Epigenetic reprogramming during human oocyte maturation and early human development

Epigenetische reprogrammering gedurende de humane eicel-maturatie en vroege ontwikkeling

Ilse Maria van den Berg

Epigenetic reprogramming during human oocyte maturation and early human development
Thesis, Erasmus University Rotterdam, the Netherlands

The research presented in this dissertation was performed at the Departments of Obstetrics & Gynaecology and Clinical Genetics, Erasmus MC Rotterdam, The Netherlands

This thesis was financially supported by the Department of Obstetrics & Gynaecology and the Department of Clinical Genetics of the Erasmus MC

Printing of this thesis was further financially supported by:

BD Biosciences
The ESHRE award 2009
The Alpha award 2008

ISBN/EAN: 978-94-6191-167-4

Cover illustration by Evelien Mouthaan, www.evelienmouthaan.blogspot.com
Title: 'Een wolk van niet weten'
Lay-out by: Legatron Electronic Publishing, Rotterdam
Printed by: Ipskamp Drukkers B.V., Enschede

Epigenetic reprogramming during human oocyte maturation and early human development

Epigenetische reprogrammering gedurende de humane eicel-maturatie en vroege ontwikkeling

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.G. Schmidt

En volgens besluit van het College van promoties.

De openbare verdediging zal plaatsvinden op
woensdag 22 februari 2012 om 15:30

door

Ilse Maria van den Berg

Geboren te Roosendaal en Nispen

Promotiecommissie

Promotoren: Prof.dr. J.S.E. Laven
Prof.dr. F.G. Grosveld

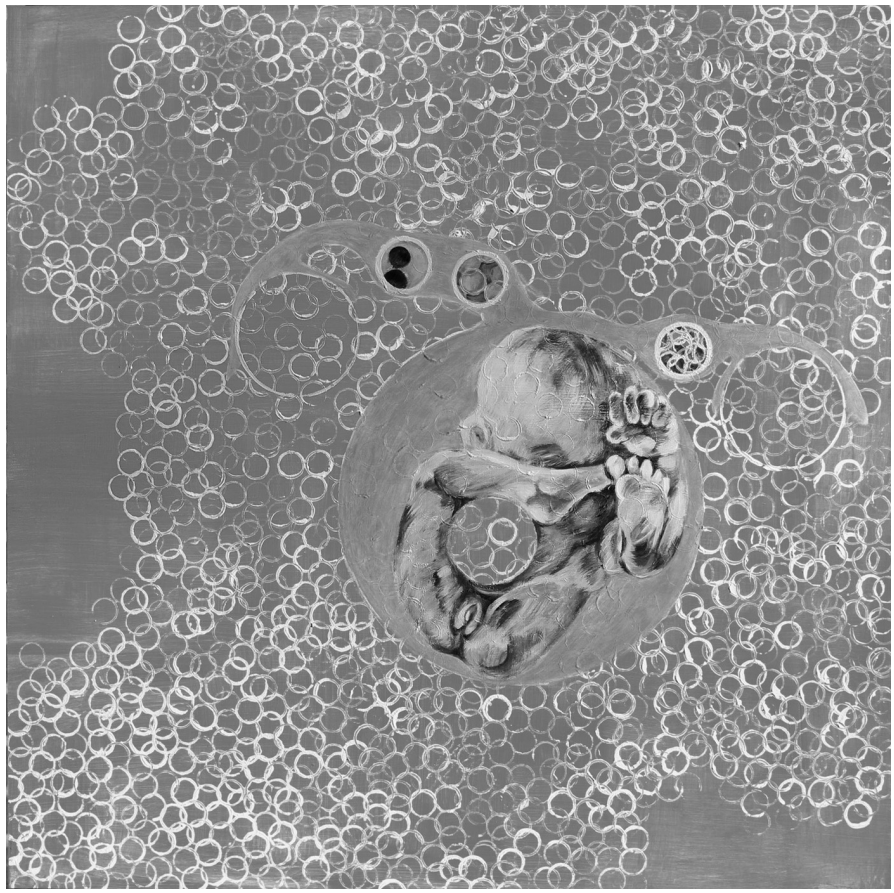
Overige leden: Dr. J. Gribnau
Dr. W.M. Baarends
Dr. R. Poot

Co-promotoren: Dr. J.H. van Doorninck
Dr. R-J. Galjaard

Paranimfen:
Sharon Hindriks
Jeroen Speksnijder

Contents

CHAPTER 1



Introduction

Circle of life

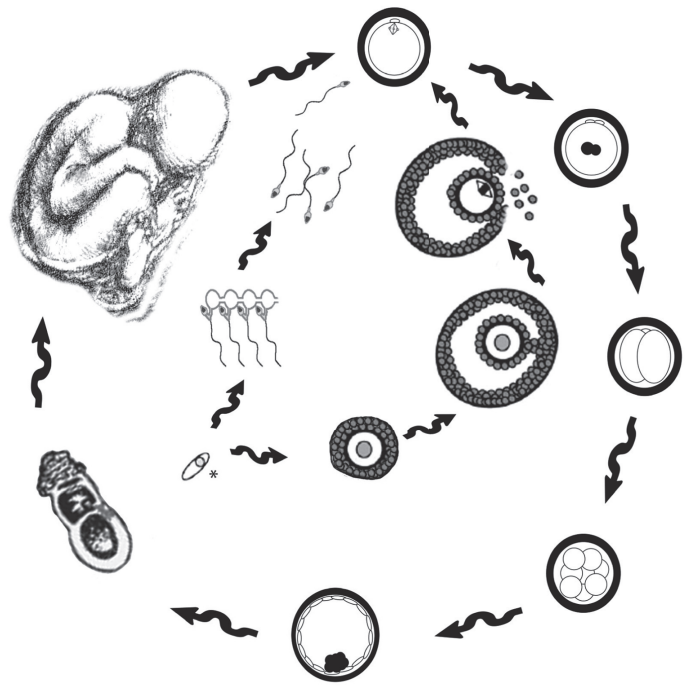


Figure 1. Schematic overview of the circle of development from gamete to gamete. Primordial germ cells (*) are produced during fetal development and mature into a gamete, either a spermatozoon or an oocyte. The next phase of the circle of life is the zygote which arises from the fertilization of an oocyte by a spermatozoon. The zygote divides into a 2-cell stage embryo which becomes an 8-cell embryo and eventually a blastocyst. The blastocyst develops into an embryo which will become a fetus.

Life starts with the fertilization of an oocyte by a spermatozoon which gives rise to a new being: the zygote (Figure 1). This zygote contains both parental DNA sets which are enclosed in separate nuclear membranes called pronuclei; the maternal pronucleus, derived from the oocyte and the paternal pronucleus, derived from the spermatozoon. The zygote is the first stage of embryonic development and divides and develops into a 2-cell embryo, followed by the morula and blastocyst stages. The human embryo, which is now a blastocyst, will implant into the womb around 7-9 days after fertilization. The time point of implantation differs between species, a mouse blastocyst implants around d4.5 while in bovines implantation takes place around d15.

After implantation the embryo grows organs and limbs, a nose, a mouth, ears and eyes. The reproductive tract is formed and the primordial germ cells migrate from the extra-embryonic mesoderm to the gonads. They eventually become either female oocytes or male sperm, after differentiation and maturation. With the fertilization of a new oocyte by a new sperm the circle of life is completed.

Male and female primordial germ cells and their progeny

Gametes are produced by gonads. Spermatozoa are formed in the testis, and oocytes are produced in the ovary. Although both the mature oocyte and spermatozoon originate from the same kind of precursor cells, the primordial germ cells (PGC's), their developmental process is strikingly different [1].

Both spermatogenesis and oogenesis have a period of arrest, however, the time point of this arrest during development differs. In the male germline, it follows immediately after the mitotic proliferation phase and the male precursors, that are now called spermatogonia B, enter a protracted period of arrest that lasts until puberty after which they will start meiosis. The oogonia also proliferate first, but contrary to spermatogonia, they enter meiosis during fetal development and arrest at the diplotene stage, now called dictyate, of the meiotic prophase around birth. Thus, the mammalian oocytes that exist at birth are the oocytes that will be fertilized in adult life.

It is perhaps not surprising that with advancing maternal age, the quality of oocytes diminishes and the long period of arrest at the phase of meiosis is associated with increased rate of chromosome division errors in human oocytes [2]. To understand how these errors could arise in mature oocytes, the development from oogonia to MII oocytes and the development of a follicle will be explained hereafter.

Oocytogenesis

Oogenesis is a dynamic and challenging process which is not yet fully understood. Around the 4^{th} week of human fetal development primordial germ cells start to migrate from the extra-embryonic mesoderm via the allantois and the adjacent yolk sac to the genital ridge where they differentiate into oogonia [1,3]. After an extensive phase of proliferation, the human oogonia enter the prophase of meiosis (box 1) around the 10^{th}–12^{th} week of gestation and are now called primary oocytes [1]. Primary oocytes develop in clusters. These clusters of oocytes, called cysts, are connected through cytoplasmic bridges. Initiation of meiosis is regulated by the

meiosis specific gene Stra8, which is transcriptionally controlled by retinoic acid [4,5]. During gestation, oocytes at different stages of the meiotic prophase can be observed simultaneously in fetal ovary [6]. Human oogonia enter leptotene around the 10^{th}–11^{th} week of gestation [6,7] and zygotene oocytes can be observed from week 11–22. Pachytene oocytes can be observed for the first time around week 12–13 and in week 14 the first diplotene oocytes can be seen. Thus altogether, it takes about four weeks for a human oocyte to complete the prophase of meiosis and arrest at dictyate.

Although mitotically dividing germ cells can be observed at birth in the female ovaries, and may possibly develop to oocytes, they are likely to be of compromised quality and it is thought that they will not populate the pool of primordial follicles [8]. However, the long accepted dogma that the generation of oocytes is restricted to the fetal period and does not occur in adult ovaries has been challenged. Johnson et al. observed large ovoid active cycling cells near the surface of mouse ovaries which express germ cell markers [9]. They proposed that these putative germ cells produce new oocytes during adult life which would mean that oogenesis is still ongoing in juvenile and adult mouse ovaries. This new theory on oocyte development was further supported by experimental data in a follow-up study from the same research group [9,10]. However, as these experiments could not be confirmed by other groups the debate is ongoing [11-14]. Although *in vitro* studies show the possibility for germ cell generation from somatic tissue and the initiation of meiosis from stem cells [15-17], the *in vivo* pathways are far from clear.

Females start with a large pool of PGCs, it peaks around 7 million at week 20 in humans, however, only 2 million remain at birth [18]. In mice, a dramatic breakdown of cysts occurs two days after birth, leaving only single oocytes with surrounding somatic cells [19]. Other species have a similar decrease in numbers of oocytes, both vertebrates as well as invertebrates, and the decrease is likely to be regulated by apoptosis. Although the underlying cellular and/or molecular cause of this loss of primary oocytes is not yet clear, studies have shown that genes in the apoptotic pathway, such as B-cell lymphoma/leukemia-2 (Bcl2), Bax and Caspase 2, are involved [20-23]. It has been postulated that this loss is a random process that continues into adult life and environmental or nutritional factors may aggravate this loss. Others have viewed it as an elimination of defective germ cells [24-27].

Follicular development

Follicular development encompasses the growth and differentiation of a primordial follicle to a Graafian follicle (Figure 2).

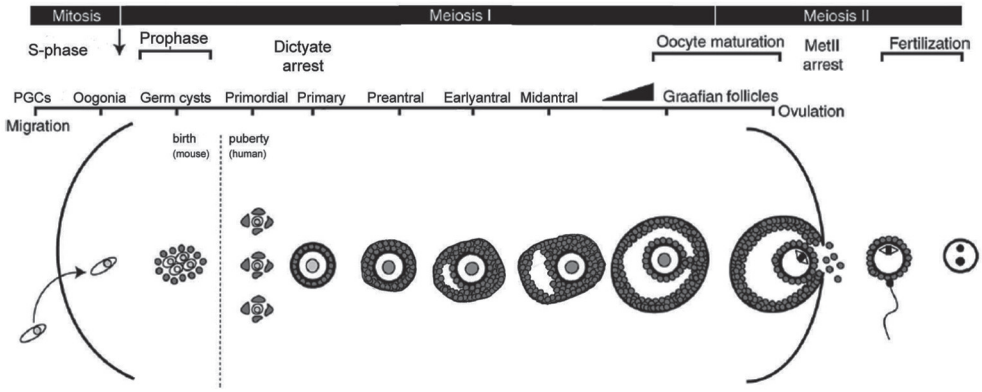


Figure 2. Schematic overview of oocyte and follicle development (adapted from [28]).

