 9 (H3K9me3) tussen het chromatine afkomstig uit de zaadcel en de eicel.

Onze resultaten laten zien dat H2ApT120 verrijkt is op de centromeren en co-lokaliseert met het CPC in alle onderzochte stadia van embryo-ontwikkeling. H3pT3 was echter verdeeld over het hele chromosoom in humane zygoten en werd pas verrijkt op de centromeren vanaf het 2-cellig stadium. Inhibitie van Aurora kinase activiteit in zygoten resulteerde in een afname van H3T3 fosforylatie en een veranderde CPC lokalisatie, zoals ook beschreven is voor somatische cellen. In zygoten was het effect echter verschillend op maternale en paternale chromosomen en lokaliseerden de CPC subunits op H3K9me3-positieve gebieden. Deze resultaten suggereren dat het mechanisme dat de lokalisatie van het CPC op de inner centromere reguleert, verschilt van dat wat beschreven is voor somatische cellen. Wij denken dat het gevonden verschil mogelijk compenseert voor de epigenetische asymmetrie tussen

de maternale en paternale chromosomen en daarmee een correcte CPC lokalisatie op de centromeren van alle chromosomen, onafhankelijk van de herkomst, bewerkstelligt.

Hoofdstuk 6

Aneuploïdie en chromosomaal mosaïcisme komen vaak voor in humane pre-implantatie IVF-embryo's en worden verondersteld bij te dragen aan de relatief lage kans op succes van IVF. De meeste van deze chromosomale afwijkingen ontstaan als gevolg van fouten in chromosoomsegregatie gedurende de eerste mitotische celdelingen. In somatische cellen zijn dit soort fouten zeldzaam, omdat cellen zijn uitgerust met diverse checkpoint mechanismen die deze fouten voorkomen.

In dit hoofdstuk wilden we meer inzicht krijgen in de mechanismen die betrokken zijn bij chromosoomsegregatie in de eerste mitotische delingen van humane embryo's. We deden een literatuurstudie en vergeleken mechanismen die alleen een rol spelen tijdens de eerste delingen in humane embryo's met mechanismen die beschreven zijn voor somatische cellen en muizenembryo's. Een van de meest opvallende verschillen is de epigenetische asymmetrie tussen maternale en paternale chromosomen in humane zygoten, iets dat ook verschilt van de situatie in muizenembryo's. We concluderen dat de unieke eigenschappen van de eerste klievingsdelingen ons in staat stellen onderscheid te maken tussen pre-implantatie embryonale mitotische celdelingen en somatische mitotische celdelingen. We veronderstellen ook dat er een paternale epigenetische bijdrage is aan de incidentie van segregatie fouten.

Hoofdstuk 7

In dit hoofdstuk vatten we de belangrijkste conclusies van de uitgevoerde studies samen en bediscussiëren we mogelijke implicaties daarvan voor de kliniek en toekomstig onderzoek.

Resumo em Português

A reprodução é o processo pelo qual todas as espécies de mamíferos se propagam. Nos seres humanos o processo reprodutivo é bastante ineficiente quando comparado com o de outros mamíferos. A probabilidade de uma mulher engravidar de modo espontâneo após relações sexuais programadas é de apenas 20-30%, um valor bastante mais baixo do que por exemplo os 70% calculados para o macaco-rhesus. Crê-se que a baixa eficácia reprodutiva dos humanos se deva ao elevado número de abortos espontâneos que ocorrem nos estádios mais precoces da gravidez, mesmo antes de esta ser detetada clinicamente. A identificação de anomalias cromossómicas em amostras de tecido provenientes de abortos espontâneos permitiu estabelecer uma correlação entre a ocorrência de anomalias cromossómicas e a baixa eficácia reprodutora dos seres humanos.

As células humanas possuem o seu material genético guardado sob a forma de cromossomas e cada célula possui 23 pares de cromossomas. A divisão de uma célula-mãe em duas novas células filhas, um processo denominado mitose, é acompanhada pela divisão igualitária do material genético de modo a garantir a manutenção da integridade genética. Falhas neste processo dão origem a aneuploidia, i.e., a geração de células com um número errado de cromossomas.