From puberty onwards, oocyte development recommences when primary oocytes are recruited for maturation and from then on each phase of follicle development can be found in the ovary. During follicle development, the granulosa cells surround the oocyte and together they form the oocyte-granulosa complex. The granulosa cells originate from the ovarian cortex and their function is to nurse and support oocyte development. Follicles can be classified into five stages based on the morphological characteristics of the granulosa cells, the appearance of the zona pellucida and the formation of the antral cavity. The five stages are: the primordial follicle, the primary follicle, the pre-antral follicle, the early-/mid-antral and the antral follicle, which is also called the Graafian follicle (reviewed in [29]). Follicular development in the human ovary culminates in either the ovulation of one, and occasionally two oocytes, or the degeneration (atresia) of the follicle and its oocyte. The follicle cells or granulosa cells are connected to the oocyte via gap junctions and through these communication and transport of molecules takes place between the oocyte and granulosa cells. Each follicular phase is characterized by transcription of stage specific genes such as Dazla, Flgα, Gdf9 and Bmp15 [30-35] and also involves endocrine regulation by hormones like the anti-müllerian hormone (AMH), gonadotropins, and estrogens ([36,37]) reviewed in [38]). An increasing body of evidence shows that estrogen-like chemicals such as Bisphenol A have negative effects on mammalian oocyte development by interfering with the endogenous hormonal regulation of follicle development [39-43]. Thus, environmental exposure to these chemicals may have consequences for oocyte development and might even have a grand-maternal effect through epigenetic interference [44]. However, phenotypic differences of the effect of Bisphenol A

on oocyte development have been described for different mouse strains [40,45]. One study showed an increase of aneuploidy in the oocytes of mice that were exposed to Bisphenol A [46], whereas Eichenlaub-Ritter et al. observed an arrest at the MI stage [45]. These differences may indicate that genotypic variation may influence the susceptibility to these compounds.

Mammalian oocyte development and follicle development are asynchronous and the oocyte remains in the dictyate stage of the prophase (Figure 2) during all the growing stages of follicle development. During this period the oocyte is transcriptionally active and does respond to environmental cues. In the growing phases of follicle development, key processes take place such as zona pellucida (ZP) formation, cumulus expansion, oocyte cytoplasmic differentiation, and nuclear maturation. Additionally, the oocyte has to stock pile mRNAs before it terminates transcription and enters the divisional stages of meiosis. This mRNA storage is needed for the first stages of embryonic development, when the embryo is transcriptionally quiescent (reviewed in [47]).

Only one antral follicle, the Graafian follicle, remains at the final stage of human follicle development. The oocyte, or occasionally two oocytes, will recommence meiosis under influence of the LH surge.

Nuclear maturation of oocytes

Nuclear maturation comprises the steps from the release from dictyate stage of prophase to the arrest at the metaphase II stage and is regulated by the cyclic activity of CDK1. High levels of cAMP are required to maintain the oocyte at the dictyate stage during follicle growth and sustain cytoplasmic maturation, a process which *in vivo* is regulated by the Gpr3 receptor [48,49]. A drop of cAMP leads to the breakdown of the nuclear envelope of the germinal vesicle (GV) and entry into the first meiotic division (MI). Thus, during *in vitro* maturation of oocytes from pre-antral follicles, the addition of cAMP analogs to the culture medium is required to prevent accelerated nuclear maturation [48,50,51].

Primary oocytes or germinal vesicle oocytes have a large nucleus which can be observed by light microscopy, the germinal vesicle. Germinal vesicle oocytes have two main chromatin configurations, initially a so called non surrounding nucleus (NSN) is present, followed by a surrounding nucleus (SN). GV oocytes with an SN organization have a heterochromatin rim that is not yet present in NSN GV oocytes and the chromatin is far more condensed in SN oocytes indicative of the onset chromatin condensation. Oocytes of the NSN configuration have high levels of transcription and are more immature than oocytes with a SN configuration which have terminated transcription. The transition in the level of chromatin condensation is associated with a timely progression of meiotic maturation and subsequent embryonic

development (reviewed in [52]). This large scale remodeling of chromatin involves the adding and removal of histone modifications (box 2). Interestingly, the chromatin of both NSN as well as SN GV oocytes have acetylated lysines in histones 3 and 4 e.g H3K9, H3K14, H4K5, H4K8, H4K12 and H4K16 [53,54]. Histone acetylation is usually a mark of transcriptionally active chromatin, although SN GV oocytes are transcriptionally silent [55,56]. This indicates that H3/H4 acetylation is not invariably associated with ongoing transcription. Germinal vesicle breakdown (GVBD) is followed by chromosome condensation. At this stage, the chromatin is deacetylated by histone deactylases (HDACs, [53,54,57-59]). Their inhibition leads to hyperacetylated chromatin in mammalian oocytes [60-62]. Class I and II HDACs are the most plausible candidates for the deacetylation of chromatin as mouse MII oocytes have high levels of mRNA of the individual HDACs and the proteins have been detected in mouse GV oocytes [63,64].

During progression from GVBD to the metaphase I stage, the chromosome homologous, which are physically attached at the sites of recombination, move to the equatorial plate and microtubules connect the homologous chromosomes to opposite spindle poles. It is crucial that the sister chromatids are connected to the same spindle pole and therefore they have to be orientated in such a way that both kinetochores of the sister chromatids form a binding unit for the microtubules (box 1). After completion of meiosis I the oocyte immediately progresses to the second meiotic division. The DNA remains condensed and a new spindle is formed. As in mitosis, the sister chromatids are now orientated amphitelically with the single chromatids attached to microtubules of the opposite spindle poles.

At this metaphase stage the oocyte arrests (MII) and awaits to be fertilized by a spermatozoon to complete the second meiotic division. A special activity, known as the cytostatic factor (CSF) maintains the metaphase II arrest in oocytes [65]. This pathway was initially investigated in frog oocytes. Masui and Markert proposed that CSF had to fulfill three criteria. First, it should appear during oocyte maturation and reach its highest level at the MII stage. Secondly, it should be able to induce an exogenous metaphase arrest upon injection into blastomeres. Lastly, a flux of Ca^{+} should inactivate CSF and release the MII arrest [65]. The first protein that was identified as a CSF candidate was the c-MOS kinase. Injection of the c-MOS protein induces MII arrest and depletion of c-MOS leads to a failure of maintaining MII arrest [66-68]. After c-MOS had been identified, more factors in the pathway that act downstream of c-MOS were found, such as the mitogen activated protein module (MAPK) which contains MEK, the ERK kinases and the ribosomal S6 kinase (RSK; reviewed in [69]). Furthermore, over-expression of the components of the SAC can induce an arrest but immune depletion of these components does not inhibit the APC/C, nor does it block an induced CSF

release by CA^+ in Xenopus [70-72]. This indicates that the APC/C activation and the subsequent cyclin B degradation do not solely depend on the proteins of the SAC and the c-MOS pathway may act independent of the SAC. Although the proteins of the c-MOS/ MAPK pathway and the members of the SAC are conserved in mammalian oocytes, the pathway involved in the MII arrest is less clear in mouse oocytes. Loss of c-MOS or MAPK/MEK does induce an arrest in mouse oocytes, however they exit MII and undergo parthenogenesis after 2–4 hrs [66,67,73,74]. This suggests that these proteins are required for the maintenance of the MII arrest, however, they are not solely responsible for its establishment.

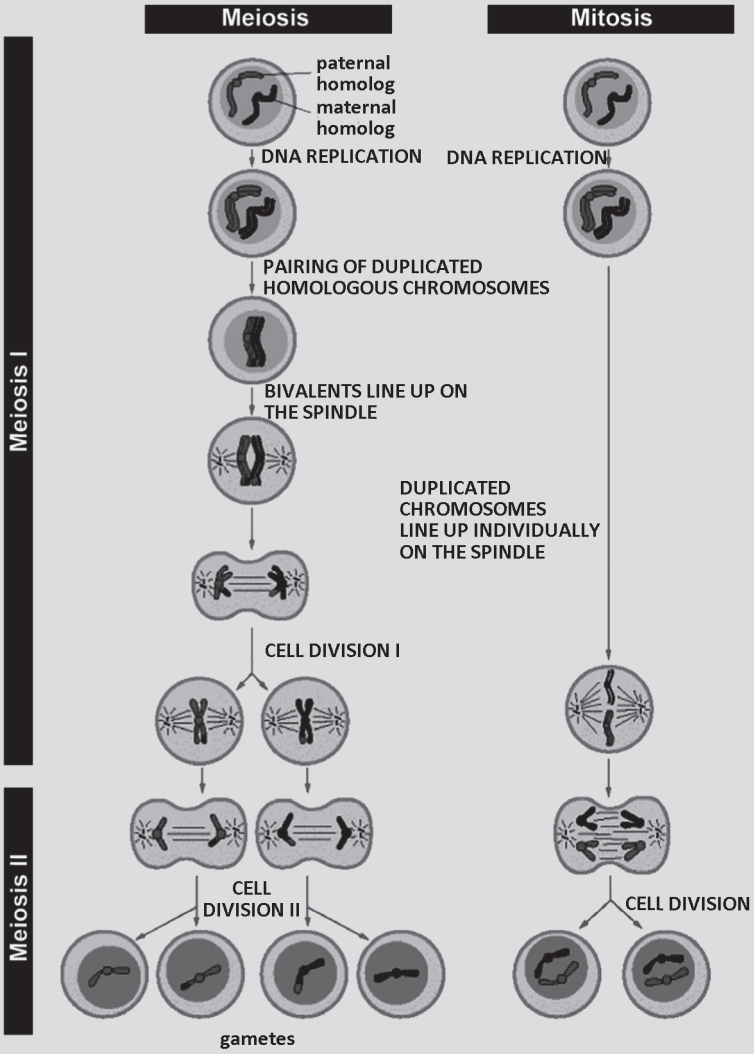
In 1991, it was already described that upon fertilization the activity of CaMKII increases, leading to cyclin B degradation [75]. However, levels of c-MOS remain high up to 30 min after the Ca^+ flux, indicating that c-MOS may regulate APC/C activation and cyclin B degradation via an intermediate kinase, one that is inactivated directly by the Ca^+ flux. EMI 2 [76-78] was identified to be the intermediate protein that inhibits APC/C (reviewed in [69,79,80]).

BOX 1

Female meiosis versus mitosis

Human somatic cells contain 23 pairs of chromosomes, thus 46 chromosomes (2n). The cell cycle of a somatic cell has two main stages, the interphase and the mitotic phase. The interphase consists of three stages: the two gap phases G1 and G2 and an intervening synthesis phase (S phase) in which the DNA is replicated after which each chromosome consists of a pair of chromatids. During the 4 mitotic phases, the chromatids of each chromosome are condensed (prophase), aligned (metaphase), segregated (anaphase) and form new nuclei (telophase) followed by cytokinesis, in which two new somatic daughter cells are formed.

Mature gametes differ from somatic cells as they contain only half of the genome, 23 chromosomes (n). This reduction of the genome is realized through a special kind of cell division called meiosis which has two dividing phases without an intervening interphase (box Figure 1). In addition, the prophase is extended and exists of four separate stadia: Leptotene, Zygotene, Pachytene and Diplotene.



Box Figure 1. Differences between mitosis and meiosis. The prophase in meiosis is protracted and during this phase, recombination between the homologous chromosomes take place. The daughter cells are haploid in meiosis whereas mitotic daughter cells are identical to the mother cell (adapted from [120]).