Apesar da elevada incidência de anomalias cromossómicas identificadas em células de embriões humanos no estádio pré-implantacional, desconhecem-se os mecanismos moleculares que estão na origem deste fenómeno. O trabalho descrito nesta tese tem por objetivo mostrar que a existência de aneuploidia e mosaicismo cromossómico é compatível com o desenvolvimento e implantação de embriões humanos, embora se desconheçam possíveis consequências para o posterior desenvolvimento e saúde do feto. Além disso, esta tese fornece as primeiras evidências dos mecanismos envolvidos na monotorização da divisão celular e segregação dos cromossomas em embriões humanos no estádio pré-implantacional.

Capítulo 1

Este primeiro capítulo serve de introdução aos assuntos abordados ao longo desta tese, fornecendo as bases de conhecimento necessárias à compreensão dos estudos aqui apresentados. Começamos por dar a conhecer ao leitor a questão das anomalias cromossómicas como um fenómeno inerente ao desenvolvimento da maioria dos embriões humanos produzidos *in vitro*, o qual é apontado como a principal causa para a baixa eficácia reprodutiva nos seres humanos. De seguida, apresentamos um resumo dos principais processos endócrinos que regulam o crescimento dos folículos e a maturação dos oócitos. Introduzimos também a questão da possível influência que a estimulação ovárica usada em pacientes de procriação medicamente assistida (PMA) poderá ter sobre a seleção natural dos folículos e constituição cromossómica dos embriões gerados. Além disso introduzimos os principais mecanismos moleculares de monitorização da divisão celular somática e apresentamos a questão do conhecimento limitado no que diz respeito a esses mesmos mecanismos de divisão durante o desenvolvimento embrionário de ratinhos e humanos.

Capítulo 2

Neste capítulo procedeu-se à revisão da literatura existente de modo a avaliar o impacto da administração de elevadas doses de gonadotrofina para estimulação ovárica a nível da maturação dos oócitos, do desenvolvimento embrionário, da epigenética de oócitos e embriões, da recetividade do endométrio e dos resultados perinatais. Da nossa análise conclui-se que o limitado conhecimento sobre estes mesmos mecanismos requer o desenvolvimento de novos estudos que permitirão reduzir os potenciais efeitos da estimulação ovárica no desenvolvimento embrionário e na implantação, o que permitirá aumentar as taxas de gravidez após PMA e garantir uma melhor saúde das futuras crianças.

Capítulo 3

Grande parte dos embriões humanos em estádios de clivagem após fertilização *in vitro* são mosaicos cromossómicos. Tal deve-se à ocorrência de erros no processo de segregação cromossómica que decorre durante as primeiras divisões celulares após formação do zigoto. Porém, pouco se sabe sobre as influências que o mosaicismo cromossómico possa ter sobre o desenvolvimento embrionário e a implantação. No capítulo 3 averiguou-se a constituição cromossómica e o potencial de desenvolvimento de embriões humanos a partir do estádio de mórula (4 dias pós-fertilização) até ao estádio de peri-implantação (8 dias pós-fertilização).

A análise de hibridação de fluorescência *in situ* (FISH) efetuada em todas as células de embriões em estádio de mórula revelou que 83% dos embriões analisados são mosaicos cromossómicos, 11% apresentam uma constituição cromossómica caótica e apenas 6% foram classificados como cromossomicamente normais. A análise de FISH em todas as células de embriões em estádio de blastocisto 5 e 8 dias pós-fertilização, revelou uma diminuição na percentagem de embriões classificados como mosaicos cromossómicos para 42% no dia 8 (estádio de peri-implantação). Além disso, identificámos uma correlação positiva significativa entre o número total de células de cada embrião e a percentagem de células cromossomicamente normais (com o número correto de cromossomas). Ou seja, quanto maior o número de células que constituem o embrião, maior a percentagem de células cromossomicamente normais. Contudo, esta correlação foi apenas observada após o estádio de cavitação, ou seja, aquando a formação de blastocistos (5 e 8 dias pós-fertilização), mas não em embriões em estado de mórula (dia 4) nem em embriões com retardamento no desenvolvimento no dia 5 pós-fertilização.

Estes resultados sugerem que o retardamento no desenvolvimento de uma proporção significativa dos embriões mosaicos cromossómicos por volta do dia 4 pós-fertilização, juntamente com a morte ou reduzida proliferação de células aneuplóides, pode ser responsável pela diminuição na proporção de células aneuplóides observada nos estádios de blastocisto 5 e 8 dias pós-fertilização.