Prophase

In the prolonged prophase the homologous chromosomes pair and recombine the two parental strands of DNA. This process of exchanging information ensures the diversity within the species and between the species. Recombination is initiated by the formation of double strands breaks (DSBs) by the enzyme SPO11 [194,195]. The first breaks can be observed in leptotene. DSBs are thought to enable the pairing process of the homologous chromosomes as the breaks are repaired by making use of the homologous chromosome as an intact template rather than the sister chromatid as is common in somatic cells that have completed S-phase. In addition, the other, less accurate, pathway of DSB repair, non-homologous end-joining (NHEJ), is repressed. Although many breaks are formed, only a minority of the DSB breaks is repaired to form a cross-over. The majority of the breaks are repaired via other pathways such as synthesis-dependent strand annealing (reviewed in [196]).

A cross-over forms a physical link between the homologous chromosomes which is called a chiasmata. Per chromosome only a few chiasmata can be observed, the number of which depends on the length of the chromosomes (reviewed in [197]). When these chiasmata are lacking or placed at certain vulnerable regions of a chromosome, this chromosome pair is predisposed to mis-segregate during the division stages [198]. During meiotic prophase, the meiotic cohesion complex [199-201] is loaded onto the chromatin [199]. It binds the sister chromatids and ensures that they stay together until they are separated during the second meiotic division.

Metaphase I and II in oocytes

The first meiotic division is initiated by the breakdown of the nuclear envelope of the germinal vesicle, a process called germinal vesicle breakdown (GVBD). The chromatin (box 2) condenses into chromosomes and extensive remodeling takes place. The lysine residues of histone 3 (H3) and histone 4 (H4) are deacetylated in the [53,54] and H3 is phosphorylated at serine 10 and 28 [202]. This remodeling of chromatin is necessary to create a stable kinetochore at the centromeres of the chromosomes. Inhibition of this process results in chromosome segregation errors due to disturbed interactions with microtubules and kinetochore interacting proteins [60,61,112-119,203,204].

Mammalian oocytes lack centrosomes and thus spindle organization and bi-orientation is differently regulated compared to somatic cells. After GVBD, many microtubule organizing centers (MTOC) can be observed in the cytoplasm [205]. The multipolar MTOCs cluster into a bi-polar barrel shaped spindle that exhibits similar characteristics as a mitotic centrosomal spindle. During this process the homologous chromosomes congress and align at the equatorial plate. A recent study shows that this process of homologue bi-orientation is highly erroneous and almost all chromosomes need more than one round of correction before they are attached amphitelicly, with each homologous chromosome attached to an opposite pole of the spindle [206]. Erroneous attached chromosomes/ chromatids, for example merotelic attachment of a homologous chromosome to both poles or syntelic attachment when both homologues are attached to the same pole, are monitored by proteins of the chromosomes passenger complex (CPC) and corrected by the mitotic centromere associated kinesin (MCAK; [207,208]). The CPC has multiple roles, namely regulating proper microtubule attachment, regulation of the spindle assembly checkpoint, localizing the Shugoshin proteins and cytokinesis. In mammalian oocytes, the CPC consists of the proteins INCENP, Aurora kinases B/C, Survivin and Borealin [209-217]. When the chromosomes are aligned properly at the equatorial plate and they are each attached to the microtubules, the oocyte progresses to anaphase. Progression to anaphase is regulated by the proteins of the anaphase-promoting complex/cyclosome (APC/C) which inactivate CDK1. The proteins of the spindle assembly check point (SAC) delay the activation of the APC/C until all chromosomes are properly attached [106,218-220]. Protection of centromeric bound REC8 by Shugoshin proteins secures the attachment of the sister chromatids until MII (reviewed in [221]). The localization of these proteins is regulated by BUB1, which in turn depends on H2A phophorylation [222]. Upon progression to anaphase I the cohesion complex is removed from the chromatid arms by Separase [223], which allows the homologous chromosomes to be separated.

Both meiotic divisions are asymmetric which enables the oocyte to retain almost all the cytoplasm. These asymmetric divisions are governed by the proteins SPIRE 1, SPIRE 2 and FORMIN 2 [224]. After extrusion of the first polar body the sister chromatids progress and align at the equatorial plate. Now, the sister chromatids have to bi-orientate at the opposite spindle poles like in mitosis. After activation of the oocyte by the fusion of the oolemma with the membrane of the spermatozoon, the second meiotic division is initiated. The centromeric bound REC8 is cleaved which allows the separation of the sister chromatids. One set of sister chromatids is extruded into the second polar body. The remaining haploid set will form the maternal pronucleus.

Why do eggs go bad?

After timed intercourse, a woman has on average a 20%–30% chance to become pregnant [81,82]. Other mammals such as rodents and rabbits, but also the more closely related monkeys, have a much higher pregnancy rate per cycle (80%–95%) [83-85]. One of the main reasons for this low pregnancy chance is most likely the high rate of aneuploidy in human oocytes. Many studies have shown that the percentage of aneuploid oocytes in women can be up to 50% and one factor that has been unequivocally associated with the high incidence of chromosome aberrations is advanced maternal age (reviewed in [86]). As mentioned before, oocytes enter the meiotic prophase around birth and have a protracted phase of developmental arrest. From puberty onwards, when a woman has her first menarche, oocyte development recommences. Thus, complete oocyte development, from the precursor cells to the mature MII oocytes, can take up to decades. It is therefore not surprising that human chromosome abnormalities, such as Down's syndrome, are in most cases maternally derived [87-89]. Human oocytes replicate and recombine their DNA many years before they enter the division stages. This means that during this long time the two chromosome homologous have to remain physically attached. The proteins responsible for this are the components of the cohesion complex. Recent studies have shown that in mouse oocytes at least two cohesins, the meiotic variants REC8 and SMC1β, are not replenished during oocyte maturation from the prophase onwards and that older mice have diminished levels of these proteins [90-96]. Because the components of the meiotic cohesion complex are conserved in mammals, these data suggest that also in human oocytes these proteins are loaded only once at the prophase of meiosis around birth, and must last for decades until the oocyte is recruited for ovulation and fertilization. Another factor that has been unequivocally associated with aneuploidy is the number and placement of the recombination sites. Studies of animal models [97] as well as data from Down syndrome patients [98,99] show that reduced cross-over formation or the placement of the cross-over at vulnerable chromosomal regions, for example in the vicinity of the centromere and telomeres, are associated with chromosome segregation errors in MI and MII ([100], reviewed in [88,101]).

Other proteins that have been associated with the high incidence of chromosome segregation errors in oocytes of women of advance maternal age are the proteins of the spindle assembly checkpoint (SAC). These proteins function as a tension sensor of microtubule-kinetochore attachment and are able to delay the transition to anaphase until all chromosomes are properly aligned. A genetic screen in yeast cells identified the genes encoding for these proteins to be essential for the arrest at metaphase when cells are challenged with spindle poisons [102-105]. These genes belong to the mitotic arrest deficiency (MAD) and budding uninhibited by benomyl (BUB) gene families and are conserved in mouse and humans. Studies

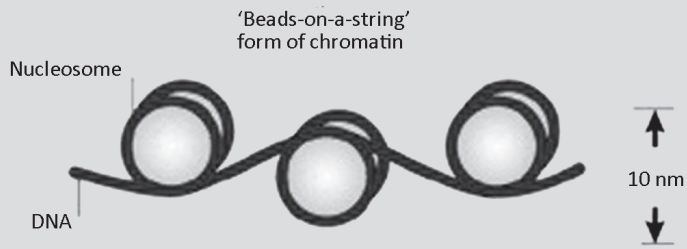
in mouse oocytes have proven that a spindle assembly checkpoint is active during the meiotic segregation stages. Oocytes respond to spindle damage by recruiting proteins of the SAC to the unattached microtubules [106]. However, oocytes that have a univalent (achiasmatic chromosomes) can evade the SAC if the sister chromatids are bi-orientated [107,108]. In addition, other data suggest that the genetic background influences the susceptibility to aneuploidy and indicate that some mouse strains only need a critical mass of chromosome alignment rather than full chromosome alignment to proceed to anaphase [109]. Thus, subtle differences in the SAC mediated response may contribute to an oocyte's susceptibility to age-related aneuploidy. It has been shown that components of the SAC are expressed in human oocytes and diminished MAD2 and BUB1 mRNA levels were observed in human oocytes of women of advanced maternal age [110]. This may indicate that these genes could be involved in the increase of aneuploidy in older women. The more pertinent data on protein levels and localization are, however, still lacking. New studies are needed to definitely determine the role of these proteins in age-related human aneuploidy.

It has become clear that correct chromosomes segregation, either in mitosis or meiosis, involves many processes and proteins that together orchestrate the equal division of homologous chromosomes. Key processes such as chromatin condensation, kinetochore formation and proper microtubule attachment require the proper interaction of many proteins both with each other but also with the chromosome. It is thought that the chromatin is remodeled in such a way that it creates an optimal platform to allow stable kinetochore-microtubule interaction. Histone acetylation and euchromatic histone modifications are associated with decondensed open chromatin to allow for gene transcription [111]. As described above, the removal of acetyl groups of lysines residues by HDACs precedes chromatin condensation at the prometaphase to metaphase transition in meiosis I [53,54,58,59]. Inhibition of HDACs by chemical agents such as trichostatin A and sodium butyrate results in hyperacetylated chromatin affecting kinetochore formation, chromosome segregation, and, after long term culture, also leads to dysregulation of gene transcription. Experimental data show that inadequate remodeling of chromatin through inhibition of HDACS during mitosis leads to aneuploidy eventually resulting in apoptosis [112-119]. *In vitro* maturation of mouse and porcine oocytes in the presence of an HDACi results in chromosome mis-segration and aneuploidy in the offspring [60-62]. Furthermore, it was observed that oocytes from older mice show residual acetylation at the MI and MII stage and an increase in chromosome misalignment compared to oocytes from young mice [60]. This inadequate remodeling of chromatin during mouse oocyte maturation may be an important factor in the etiology of age related aneuploidy. Chapter 2 of this thesis describes the kinetics of chromatin remodeling,

in particular the deacetylation of lysine residues on H4, during human oocyte maturation and provides evidence that residual acetylation may be correlated with advanced maternal age and chromosome misalignment in human oocytes.

BOX 2

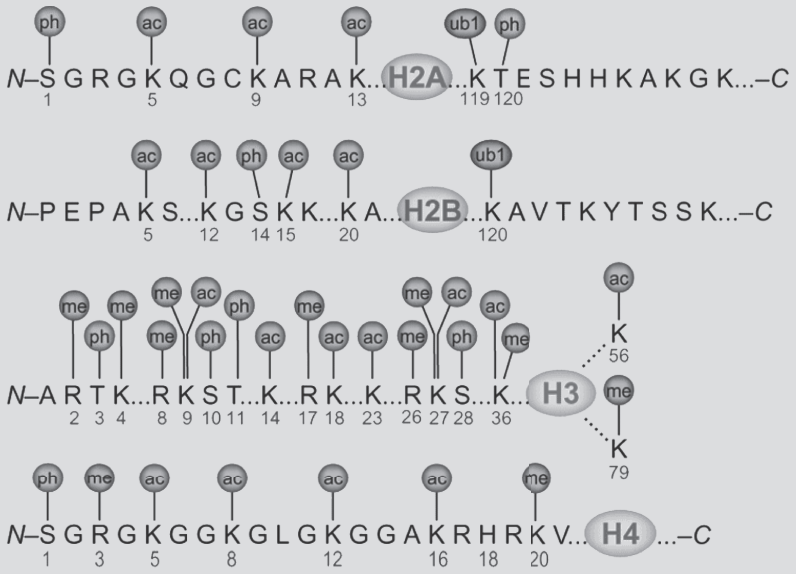
About DNA, epigenetics and chromatin



Box Figure 2. Beads on a string (adapted from [225]).

In the nucleus, the DNA is wrapped around an octamere of proteins, called the nucleosome (box Figure 2). Around each nucleosome about 150 basepairs of DNA are wrapped. Nucleosomes are separated by 10-80 bp of DNA, depending on the species and the tissue. The core of the nucleosome contains five very basic proteins that neutralize the negative charge of the DNA, the histone proteins. They are histone 2a (H2A) and histone 2B (H2B) which form heterodimers, histone 3 (H3) and histone 4 (H4) which form heterotetrameres. One molecule of histone H1 is bound to the DNA on the edge of most nucleosomes. The histones form the basic subunits of the chromatin and they can be either replaced by variant histones or they can be post-translationally modified by covalently bound small molecule groups (Box Figure 3). These histone variants and modifications play a very important role in creating a specific chromatin structure which allows the associated DNA, ranging from the promoter region of individual genes to complete intergenic repeat regions, to be accessible or inaccessible to protein complexes interacting with the chromatin or directly with DNA.

Catalyzing enzymes such as histone acetyl transferases (HATs), histone methyltransferase (HMTs) and histone deacetylases (HDACs) are components of transcriptional regulatory complexes and can modify the tails of all 5 histones, which stick out of the spherical core. The combination of histone modifications and variants results in a dynamic code, "the histone signature" [111], which is related to the chromatin structure of the DNA of a specific gene, a region or a phase of the cell cycle. Recently, novel sites of histone post-translational modifications (PTMs) and a new type of histone modification, histone lysine crotonylation (Kcr), involved in spermatogenesis, have been described [226] and it was suggested that new PMTs will be discovered. Dysregulation of chromatin can result in up- or downregulation of transcription of genes but also causes disruption of the higher order chromatin structure which affects chromatin interacting protein complexes. For example, acetylated lysines of H4 are associated with DNA transcription and repair during interphase; however, hyperacetylated chromatin during the metaphase of mitosis and meiosis causes chromosome division errors leading to aneuploidy.



Box Figure 3. Examples of post translational histone modifications on the amino acids (numbers) of the histone proteins (adapted from [227]).

Spermatogenesis

Spermatogenesis is the male counterpart of oogenesis. It consists of three stages: i) mitotic proliferation, ii) meiosis and iii) spermiogenesis. Unlike oogenesis, spermatogenesis is a continuous process in adults. The testes contain undifferentiated spermatogonial stem cells that can proliferate and generate new daughter cells throughout life. Spermatogenesis initiates at puberty and the spermatogonia enter the meiotic prophase [120].

The stages of meiosis are basically the same in spermatocytes and oocytes, however, there are some differences, and in this context the differential behavior of the sex chromosomes during male and female gametogenesis is most relevant. During prophase (box 1), the homologous chromosomes pair and synapse to allow cross-over formation. In contrast to females that have two X chromosomes, the sex chromosomes of males, the X and Y, do not share full sequence homology. Although transient pairing of the heterologous regions occurs in early pachytene, at midpachytene only the pseudo autosomal regions (PAR), where the X and Y chromosome share sequence homology, remain synapsed [121]. The unsynapsed regions trigger a silencing mechanism, known as meiotic sex chromosome inactivation (MSCI). This mechanism involves extensive remodeling of chromatin including incorporation of histone variants and the addition of specific histone modifications. This results in the formation of a heterochromatic compartment at the periphery of the nucleus, known as the sex body or XY body (reviewed in [122,123]). In round spermatids of the mouse, the X and Y chromosome are thought to remain largely inactive, forming the so called post-meiotic sex chromatin (PMSC) [124], although several single-copy and multi-copy genes are reactivated [125,126]. MSCI and PMSC have been associated with imprinted X chromosome inactivation in the mouse pre-implantation embryo [127], although this is still controversial [128]. For a correct progression through male meiosis, MSCI is essential as mutants in which MSCI is incomplete, are infertile due to toxic effects of genes expressed by the X and Y chromosome [129-132].

After completion of meiosis, the spermatids undergo morphological changes as they mature into spermatozoa. The chromatin also undergoes drastic remodeling via the histone-to-protamine replacement process, which allows the DNA to be highly compacted [133,134]. The percentage of remaining histones differs per species: in human sperm around 15% of histone proteins are retained while in mice this percentage is only around 1% [135-137].

To be able to fertilize an oocyte, the spermatozoon must undergo a final maturation step, known as the capacitation process [138], which occurs in the female genital tract [139].

The zygote: Fertilization and pronuclei formation

Upon fertilization the oocyte needs to remodel the maternal and paternal chromatin in preparation for transcriptional activation, a process to which the spermatozoon does not contribute any proteins. When an oocyte ovulates, it has a window of around 36 hrs to be fertilized before it reaches the uterus, where it degenerates and is taken up by the endometrium. When a spermatozoon reaches the oocyte it binds to the zona pellucida and undergoes the acrosome reaction. This reaction involves the release of the contents of the sperm acrosome by exocytosis into the oocyte cytoplasm. This generates a Ca^{+} flux within the oocyte which triggers it into anaphase and finishing the second meiotic division by extruding the second polar body.

Pronuclear formation of the parental genomes occurs simultaneously in most human oocytes [140]. The parental pronuclei (PN) are quite different with respect to their chromatin. As mentioned above, a spermatozoon has to pack its DNA into a small, aerodynamic sperm head, and to accomplish this, the histones are largely replaced by smaller highly positively charged protamines.

Approximately three hours after the spermatozoon has entered the oocyte cytoplasm, the pronuclei start to form. The sperm head decondenses and the protamines are removed to be replaced by histones. These new histones are not yet modified in the same way as the histones on the maternal pronucleus [141,142]. Thus at the time of pronuclei formation, the two parental chromatin structures are quite different and show asymmetric patterns of mainly heterochromatin related histone modifications, such as H3K9me2 which is present in the maternal pronucleus but absent in the male PN. This asymmetry can still be observed in the next stage of pre-implantation development. Mouse 2 cell stage embryos still have nuclei that are not completely evenly stained for histone H3 K9 dimethylation when compared to somatic nuclei or nuclei of later stage blastomeres [143,144]. This difference in staining was not observed in the nuclei of 2 cell stage androgenetic embryos (see glossary for explanation), which have no staining of H3K9me2 or parthenogenetic embryos, which have evenly stained nuclei. This asymmetry gradually disappears during the subsequent stages following zygotic genome activation indicating that from that stage onwards the responsible methyltransferases are active. As will be described in chapter three, our data on human tri-pronuclear zygotes suggest that human embryos have a similar differential pattern of histone modifications on the male and female pronuclei as mouse zygotes.

Abnormal pronuclei formation

Artificial reproductive technology (ART) has provided much new knowledge on human early embryonic development. As conception takes place outside the body, the first developmental stages of an embryo can be followed *in vitro.* After approximately 16–20 hrs post insemination (hpi) two nuclear structures can be observed under a light microscope. Occasionally, the zygote contains more than two pronuclei. Even after intra cytoplasmic sperm injection (ICSI), where only a single spermatozoon is injected directly into the ooplasm, three pronuclei can sometimes be observed. After IVF, around 5% to 8% of the zygotes contains an extra pronucleus and in around 2.5 % and 6% of the zygotes derived after ICSI, a third pronucleus can be seen [145-147].

Another abnormal pronuclear pattern is the presence of only a single pronucleus. This single PN after conventional insemination is in most cases larger than one of the two pronuclei in normal fertilized oocytes, indicative of the presence of more than one parental genome ([148] and own observations).

Abnormal fertilization can also occur after natural conception. Oocytes that are fertilized by two spermatozoa can become embryos and even implant in the uterus. They are, of course, genetically aberrant and do not develop into a normal fetus. They form many extra-embryonic grapelike tissues and are called hydatidiform moles. In case of a triploid embryo with two sets of the paternal genome and one maternal genome, embryonic structures are formed and this is called a partial hydatidiform mole. In contrast, a complete hydatidiform mole (CHM) exclusively exists of paternal genetic material. These androgenetic embryos are most likely the result of the fertilization of an oocyte without DNA, a so called empty egg by a single sperm that is reduplicated. In these cases the embryo will be homozygous and either XX or YY. However, 4% of the CHM are heterozygous XY and most likely originated from fertilization by two spermatozoa [149]. Complete and partial hydatidiform moles usually end in a spontaneous abortion, however, the cells are sometimes invasive and can cause a tumor-like phenotype in the uterus.

It is evident that in ART treatments, tri-pronuclear zygotes are not safe to transfer back into the uterus even as the pre-implantation embryo can develop seemingly normal until the blastocyst stage. The transfer of embryos developed from mono-pronuclear zygotes is sometimes considered in cases where no normal zygotes are available for transfer into the uterus. Identifying the sex and chromosome constitution of the embryo might give more information on the presence of a diploid genome indicating that fertilization may have occurred. However, the presence of either an XX or XY chromosome combination can in fact originate from an abnormal fertilization and does not provide conclusive evidence of a

normal fertilization. As mentioned above, a mono-pronuclear zygote could be the result of the fertilization of an empty egg by two spermatozoa [149-151]. In chapter 3 of this thesis we describe the parental contribution of the gametes in mono-pronuclear zygotes derived from IVF and ICSI treatments.

Embryogenesis/pre-implantation development

At the first cell division the zygote becomes a 2-cell embryo. The pre-implantation divisions differ from normal somatic divisions in that there is no cellular growth after cytokinesis. Thus, the first divisions result in daughter cells of half of the size of the parental cell and are called cleavage divisions. As mentioned before, the oocyte has stockpiled mRNA during oocyte maturation and the pre-implantation embryo is dependent on this maternal supply until its own genome is activated. In the mouse, embryonic genome activation occurs in three waves. The first minor wave occurs at the zygote stage followed by the major wave at the two cell stage (reviewed in [152]) that activates most genes. The third minor wave occurs at the cleavage stages between the two cell stage and the blastocyst stage [153]. Human embryos start to activate their genome at a later time point than mouse embryos, approximately between the fourth and eight cell stage [154].

In both mouse and human embryos, but also in other mammals, the first morphologic cell differentiation occurs between the 8 and 16 cell stage at the time of compaction. The outer cells of the embryo will take part in the formation of the extra-embryonic lineage whereas the inner cells will mainly constitute the epiblast. At the blastocyst stage this distinction in cell allocation and differentiation is most prominent as the inner cells form a small clump of cells, the inner cell mass (ICM). The outer cells form the outside layer, the trophectoderm (TE), enclosing the blastocoelic fluid. The trophectoderm will form the future extra embryonic tissues such as the chorion and the placenta. The ICM will eventually form all the cells of the tissues and organs of the embryo. The ICM cells can differentiate into every somatic cell type and this special characteristic is called pluripotency. Genes that are associated with this state of pre-differentiation are called pluripotency genes and they encode for proteins such as NANOG, SOX2 and OCT3/4 [155-158]. Cells of the ICM have high levels of these proteins which is a mark for their undifferentiated state although the protein levels vary between individual cells. At day 3.5 the ICM of a mouse embryo contains cells which express both epiblast markers such as NANOG as well as primitive endoderm (PrE) markers such as Gata4. Following further development to the hypoblast stage (mouse 5.5 dpc.) NANOG is only expressed by the future epiblast cells [159,160]. In mouse blastocysts this expression is mirrored by the protein CDX2 that is exclusively present in the TE cells or the GATA4/GATA6 proteins present in the PrE cells

[161-163]. Around this time the mouse embryo will implant in the uterus. In humans, the exact time point of implantation is not possible to determine *in vivo*. However, *in vitro* models, using a layer of endometrial stromal and epithelial cells have shown that "implantation" takes place *in vitro* between days 7.5 and 9 post fertilization [164-166].

During pre-implantation development, female and male embryos are morphologically the same, however, they are of course genetically different. Female mammals have to compensate the expression of X-linked genes that males are lacking on their Y chromosome [167]. Therefore, the expression of the majority of genes is silenced on one of the X chromosomes through an elaborate epigenetic process called X chromosome inactivation (XCI; box 3). XCI comprises all the features of epigenetic regulation, such as transcriptional regulation by non-coding RNAs, chromatin remodeling and DNA methylation. In the mouse XCI is established in three steps during embryonic development [127,128,160,168-171].