Capítulo 4

O trabalho apresentado neste capítulo teve por objetivo a caracterização dos mecanismos moleculares que estão subjacentes aos frequentes erros de segregação cromossómica verificados em embriões humanos gerados por fertilização *in vitro* (IVF) durante os estádios

de clivagem.

O *chromosomal passenger complex* (CPC) é um complexo proteico que participa num mecanismo de monitorização da divisão celular denominado *checkpoint* mitótico. Este mecanismo permite verificar e corrigir a ligação dos cromossomas aos microtúbulos do fuso, a qual é essencial à correta segregação de cromossomas de modo a garantir a manutenção da integridade genética após a divisão celular. Em células somáticas o CPC é constituído por 3 subunidades não catalíticas, INCENP, Borealin e Survivin; e uma catalítica, Aurora B. A elevada frequência de erros na segregação de cromossomas durante as primeiras divisões celulares em embriões humanos levou a que alguns cientistas postulassem que este fenómeno dever-se-ia à ausência de mecanismos de monitorização da divisão celular durante os estádios de clivagem do desenvolvimento embrionário. Porém, os nossos dados mostram que o CPC está presente tanto em oócitos como em embriões humanos, desde o estádio de zigoto até ao de blastocisto. Além disso, mostram também a presença de diferenças na subunidade catalítica do CPC. Enquanto Aurora B e C estão presentes em oócitos humanos, após fertilização e durante as três primeiras divisões embrionárias Aurora C passa a ser a cinase dominante. Mais tarde, por volta do estádio de mórula detetámos a presença de ambas as proteínas Aurora B e C, bem como um acréscimo nos níveis de RNA mensageiro das mesmas. Subsequentemente, no estádio de blastocisto, apenas Aurora B foi detetada tanto a nível da proteína como do RNA mensageiro.

Os nossos resultados sugerem que, ao contrário do que acontece em células somáticas, Aurora C desempenha uma função fundamental no funcionamento do CPC durante o desenvolvimento embrionário pré-implantacional, desde o estádio de zigoto ao de mórula (dias 1 e 4 pós-fertilização, respetivamente). Contudo, a presença de Aurora C por si só não permite explicar a elevada ocorrência de erros de segregação cromossómica durante estes estádios. O estudo de possíveis diferenças funcionais entre Aurora B e C, bem como de distúrbios no equilíbrio dos níveis de expressão dos RNA mensageiros de Aurora B e C entre o estádio de zigoto e mórula poderá ajudar a uma melhor identificação de fatores cruciais.

Capítulo 5

De modo a dar continuidade à pesquisa dos processos moleculares que poderão estar na origem dos erros de segregação de cromossomas, procedemos então ao estudo do mecanismo que regula a localização do CPC nos centrómeros de embriões humanos. Em células somáticas, o funcionamento do CPC depende da sua localização exata no centrómero interno. Essa localização é determinada por duas modificações de histonas do DNA centromérico: fosforilação da histona H3 na Treonina 3 (H3pT3) e fosforilação da histona H2A na Treonina 120 (H2ApT120), efetuadas pelas cinases Haspin e Bub1, respetivamente. Porém, desconhece-se se este mesmo mecanismo é conservado durante as primeiras divisões embrionárias em humanos. O nosso trabalho teve como objetivo o estudo das referidas fosforilações em embriões humanos durante os estádios de pré-implantação. Além disso, estabelecemos um paralelo entre os níveis de H3pT3 e H2ApT120 e os níveis de trimetilação da histona H3 na Lisina 9 (H3K9me3), os quais diferem significativamente entre a cromatina de origem paterna (derivada do espermatozoide) e a de origem materna (derivada

do oócito) aquando da primeira divisão celular.

Os nossos resultados mostram a concentração de H2ApT120 ao nível dos centrómeros, co-localizando-se com o CPC em todos os estádios de desenvolvimento embrionário humano investigados (zigoto, 2 células, 8 células e blastocisto), o que está de acordo com o padrão de distribuição em células somáticas. Em contrapartida, a detecção de H3pT3 revelou uma distribuição generalizada à totalidade do comprimento dos cromossomas em zigotos humanos. Porém, em embriões de duas células e subsequentes estádios de desenvolvimento, H3pT3 concentrou-se nos centrómeros, tal como acontece em células somáticas. A inibição da atividade catalítica de Aurora B e C em zigotos humanos resultou na diminuição dos níveis de fosforilação de H3T3 e alteração da localização do CPC, à semelhança do que fora anteriormente descrito em células somáticas. Contudo, este efeito revelou-se diferente entre os cromossomas de origem paterna e materna, já que a localização do CPC passou a coincidir com as regiões marcadas pela H3K9me3.