In the mouse pre-implantation embryo, the first step of XCI takes place at the two cell stage when the first signs of XCI can be observed, namely expression of the X inactive specific transcript gene (Xist) [172-174]. During the first stages of murine pre-implantation development the paternal X (Xp) preferentially initiates XCI. The Xp exhibits the most important hallmarks of XCI, such as coating of the inactive X chromosome by *Xist* RNA *in cis*, exclusion of the transcription machinery, loss of euchromatin histone modifications, accumulation of heterochromatin histone marks and silencing of individual genes. This preferential silencing of the paternal X chromosome is called imprinted XCI. DNA methylation of silenced genes is not established at the pre-implantation stage. This lack of DNA methylation may reflect the incomplete status of XCI at this early stage of XCI [175]. Deletion of the key genes of XCI, Xist and Tsix, is lethal only after implantation development and this raises the question whether functional dosage compensation is required during normal pre-implantation development [147,148]. FISH data examining *XIST* cloud formation, as an indicator of XCI, in parthenogenetic, gynogenetic and androgenetic pre-implantation embryos show that at the morula stage a counting mechanism is initiated which indicates that imprinted XCI can be overridden [176,177]. This counting mechanism explains why XpO mice are viable although they are smaller as their wildtype counterparts [178-180].

It was initially postulated that the paternal X enters the oocyte in a pre-inactivated state. This was thought to occur by the mechanism of meiotic sex chromosome inactivation (MSCI) followed by PMSC, which take place during spermatogenesis [127]. However, as there is expression of the paternal X during pre-implantation development this theory was rejected. A study in mouse oocytes showed that the maternal X (Xm) acquires the mark that prevents it from being inactivated during maturation [181] most likely by methylation of the Xist gene

on the Xm. This is mirrored by the unmethylated status of Xist on the Xp which may allow the preferentially expression of paternal *Xist* RNA from the two cell stage onward [182-184]. However, these data on the parental specific methylation were not completely reproducible [185].

The second step of XCI takes place in the blastocyst embryo. The cells of the trophectoderm secure the imprinted mark on the paternal X [186,187], whereas the ICM cells will reactivate the Xp [128,168]. At the time of implantation, the cells of the now epiblast initiate random XCI by inactivating either the maternal or the paternal X chromosome in the third step of XCI. Different models have been proposed to explain the kinetics of XCI (box 3). Undifferentiated mouse ES cells, which are derived from the ICM [188,189], still have two active X chromosomes and are commonly used as a model to study the process and the genes that are involved in the initiation of random XCI (reviewed in [190]).

BOX 3

Random XCI

In mouse blastocyst embryos, the cells of the developing ICM reactivate the paternal X whereas the cells of the trophectoderm maintain the Xp silenced [127,128,168,228]. After reactivation, just after implantation, the cells of the ICM start random XCI which results in silencing of either the maternal or paternal X chromosome. Mouse embryonic stem cells (mES cells) originate from the ICM and can be used to recapitulate the steps that are taken in random XCI. The use of mES cells has provided in depth knowledge of the complicated process of XCI. Many genes and proteins have been discovered and different models have been proposed to explain how a cell ends up with one inactive X and one active X chromosome (reviewed in [229,230]).

Models of XCI

A diploid set of autosomes always has one active X chromosome and every extra X chromosome will be inactivated. This indicates that a cell somehow “counts” how many X chromosomes there are per diploid set of autosomes and then “chooses” which X to inactivate. Different models have been proposed to explain this counting and choice mechanism [231-237]. However, it has become clear in recent years that XCI is best explained by the so called “stochastic” model.

The stochastic model

Observations in female ES cell lines with different X:autosomes ratios revealed that at some time points during the initiation of XCI, different numbers of inactive X chromosomes per set of autosomes can be observed; XX cells with two active X chromosomes, instead of the correct pattern of one Xi and one Xa and cells with two Xi's [238]. This pattern can be explained when it is assumed that every X chromosome has a certain chance to be inactivated. The probability to be inactivated depends on the nuclear concentration of trans-acting factors that regulate *Xist* and *Tsix* transcription upon differentiation [239-242]. During differentiation, these trans-acting factors upregulate Xist and/or down regulate Tsix, thereby increasing the chance of inactivation which leads in most cases to the random inactivation of one X-chromosome. Cells that will inactivate both X chromosomes will die and cells without an Xi will go through another round of XCI or die. Most patterns of XCI observed in wild type ES cells as well as in cells that carry XCI related mutations can be explained by this model [243].

Initial data had suggested that XCI did not exist in human pre-implantation embryos. This was based on nested RT-PCR data of *XIST* RNA from male and female human pre-implantation embryos [191,192]. However, single cell FISH data from mouse pre-implantation embryos suggested that also mouse male embryos have a small pinpoint expression of *XIST* RNA from the maternal X [193]. We decided to study XCI on a single cell level in human pre-implantation embryos because the nested RT-PCR data do not allow reliable discrimination between pinpoint *XIST* expression and clouds of *XIST* RNA. In this thesis we provide evidence that human female embryos initiate XCI during pre-implantation development as the main characteristics of XCI are present in female embryos (chapter 4.1). However, whether human female embryos establish XCI using the same mechanism as mouse embryos has yet to be determined.

Although the first important discoveries in the field of XCI were made in both humans and in mice, most subsequent studies on the genetic elements that control XCI have been done in the mouse. Chapter 4.2 of this thesis reviews all the steps in XCI during embryonic development in mice and men and in that we propose that a distinction should be made between X chromosome remodeling (XCR) and actual X chromosome inactivation (XCI).

Thesis aim and outline

The aim of this thesis was to gain more insight in specific epigenetic changes that take place In human oocytes, zygotes and early embryos.

The work described in this thesis can be divided into three topics: 1. Epigenetic regulation of the chromatin structure during human oocyte maturation. 2. Epigenetic features that distinguish the parental pronuclei in abnormal fertilized human and mouse zygotes, and 3. Initiation of the epigenetic process of X chromosome inactivation in human pre-implantation embryos. The topics are described in three chapters (2-3-4) while the findings of the conducted research are discussed in the final chapter (5).

Chapter 2

Defective regulation of chromatin remodeling during human oocyte maturation

This chapter focuses on the epigenetic regulation of chromatin remodeling during human oocyte maturation. It describes the pattern of histone acetylation during the last three stages of human oocyte maturation: the germinal vesicle (GV) stage, the metaphase of meiosis I (MI) and the metaphase of the second meiotic division (MII). It has been described for different mammals that the chromatin is deacetylated during maturation from the GV stage to the MII stage. The findings presented in this chapter show that also during human oocyte maturation, chromatin is deacetylated for most of the investigated lysine residues of histone 4. However, it was observed that a large percentage of the *in vivo* matured human oocytes still showed acetylated chromatin. This residual acetylation was found to be associated with a higher maternal age and a higher incidence of chromosome misalignment.

Chapter 3

Difference between the parental chromatin in pronuclei of human and mouse zygotes

This chapter describes the work on abnormal fertilized human oocytes and their chromatin constitution. The observation that the maternal and paternal chromatin in tri-pronuclear zygotes are differentially modified allowed us to use it as a tool to determine the parental origin of the single pronucleus in mono-pronuclear oocytes.

Chapter 4

X chromosome inactivation in pre-implantation embryos

The work described in chapter 4.1 shows that female human pre-implantation embryos initiate the process of X chromosome inactivation.

Chapter 4.2 provides a detailed overview on the current knowledge on initiation of the process of XCI in the earliest stages of mouse and human development. It discusses the most recent findings and raises questions for future research.

Chapter 5

General discussion

In this chapter the findings described in the preceding chapters are discussed in light of the current knowledge of the research field.

References

1. Schoenwolf, G., et al., *Larsen's Human Embryology*. 4th ed. 2008. 712.
2. Hassold, T., H. Hall, and P. Hunt, *The origin of human aneuploidy: where we have been, where we are going.* Hum Mol Genet, 2007. **16 Spec No. 2**: p. R203-8.
3. Ginsburg, M., M.H. Snow, and A. McLaren, *Primordial germ cells in the mouse embryo during gastrulation.* Development, 1990. **110**(2): p. 521-8.
4. Anderson, E.L., et al., *Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice.* Proceedings of the National Academy of Sciences, 2008. **105**(39): p. 14976-14980.
5. Menke, D.B., J. Koubova, and D.C. Page, *Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave.* Developmental Biology, 2003. **262**(2): p. 303-312.
6. Garcia, M., et al., *Development of the first meiotic prophase stages in human fetal oocytes observed by light and electron microscopy.* Hum Genet, 1987. **77**(3): p. 223-32.
7. Kurilo, L.F., *Oogenesis in antenatal development in man.* Hum Genet, 1981. **57**(1): p. 86-92.
8. Baker, T.G., *A Quantitative and Cytological Study of Germ Cells in Human Ovaries.* Proc R Soc Lond B Biol Sci, 1963. **158**: p. 417-33.
9. Johnson, J., et al., *Germline stem cells and follicular renewal in the postnatal mammalian ovary.* Nature, 2004. **428**(6979): p. 145-50.
10. Johnson, J., et al., *Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood.* Cell, 2005. **122**(2): p. 303-15.
11. Ainsworth, C., *Bone cells linked to creation of fresh eggs in mammals.* Nature, 2005. **436**(7051): p. 609.
12. Notarianni, E., *Reinterpretation of evidence advanced for neo-oogenesis in mammals, in terms of a finite oocyte reserve.* J Ovarian Res, 2011. **4**(1): p. 1.
13. Powell, K., *Born or made? Debate on mouse eggs reignites.* Nature, 2006. **441**(7095): p. 795.
14. Telfer, E.E., et al., *On regenerating the ovary and generating controversy.* Cell, 2005. **122**(6): p. 821-2.
15. Zou, K., et al., *Production of offspring from a germline stem cell line derived from neonatal ovaries.* Nat Cell Biol, 2009. **11**(5): p. 631-636.
16. Virant-Klun, I., et al., *Parthenogenetic embryo-like structures in the human ovarian surface epithelium cell culture in postmenopausal women with no naturally present follicles and oocytes.* Stem Cells Dev, 2009. **18**(1): p. 137-49.
17. Bukovsky, A., M. Svetlikova, and M.R. Caudle, *Oogenesis in cultures derived from adult human ovaries.* Reprod Biol Endocrinol, 2005. **3**: p. 17.
18. Gondos, B., P. Bhiraleus, and C.J. Hobel, *Ultrastructural observations on germ cells in human fetal ovaries.* Am J Obstet Gynecol, 1971. **110**(5): p. 644-52.
19. Pepling, M.E. and A.C. Spradling, *Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles.* Dev Biol, 2001. **234**(2): p. 339-51.
20. Ratts, V.S., et al., *Ablation of bcl-2 gene expression decreases the numbers of oocytes and primordial follicles established in the post-natal female mouse gonad.* Endocrinology, 1995. **136**(8): p. 3665-8.
21. Flaws, J.A., et al., *Effect of bcl-2 on the primordial follicle endowment in the mouse ovary.* Biol Reprod, 2001. **64**(4): p. 1153-9.
22. Bergeron, L., et al., *Defects in regulation of apoptosis in caspase-2-deficient mice.* Genes Dev, 1998. **12**(9): p. 1304-14.

23. Perez, G.I., et al., *Prolongation of ovarian lifespan into advanced chronological age by Bax-deficiency.* Nat Genet, 1999. **21**(2): p. 200-3.
24. Morita, Y. and J.L. Tilly, *Oocyte apoptosis: like sand through an hourglass.* Dev Biol, 1999. **213**(1): p. 1-17.
25. Peters, P. and K.P. McNatty, *The ovary*. 1980: University of California Press.
26. Coucouvanis, E.C., et al., *Evidence That the Mechanism of Prenatal Germ Cell Death in the Mouse Is Apoptosis.* Experimental Cell Research, 1993. **209**(2): p. 238-247.
27. Krakauer, D.C. and A. Mira, *Mitochondria and germ-cell death.* Nature, 1999. **400**(6740): p. 125-6.
28. Jones, K.T., *Meiosis in oocytes: predisposition to aneuploidy and its increased incidence with age.* Hum Reprod Update, 2008. **14**(2): p. 143-58.
29. Picton, H.M., *Activation of follicle development: the primordial follicle.* Theriogenology, 2001. **55**(6): p. 1193-210.
30. Carabatsos, M.J., et al., *Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice.* Dev Biol, 1998. **204**(2): p. 373-84.
31. Dong, J., et al., *Growth differentiation factor-9 is required during early ovarian folliculogenesis.* Nature, 1996. **383**(6600): p. 531-5.
32. Elvin, J.A., et al., *Paracrine actions of growth differentiation factor-9 in the mammalian ovary.* Mol Endocrinol, 1999. **13**(6): p. 1035-48.
33. Ruggiu, M., et al., *The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis.* Nature, 1997. **389**(6646): p. 73-7.
34. Soyal, S.M., A. Amleh, and J. Dean, *FIGalpha, a germ cell-specific transcription factor required for ovarian follicle formation.* Development, 2000. **127**(21): p. 4645-54.
35. Yan, C., et al., *Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function.* Mol Endocrinol, 2001. **15**(6): p. 854-66.
36. Bouman, L.N., J.A. Bernards, and H.W.G.M. Boddeke, *Medische Fysiology*. 2nd ed. 2008, Houten: Bohn Stafleu van Loghum. 831.
37. Kumar, T.R., et al., *Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility.* Nat Genet, 1997. **15**(2): p. 201-4.
38. Visser, J.A. and A.P.N. Themmen, *Anti-Müllerian hormone and folliculogenesis.* Molecular and Cellular Endocrinology, 2005. **234**(1-2): p. 81-86.
39. Can, A., O. Semiz, and O. Cinar, *Bisphenol-A induces cell cycle delay and alters centrosome and spindle microtubular organization in oocytes during meiosis.* Mol Hum Reprod, 2005. **11**(6): p. 389-96.
40. Hunt, P.A., et al., *Bisphenol a exposure causes meiotic aneuploidy in the female mouse.* Curr Biol, 2003. **13**(7): p. 546-53.
41. Lenie, S., et al., *Continuous exposure to bisphenol A during in vitro follicular development induces meiotic abnormalities.* Mutat Res, 2008. **651**(1-2): p. 71-81.
42. Mlynarcikova, A., et al., *Effects of selected endocrine disruptors on meiotic maturation, cumulus expansion, synthesis of hyaluronan and progesterone by porcine oocyte-cumulus complexes.* Toxicol *In Vitro*, 2009. **23**(3): p. 371-7.
43. Brieno-Enriquez, M.A., et al., *Human meiotic progression and recombination are affected by Bisphenol A exposure during in vitro human oocyte development.* Hum Reprod, 2011. **26**(10): p. 2807-18.
44. Susiarjo, M., et al., *Bisphenol A exposure in utero disrupts early oogenesis in the mouse.* PLoS Genet, 2007. **3**(1): p. e5.

45. Eichenlaub-Ritter, U., et al., *Exposure of mouse oocytes to bisphenol A causes meiotic arrest but not aneuploidy.* Mutat Res, 2008. **651**(1-2): p. 82-92.
46. Hunt, P.A., et al., *Bisphenol A Exposure Causes Meiotic Aneuploidy in the Female Mouse.* Current Biology, 2003. **13**(7): p. 546-553.
47. Li, L., P. Zheng, and J. Dean, *Maternal control of early mouse development.* Development, 2010. **137**(6): p. 859-70.
48. Mehlmann, L.M., *Oocyte-specific expression of Gpr3 is required for the maintenance of meiotic arrest in mouse oocytes.* Dev Biol, 2005. **288**(2): p. 397-404.
49. Hinckley, M., et al., *The G-protein-coupled receptors GPR3 and GPR12 are involved in cAMP signaling and maintenance of meiotic arrest in rodent oocytes.* Developmental Biology, 2005. **287**(2): p. 249-261.
50. Eppig, J.J. and A.C. Schroeder, *Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization in vitro.* Biol Reprod, 1989. **41**(2): p. 268-76.
51. Hartshorne, G.M., I.L. Sargent, and D.H. Barlow, *Growth rates and antrum formation of mouse ovarian follicles in vitro in response to follicle-stimulating hormone, relaxin, cyclic AMP and hypoxanthine.* Hum Reprod, 1994. **9**(6): p. 1003-12.
52. De La Fuente, R., *Chromatin modifications in the germinal vesicle (GV) of mammalian oocytes.* Dev Biol, 2006. **292**(1): p. 1-12.
53. Endo, T., et al., *Changes in histone modifications during in vitro maturation of porcine oocytes.* Mol Reprod Dev, 2005. **71**(1): p. 123-8.
54. Kim, J.M., et al., *Changes in histone acetylation during mouse oocyte meiosis.* J Cell Biol, 2003. **162**(1): p. 37-46.
55. Bouniol-Baly, C., et al., *Differential transcriptional activity associated with chromatin configuration in fully grown mouse germinal vesicle oocytes.* Biol Reprod, 1999. **60**(3): p. 580-7.
56. Miyara, F., et al., *Chromatin configuration and transcriptional control in human and mouse oocytes.* Mol Reprod Dev, 2003. **64**(4): p. 458-70.
57. Maalouf, W.E., R. Alberio, and K.H. Campbell, *Differential acetylation of histone H4 lysine during development of in vitro fertilized, cloned and parthenogenetically activated bovine embryos.* Epigenetics, 2008. **3**(4): p. 199-209.
58. Nagashima, T., et al., *Histone acetylation and subcellular localization of chromosomal protein BRD4 during mouse oocyte meiosis and mitosis.* Mol Hum Reprod, 2007. **13**(3): p. 141-8.
59. Tang, L.S., et al., *Dynamic changes in histone acetylation during sheep oocyte maturation.* J Reprod Dev, 2007. **53**(3): p. 555-61.
60. Akiyama, T., M. Nagata, and F. Aoki, *Inadequate histone deacetylation during oocyte meiosis causes aneuploidy and embryo death in mice.* Proc Natl Acad Sci U S A, 2006. **103**(19): p. 7339-44.
61. Wang, Q., et al., *Histone deacetylation is required for orderly meiosis.* Cell Cycle, 2006. **5**(7): p. 766-74.
62. De La Fuente, R., et al., *ATRX, a member of the SNF2 family of helicase/ATPases, is required for chromosome alignment and meiotic spindle organization in metaphase II stage mouse oocytes.* Dev Biol, 2004. **272**(1): p. 1-14.
63. Yoshida, N., et al., *Epigenetic discrimination by mouse metaphase II oocytes mediates asymmetric chromatin remodeling independently of meiotic exit.* Dev Biol, 2007. **301**(2): p. 464-77.
64. Ma, P. and R.M. Schultz, *Histone deacetylase 1 (HDAC1) regulates histone acetylation, development, and gene expression in preimplantation mouse embryos.* Dev Biol, 2008. **319**(1): p. 110-20.
65. Masui, Y. and C.L. Markert, *Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes.* J Exp Zool, 1971. **177**(2): p. 129-45.

66. Colledge, W.H., et al., *Disruption of c-mos causes parthenogenetic development of unfertilized mouse eggs.* Nature, 1994. **370**(6484): p. 65-8.

67. Hashimoto, N., et al., *Parthenogenetic activation of oocytes in c-mos-deficient mice.* Nature, 1994. **370**(6484): p. 68-71.

68. O'Keefe, S.J., et al., *Microinjection of antisense c-mos oligonucleotides prevents meiosis II in the maturing mouse egg.* Proc Natl Acad Sci U S A, 1989. **86**(18): p. 7038-42.

69. Wu, J.Q. and S. Kornbluth, *Across the meiotic divide - CSF activity in the post-Emi2/XErp1 era.* J Cell Sci, 2008. **121**(Pt 21): p. 3509-14.

70. Grimison, B., et al., *Metaphase arrest by cyclin E-Cdk2 requires the spindle-checkpoint kinase Mps1.* Curr Biol, 2006. **16**(19): p. 1968-73.

71. Schwab, M.S., et al., *Bub1 is activated by the protein kinase p90(Rsk) during Xenopus oocyte maturation.* Curr Biol, 2001. **11**(3): p. 141-50.

72. Tunquist, B.J., et al., *Spindle checkpoint proteins Mad1 and Mad2 are required for cytostatic factor-mediated metaphase arrest.* J Cell Biol, 2003. **163**(6): p. 1231-42.

73. Phillips, K.P., et al., *Inhibition of MEK or cdc2 kinase parthenogenetically activates mouse eggs and yields the same phenotypes as Mos(-/-) parthenogenotes.* Dev Biol, 2002. **247**(1): p. 210-23.

74. Verlhac, M.H., et al., *Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse.* Development, 1996. **122**(3): p. 815-22.

75. Lorca, T., et al., *Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of Xenopus eggs.* Nature, 1993. **366**(6452): p. 270-3.

76. Schmidt, A., et al., *Xenopus polo-like kinase Plx1 regulates XErp1, a novel inhibitor of APC/C activity.* Genes Dev, 2005. **19**(4): p. 502-13.

77. Suzuki, T., et al., *Mouse Emi2 as a distinctive regulatory hub in second meiotic metaphase.* Development, 2010. **137**(19): p. 3281-91.

78. Tung, J.J., et al., *A role for the anaphase-promoting complex inhibitor Emi2/XErp1, a homolog of early mitotic inhibitor 1, in cytostatic factor arrest of Xenopus eggs.* Proc Natl Acad Sci U S A, 2005. **102**(12): p. 4318-23.

79. Madgwick, S. and K.T. Jones, *How eggs arrest at metaphase II: MPF stabilisation plus APC/C inhibition equals Cytostatic Factor.* Cell Div, 2007. **2**: p. 4.

80. Schmidt, A., et al., *Cytostatic factor: an activity that puts the cell cycle on hold.* J Cell Sci, 2006. **119**(Pt 7): p. 1213-8.

81. Evers, J.L., *Female subfertility.* Lancet, 2002. **360**(9327): p. 151-9.

82. Taylor, A., *ABC of subfertility: extent of the problem.* BMJ, 2003. **327**(7412): p. 434-6.

83. Dukelow, W.R., et al., *Preimplantation development of the primate embryo after in vitro fertilization.* J Exp Zool, 1983. **228**(2): p. 215-21.

84. Foote, R.H. and E.W. Carney, *Factors limiting reproductive efficiency in selected laboratory animals.* Ann N Y Acad Sci, 1988. **541**: p. 683-96.

85. Ghosh, D., et al., *Serum concentrations of oestradiol-17beta, progesterone, relaxin and chorionic gonadotrophin during blastocyst implantation in natural pregnancy cycle and in embryo transfer cycle in the rhesus monkey.* Hum Reprod, 1997. **12**(5): p. 914-20.

86. Hassold, T. and P. Hunt, *To err (meiotically) is human: the genesis of human aneuploidy.* Nat Rev Genet, 2001. **2**(4): p. 280-91.

87. Yoon, P.W., et al., *Advanced maternal age and the risk of Down syndrome characterized by the meiotic stage of chromosomal error: a population-based study.* Am J Hum Genet, 1996. **58**(3): p. 628-33.

88. Hassold, T. and S. Sherman, *Down syndrome: genetic recombination and the origin of the extra chromosome 21.* Clin Genet, 2000. **57**(2): p. 95-100.

89. Stewart, G.D., et al., *Trisomy 21 (Down syndrome): studying nondisjunction and meiotic recombination by using cytogenetic and molecular polymorphisms that span chromosome 21.* Am J Hum Genet, 1988. **42**(2): p. 227-36.

90. Jessberger, R., *Deterioration without replenishment--the misery of oocyte cohesin.* Genes Dev, 2010. **24**(23): p. 2587-91.