Estes resultados sugerem que em zigotos humanos os mecanismos que definem a localização do CPC no centrómero interno diferem em parte daqueles até agora descritos em células somáticas. Assim sendo, sugerimos que as diferenças identificadas façam parte de um mecanismo de compensação da assimetria epigenética verificada entre os cromossomas de origem materna e paterna, assegurando a correta localização do CPC nos centrómeros de todos os cromossomas, independentemente da sua origem parental.

Capítulo 6

A aneuploidia e o mosaicismo cromossómico são frequentemente identificados em embriões humanos pré-implantacionais gerados por IVF e pensa-se que possam contribuir para as relativas baixas taxas de sucesso da PMA. A maioria destas anomalias cromossómicas surge devido a erros na segregação dos cromossomas durante as primeiras divisões mitóticas. Em células somáticas os erros de segregação são raros, pois as células estão equipadas com mecanismos de monotorização da divisão celular que permitem a deteção e correção de erros durante a segregação dos cromossomas.

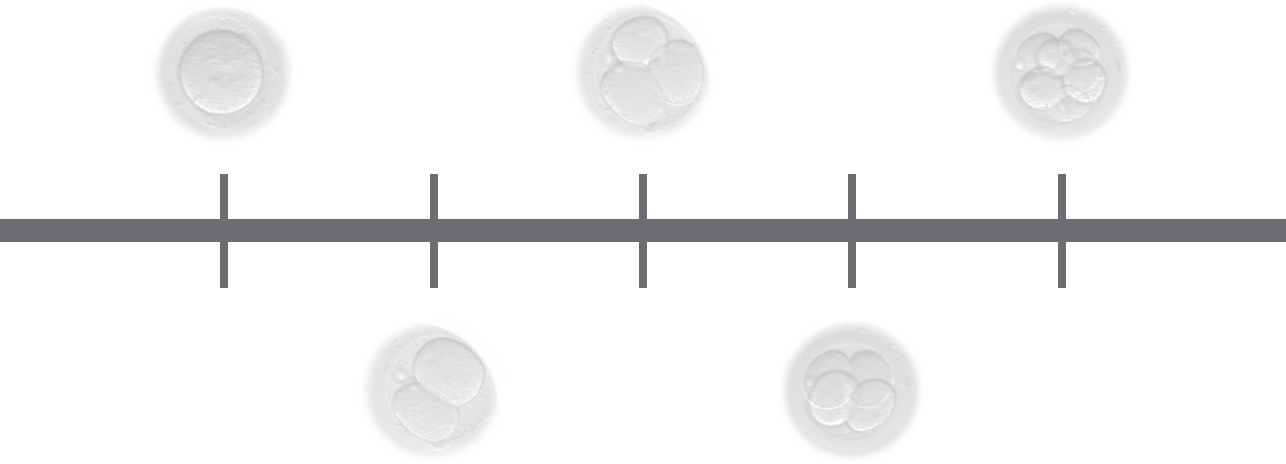
Este capítulo tem por objetivo proporcionar uma melhor compreensão da maquinaria do ciclo celular que está envolvida na segregação de cromossomas durante a primeira divisão mitótica em embriões humanos. Para tal, revimos a literatura corrente e inventariamos uma série de mecanismos que ocorrem exclusivamente durante a primeira divisão celular em humanos em comparação com as divisões somáticas e divisões embrionárias de ratinho. Uma das diferenças mais notórias é a acentuada assimetria epigenética detetada nos zigotos humanos, a qual difere da observada em zigotos de ratinho.

Neste capítulo concluímos que as características únicas da primeira divisão embrionária diferenciam-na das divisões somáticas. Além disso, sugerimos ainda que a herança epigenética paterna possa contribuir para a elevada frequência de erros verificados durante a segregação de cromossomas em embriões humanos.

Capítulo 7

Neste capítulo resumimos as principais conclusões extraídas de cada estudo apresentado

nesta tese e discutimos as implicações para a prática clínica e futura investigação.

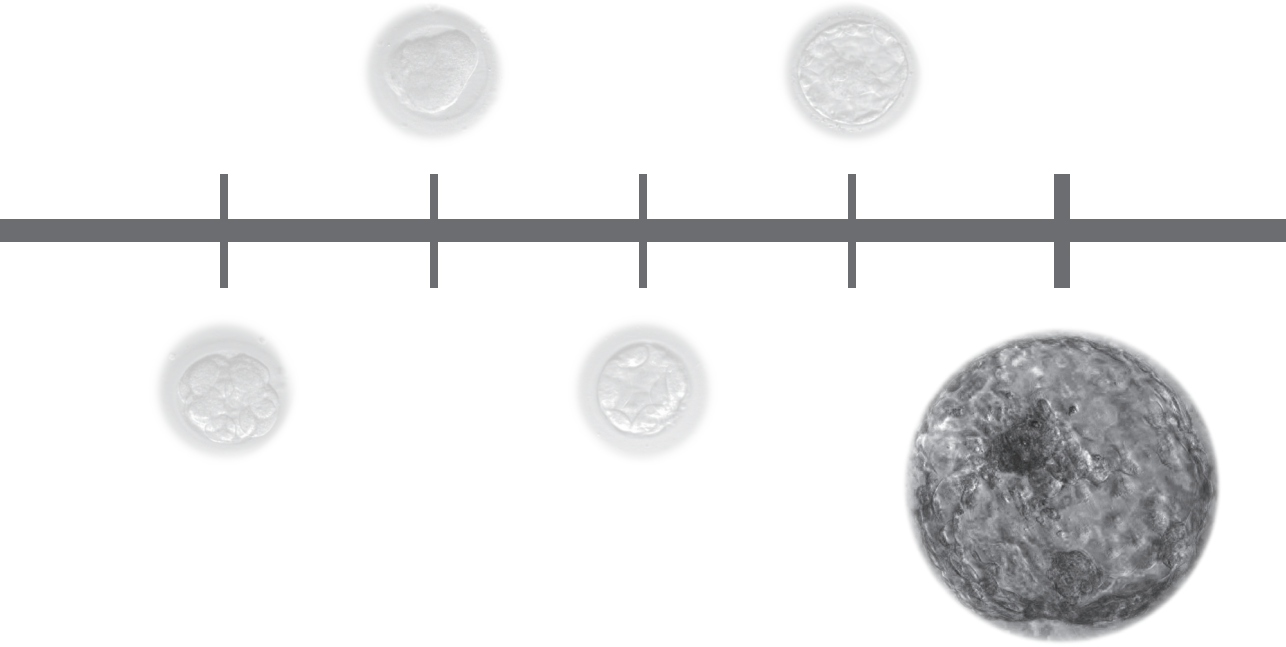


Chapter 10

Acknowledgements

List of publications

Curriculum Vitae



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List of publications

Avo Santos M, van de Werken C, de Vries M, Jahr H, Vromans MJ, Laven JS, Fauser BC, Kops GJ, Lens SM, Baart EB. *A role for Aurora C in the chromosomal passenger complex during human preimplantation embryo development*. Hum Reprod. 2011 Jul;26(7):1868-81.

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Curriculum Vitae

Margarida da Avó Ribeiro dos Santos was born on the 8th July in Alcobaça, Portugal. In June 2001 she completed her secondary school studies at Escola Secundária D. Pedro I in Alcobaça, Portugal. In the same year she started her studies in Microbiology and Genetics at the Faculty of Sciences in Lisbon. In 2004 she attended the *Universidad Complutense de Madrid*, as part of a six-month European Union Erasmus program. In July 2005 she completed the degree in Microbiology and Genetics. After graduation she worked for a year at *Instituto Gulbenkian de Ciência*, Oeiras, Portugal, under the supervision of Dr. Joaquín Léon, where she focused her research on vertebrate limb development. In September 2006 Margarida moved to London, where a year later she received the degree of Masters in Science in Reproductive and Developmental Biology from the Imperial College London. In 2007 she was awarded a PhD fellowship from the Portuguese Foundation for Science and Technology (FCT) to finance her PhD studies in The Netherlands. From January 2008 until August 2012 she worked on the research described in this thesis performed at the Department of Reproductive Medicine and Gynecology of the University Medical Center Utrecht and at the Department of Obstetrics and Gynecology of the Erasmus Medical Center Rotterdam, under the supervision of Prof. Dr. B. C. Fauser, Prof. Dr. J. S. Laven and Dr. E. B. Baart. Margarida has recently joined the IVF laboratory at the Diaconessenhuis Voorburg, The Netherlands, where she is working as clinical embryologist in training.