91. Lister, L.M., et al., *Age-related meiotic segregation errors in mammalian oocytes are preceded by depletion of cohesin and Sgo2.* Curr Biol, 2010. **20**(17): p. 1511-21.

92. Liu, L. and D.L. Keefe, *Defective cohesin is associated with age-dependent misaligned chromosomes in oocytes.* Reprod Biomed Online, 2008. **16**(1): p. 103-12.

93. Revenkova, E., et al., *Oocyte cohesin expression restricted to predictyate stages provides full fertility and prevents aneuploidy.* Curr Biol, 2010. **20**(17): p. 1529-33.

94. Tachibana-Konwalski, K., et al., *Rec8-containing cohesin maintains bivalents without turnover during the growing phase of mouse oocytes.* Genes Dev, 2010. **24**(22): p. 2505-16.

95. Hodges, C.A., et al., *SMC1beta-deficient female mice provide evidence that cohesins are a missing link in age-related nondisjunction.* Nat Genet, 2005. **37**(12): p. 1351-5.

96. Chiang, T., et al., *Evidence that weakened centromere cohesion is a leading cause of age-related aneuploidy in oocytes.* Curr Biol, 2010. **20**(17): p. 1522-8.

97. Koehler, K.E., et al., *Spontaneous X chromosome MI and MII nondisjunction events in Drosophila melanogaster oocytes have different recombinational histories.* Nat Genet, 1996. **14**(4): p. 406-14.

98. Warren, A.C., et al., *Evidence for reduced recombination on the nondisjoined chromosomes 21 in Down syndrome.* Science, 1987. **237**(4815): p. 652-4.

99. Sherman, S.L., et al., *Trisomy 21: association between reduced recombination and nondisjunction.* Am J Hum Genet, 1991. **49**(3): p. 608-20.

100. Lamb, N.E., et al., *Susceptible chiasmate configurations of chromosome 21 predispose to non-disjunction in both maternal meiosis I and meiosis II.* Nat Genet, 1996. **14**(4): p. 400-5.

101. Orr-Weaver, T., *Meiotic nondisjunction does the two-step.* Nat Genet, 1996. **14**(4): p. 374-6.

102. Hoyt, M.A., L. Totis, and B.T. Roberts, *S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function.* Cell, 1991. **66**(3): p. 507-17.

103. Li, R., et al., *The mitotic feedback control gene MAD2 encodes the alpha-subunit of a prenyltransferase.* Nature, 1993. **366**(6450): p. 82-4.

104. Li, R. and A.W. Murray, *Feedback control of mitosis in budding yeast.* Cell, 1991. **66**(3): p. 519-31.

105. Rabitsch, K.P., et al., *A screen for genes required for meiosis and spore formation based on whole-genome expression.* Curr Biol, 2001. **11**(13): p. 1001-9.

106. Wassmann, K., T. Niault, and B. Maro, *Metaphase I arrest upon activation of the Mad2-dependent spindle checkpoint in mouse oocytes.* Curr Biol, 2003. **13**(18): p. 1596-608.

107. Kouznetsova, A., et al., *Bi-orientation of achiasmatic chromosomes in meiosis I oocytes contributes to aneuploidy in mice.* Nat Genet, 2007. **39**(8): p. 966-968.

108. LeMaire-Adkins, R., K. Radke, and P.A. Hunt, *Lack of checkpoint control at the metaphase/anaphase transition: a mechanism of meiotic nondisjunction in mammalian females.* J Cell Biol, 1997. **139**(7): p. 1611-9.

109. Nagaoka, S.I., et al., *Oocyte-specific differences in cell-cycle control create an innate susceptibility to meiotic errors.* Curr Biol, 2011. **21**(8): p. 651-7.

110. Steuerwald, N., et al., *Association between spindle assembly checkpoint expression and maternal age in human oocytes.* Mol Hum Reprod, 2001. **7**(1): p. 49-55.

111. Jenuwein, T. and C.D. Allis, *Translating the histone code.* Science, 2001. **293**(5532): p. 1074-80.

112. Cimini, D., et al., *Histone hyperacetylation in mitosis prevents sister chromatid separation and produces chromosome segregation defects.* Mol Biol Cell, 2003. **14**(9): p. 3821-33.

113. Ekwall, K., et al., *Transient inhibition of histone deacetylation alters the structural and functional imprint at fission yeast centromeres.* Cell, 1997. **91**(7): p. 1021-32.

114. Robbins, A.R., et al., *Inhibitors of histone deacetylases alter kinetochore assembly by disrupting pericentromeric heterochromatin.* Cell Cycle, 2005. **4**(5): p. 717-26.

115. Ma, Y., et al., *Inhibition of protein deacetylation by trichostatin A impairs microtubule-kinetochore attachment.* Cell Mol Life Sci, 2008. **65**(19): p. 3100-9.

116. Taddei, A., et al., *Reversible disruption of pericentric heterochromatin and centromere function by inhibiting deacetylases.* Nat Cell Biol, 2001. **3**(2): p. 114-20.

117. Magnaghi-Jaulin, L., et al., *Histone deacetylase inhibitors induce premature sister chromatid separation and override the mitotic spindle assembly checkpoint.* Cancer Res, 2007. **67**(13): p. 6360-7.

118. Shin, H.-J., et al., *Inhibition of histone deacetylase activity increases chromosomal instability by the aberrant regulation of mitotic checkpoint activation.* Oncogene, 2003. **22**(25): p. 3853-3858.

119. Stevens, F.E., et al., *Histone deacetylase inhibitors induce mitotic slippage.* Oncogene, 2008. **27**(10): p. 1345-54.

120. Alberts, B., et al., *Molecular Biology Of The Cell*. Vol. 4. 2002.

121. Tres, L.L., *Extensive pairing of the XY bivalent in mouse spermatocytes as visualized by whole-mount electron microscopy.* J Cell Sci, 1977. **25**: p. 1-15.

122. Inagaki, A., S. Schoenmakers, and W.M. Baarends, *DNA double strand break repair, chromosome synapsis and transcriptional silencing in meiosis.* Epigenetics, 2010. **5**(4): p. 255-66.

123. Turner, J.M., *Meiotic sex chromosome inactivation.* Development, 2007. **134**(10): p. 1823-31.

124. Namekawa, S.H., et al., *Postmeiotic sex chromatin in the male germline of mice.* Curr Biol, 2006. **16**(7): p. 660-7.

125. Mueller, J.L., et al., *The mouse X chromosome is enriched for multicopy testis genes showing postmeiotic expression.* Nat Genet, 2008. **40**(6): p. 794-9.

126. Mulugeta Achame, E., et al., *The ubiquitin-conjugating enzyme HR6B is required for maintenance of X chromosome silencing in mouse spermatocytes and spermatids.* BMC Genomics, 2010. **11**: p. 367.

127. Huynh, K.D. and J.T. Lee, *Inheritance of a pre-inactivated paternal X chromosome in early mouse embryos.* Nature, 2003. **426**(6968): p. 857-62.

128. Okamoto, I. and E. Heard, *The dynamics of imprinted X inactivation during preimplantation development in mice.* Cytogenet Genome Res, 2006. **113**(1-4): p. 318-24.

129. Fernandez-Capetillo, O., et al., *H2AX is required for chromatin remodeling and inactivation of sex chromosomes in male mouse meiosis.* Dev Cell, 2003. **4**(4): p. 497-508.

130. Turner, J.M., et al., *BRCA1, histone H2AX phosphorylation, and male meiotic sex chromosome inactivation.* Curr Biol, 2004. **14**(23): p. 2135-42.

131. Bellani, M.A., et al., *SPO11 is required for sex-body formation, and Spo11 heterozygosity rescues the prophase arrest of Atm-/- spermatocytes.* J Cell Sci, 2005. **118**(Pt 15): p. 3233-45.

132. Royo, H., et al., *Evidence that meiotic sex chromosome inactivation is essential for male fertility.* Curr Biol, 2010. **20**(23): p. 2117-23.

133. Meistrich, M.L., et al., *Roles of transition nuclear proteins in spermiogenesis.* Chromosoma, 2003. **111**(8): p. 483-8.

134. Rousseaux, S., et al., *Establishment of male-specific epigenetic information.* Gene, 2005. **345**(2): p. 139-53.

135. Tanphaichitr, N., et al., *Basic nuclear proteins in testicular cells and ejaculated spermatozoa in man.* Exp Cell Res, 1978. **117**(2): p. 347-56.

136. Bench, G.S., et al., *DNA and total protamine masses in individual sperm from fertile mammalian subjects.* Cytometry, 1996. **23**(4): p. 263-71.

137. Gatewood, J.M., et al., *Isolation of four core histones from human sperm chromatin representing a minor subset of somatic histones.* J Biol Chem, 1990. **265**(33): p. 20662-6.

138. Visconti, P.E., et al., *Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway.* Development, 1995. **121**(4): p. 1139-50.

139. Lambert, H., et al., *Sperm capacitation in the human female reproductive tract.* Fertil Steril, 1985. **43**(2): p. 325-7.

140. Payne, D., et al., *Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography.* Hum Reprod, 1997. **12**(3): p. 532-41.

141. van der Heijden, G.W., et al., *Asymmetry in histone H3 variants and lysine methylation between paternal and maternal chromatin of the early mouse zygote.* Mech Dev, 2005. **122**(9): p. 1008-22.

142. Lepikhov, K. and J. Walter, *Differential dynamics of histone H3 methylation at positions K4 and K9 in the mouse zygote.* BMC Dev Biol, 2004. **4**: p. 12.

143. Yeo, S., et al., *Methylation changes of lysine 9 of histone H3 during preimplantation mouse development.* Mol Cells, 2005. **20**(3): p. 423-8.

144. Liu, H., J.M. Kim, and F. Aoki, *Regulation of histone H3 lysine 9 methylation in oocytes and early pre-implantation embryos.* Development, 2004. **131**(10): p. 2269-80.

145. Pieters, M.H., et al., *Triploidy after in vitro fertilization: cytogenetic analysis of human zygotes and embryos.* J Assist Reprod Genet, 1992. **9**(1): p. 68-76.

146. Porter, R., et al., *Estimation of second polar body retention rate after conventional insemination and intracytoplasmic sperm injection: in vitro observations from more than 5000 human oocytes.* J Assist Reprod Genet, 2003. **20**(9): p. 371-6.

147. Macas, E., et al., *The chromosomal complements of multipronuclear human zygotes resulting from intracytoplasmic sperm injection.* Hum Reprod, 1996. **11**(11): p. 2496-501.

148. Otsu, E., et al., *Developmental potential and chromosomal constitution of embryos derived from larger single pronuclei of human zygotes used in in vitro fertilization.* Fertil Steril, 2004. **81**(3): p. 723-4.

149. Lawler, S.D., *Genetic studies on hydatidiform moles.* Adv Exp Med Biol, 1984. **176**: p. 147-61.

150. Fisher, R.A., D.M. Sheppard, and S.D. Lawler, *Two patients with complete hydatidiform mole with 46,XY karyotype.* Br J Obstet Gynaecol, 1984. **91**(7): p. 690-3.

151. Wake, N., et al., *Malignant potential of homozygous and heterozygous complete moles.* Cancer Res, 1984. **44**(3): p. 1226-30.

152. Latham, K.E. and R.M. Schultz, *Embryonic genome activation.* Front Biosci, 2001. **6**: p. D748-59.

153. Hamatani, T., et al., *Dynamics of global gene expression changes during mouse preimplantation development.* Dev Cell, 2004. **6**(1): p. 117-31.

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

155. Nichols, J., et al., *Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4.* Cell, 1998. **95**(3): p. 379-91.

156. Avilion, A.A., et al., *Multipotent cell lineages in early mouse development depend on SOX2 function.* Genes & Development, 2003. **17**(1): p. 126-140.

157. Mitsui, K., et al., *The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells.* Cell, 2003. **113**(5): p. 631-42.

158. Chambers, I., et al., *Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells.* Cell, 2003. **113**(5): p. 643-55.

159. Chazaud, C., et al., *Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway.* Dev Cell, 2006. **10**(5): p. 615-24.

160. Silva, J., et al., *Nanog is the gateway to the pluripotent ground state.* Cell, 2009. **138**(4): p. 722-37.

161. Strumpf, D., et al., *Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst.* Development, 2005. **132**(9): p. 2093-2102.

162. Morrisey, E.E., et al., *GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo.* Genes Dev, 1998. **12**(22): p. 3579-90.

163. Cai, K.Q., et al., *Dynamic GATA6 expression in primitive endoderm formation and maturation in early mouse embryogenesis.* Dev Dyn, 2008. **237**(10): p. 2820-9.

164. Simon, C., et al., *Coculture of human embryos with autologous human endometrial epithelial cells in patients with implantation failure.* J Clin Endocrinol Metab, 1999. **84**(8): p. 2638-46.

165. Bentin-Ley, U. and A. Lopata, *In vitro models of human blastocyst implantation.* Baillieres Best Pract Res Clin Obstet Gynaecol, 2000. **14**(5): p. 765-74.

166. Bentin-Ley, U., et al., *Isolation and culture of human endometrial cells in a three-dimensional culture system.* J Reprod Fertil, 1994. **101**(2): p. 327-32.

167. Lyon, M.F., *Gene action in the X-chromosome of the mouse (Mus musculus L.).* Nature, 1961. **190**: p. 372-3.

168. Mak, W., et al., *Reactivation of the paternal X chromosome in early mouse embryos.* Science, 2004. **303**(5658): p. 666-9.

169. Rastan, S., *Timing of X-chromosome inactivation in postimplantation mouse embryos.* J Embryol Exp Morphol, 1982. **71**: p. 11-24.

170. Sugawara, O., N. Takagi, and M. Sasaki, *Correlation between X-chromosome inactivation and cell differentiation in female preimplantation mouse embryos.* Cytogenet Cell Genet, 1985. **39**(3): p. 210-9.

171. Takagi, N., O. Sugawara, and M. Sasaki, *Regional and temporal changes in the pattern of X-chromosome replication during the early post-implantation development of the female mouse.* Chromosoma, 1982. **85**(2): p. 275-86.

172. Brown, C.J., et al., *A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome.* Nature, 1991. **349**(6304): p. 38-44.

173. Penny, G.D., et al., *Requirement for Xist in X chromosome inactivation.* Nature, 1996. **379**(6561): p. 131-7.

174. Plath, K., et al., *Xist RNA and the mechanism of X chromosome inactivation.* Annu Rev Genet, 2002. **36**: p. 233-78.

175. Kalantry, S., et al., *Evidence of Xist RNA-independent initiation of mouse imprinted X-chromosome inactivation.* Nature, 2009. **460**(7255): p. 647-51.

176. Kay, G.F., et al., *Imprinting and X chromosome counting mechanisms determine Xist expression in early mouse development.* Cell, 1994. **77**(5): p. 639-50.

177. Okamoto, I., S. Tan, and N. Takagi, *X-chromosome inactivation in XX androgenetic mouse embryos surviving implantation.* Development, 2000. **127**(19): p. 4137-45.

178. Burgoyne, P.S., P.P. Tam, and E.P. Evans, *Retarded development of XO conceptuses during early pregnancy in the mouse.* J Reprod Fertil, 1983. **68**(2): p. 387-93.

179. Burgoyne, P.S., E.P. Evans, and K. Holland, *XO monosomy is associated with reduced birthweight and lowered weight gain in the mouse.* J Reprod Fertil, 1983. **68**(2): p. 381-5.

180. Burgoyne, P.S., O.A. Ojarikre, and J.M. Turner, *Evidence that postnatal growth retardation in XO mice is due to haploinsufficiency for a non-PAR X gene.* Cytogenet Genome Res, 2002. **99**(1-4): p. 252-6.

181. Tada, T., et al., *Imprint switching for non-random X-chromosome inactivation during mouse oocyte growth.* Development, 2000. **127**(14): p. 3101-5.

182. Ariel, M., et al., *Gamete-specific methylation correlates with imprinting of the murine Xist gene.* Nat Genet, 1995. **9**(3): p. 312-5.

183. Norris, D.P., et al., *Evidence that random and imprinted Xist expression is controlled by preemptive methylation.* Cell, 1994. **77**(1): p. 41-51.

184. Zuccotti, M. and M. Monk, *Methylation of the mouse Xist gene in sperm and eggs correlates with imprinted Xist expression and paternal X-inactivation.* Nat Genet, 1995. **9**(3): p. 316-20.

185. McDonald, L.E., C.A. Paterson, and G.F. Kay, *Bisulfite genomic sequencing-derived methylation profile of the xist gene throughout early mouse development.* Genomics, 1998. **54**(3): p. 379-86.

186. Kalantry, S., et al., *The Polycomb group protein Eed protects the inactive X-chromosome from differentiation-induced reactivation.* Nat Cell Biol, 2006. **8**(2): p. 195-202.

187. Wang, J., et al., *Imprinted X inactivation maintained by a mouse Polycomb group gene.* Nat Genet, 2001. **28**(4): p. 371-5.

188. Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos.* Nature, 1981. **292**(5819): p. 154-156.

189. Martin, G.R., *Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells.* Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7634-8.

190. Barakat, T.S. and J. Gribnau, *X chromosome inactivation and embryonic stem cells.* Adv Exp Med Biol, 2010. **695**: p. 132-54.

191. Ray, P.F., R.M. Winston, and A.H. Handyside, *XIST expression from the maternal X chromosome in human male preimplantation embryos at the blastocyst stage.* Hum Mol Genet, 1997. **6**(8): p. 1323-7.

192. Daniels, R., et al., *XIST expression in human oocytes and preimplantation embryos.* Am J Hum Genet, 1997. **61**(1): p. 33-9.

193. Sheardown, S.A., et al., *Stabilization of Xist RNA mediates initiation of X chromosome inactivation.* Cell, 1997. **91**(1): p. 99-107.

194. Keeney, S., C.N. Giroux, and N. Kleckner, *Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family.* Cell, 1997. **88**(3): p. 375-84.

195. Bergerat, A., et al., *An atypical topoisomerase II from Archaea with implications for meiotic recombination.* Nature, 1997. **386**(6623): p. 414-7.

196. Cohen, P.E., S.E. Pollack, and J.W. Pollard, *Genetic analysis of chromosome pairing, recombination, and cell cycle control during first meiotic prophase in mammals.* Endocr Rev, 2006. **27**(4): p. 398-426.

197. Kauppi, L., A.J. Jeffreys, and S. Keeney, *Where the crossovers are: recombination distributions in mammals.* Nat Rev Genet, 2004. **5**(6): p. 413-24.

198. Lamb, N., et al., *Susceptible chiasmate configurations of chromosome 21 predispose to non-disjunction in both maternal meiosis I and meiosis II.* Nat Genet, 1996. **14**(4): p. 400-405.

199. Revenkova, E., et al., *Novel meiosis-specific isoform of mammalian SMC1.* Mol Cell Biol, 2001. **21**(20): p. 6984-98.

200. Watanabe, Y. and P. Nurse, *Cohesin Rec8 is required for reductional chromosome segregation at meiosis.* Nature, 1999. **400**(6743): p. 461-4.

201. Prieto, I., et al., *Mammalian STAG3 is a cohesin specific to sister chromatid arms in meiosis I.* Nat Cell Biol, 2001. **3**(8): p. 761-6.

202. Swain, J.E., et al., *Proper chromatin condensation and maintenance of histone H3 phosphorylation during mouse oocyte meiosis requires protein phosphatase activity.* Biol Reprod, 2007. **76**(4): p. 628-38.

203. Eot-Houllier, G., et al., *Histone deacetylase 3 is required for centromeric H3K4 deacetylation and sister chromatid cohesion.* Genes Dev, 2008. **22**(19): p. 2639-44.

204. Baumann, C., M.M. Viveiros, and R. De La Fuente, *Loss of maternal ATRX results in centromere instability and aneuploidy in the mammalian oocyte and pre-implantation embryo.* PLoS Genet, 2010. **6**(9).

205. Schuh, M. and J. Ellenberg, *Self-Organization of MTOCs Replaces Centrosome Function during Acentrosomal Spindle Assembly in Live Mouse Oocytes.* Cell, 2007. **130**(3): p. 484-498.

206. Kitajima, T.S., M. Ohsugi, and J. Ellenberg, *Complete kinetochore tracking reveals error-prone homologous chromosome biorientation in Mammalian oocytes.* Cell, 2011. **146**(4): p. 568-81.

207. Illingworth, C., et al., *MCAK regulates chromosome alignment but is not necessary for preventing aneuploidy in mouse oocyte meiosis I.* Development, 2010. **137**(13): p. 2133-8.

208. Vogt, E., et al., *MCAK is present at centromeres, midspindle and chiasmata and involved in silencing of the spindle assembly checkpoint in mammalian oocytes.* Mol Hum Reprod, 2010. **16**(9): p. 665-84.

209. Swain, J.E., et al., *Regulation of spindle and chromatin dynamics during early and late stages of oocyte maturation by aurora kinases.* Mol Hum Reprod, 2008. **14**(5): p. 291-9.

210. Sharif, B., et al., *The chromosome passenger complex is required for fidelity of chromosome transmission and cytokinesis in meiosis of mouse oocytes.* Journal of Cell Science, 2010. **123**(24): p. 4292-4300.

211. Sun, S.C., et al., *Borealin regulates bipolar spindle formation but may not act as chromosomal passenger during mouse oocyte meiosis.* Front Biosci (Elite Ed), 2010. **2**: p. 991-1000.

212. Yamamoto, T.M., A.L. Lewellyn, and J.L. Maller, *Regulation of the Aurora B chromosome passenger protein complex during oocyte maturation in Xenopus laevis.* Mol Cell Biol, 2008. **28**(12): p. 4196-203.

213. Colombié, N., et al., *Dual roles of Incenp crucial to the assembly of the acentrosomal metaphase spindle in female meiosis.* Development, 2008. **135**(19): p. 3239-3246.

214. Shuda, K., et al., *Aurora kinase B modulates chromosome alignment in mouse oocytes.* Molecular Reproduction and Development, 2009. **76**(11): p. 1094-1105.

215. Sun, S.-C., et al., *Perturbation of survivin expression affects chromosome alignment and spindle checkpoint in mouse oocyte meiotic maturation.* Cell Cycle, 2009. **8**(20): p. 3365-3372.

216. Vogt, E., A. Kipp, and U. Eichenlaub-Ritter, *Aurora kinase B, epigenetic state of centromeric heterochromatin and chiasma resolution in oocytes.* Reprod Biomed Online, 2009. **19**(3): p. 352-68.

217. Wang, K., et al., *Survivin is a critical regulator of spindle organization and chromosome segregation during rat oocyte meiotic maturation.* Zygote, 2010: p. 1-7.

218. Li, M., et al., *Bub3 is a spindle assembly checkpoint protein regulating chromosome segregation during mouse oocyte meiosis.* PLoS One, 2009. **4**(11): p. e7701.

219. McGuinness, B.E., et al., *Regulation of APC/C activity in oocytes by a Bub1-dependent spindle assembly checkpoint.* Curr Biol, 2009. **19**(5): p. 369-80.

220. Niault, T., et al., *Changing Mad2 levels affects chromosome segregation and spindle assembly checkpoint control in female mouse meiosis I.* PLoS One, 2007. **2**(11): p. e1165.

221. Clift, D. and A.L. Marston, *The role of shugoshin in meiotic chromosome segregation.* Cytogenet Genome Res, 2011. **133**(2-4): p. 234-42.

222. Kawashima, S.A., et al., *Phosphorylation of H2A by Bub1 prevents chromosomal instability through localizing shugoshin.* Science, 2010. **327**(5962): p. 172-7.

223. Kudo, N.R., et al., *Role of cleavage by separase of the Rec8 kleisin subunit of cohesin during mammalian meiosis I.* J Cell Sci, 2009. **122**(Pt 15): p. 2686-98.

224. Pfender, S., et al., *Spire-type actin nucleators cooperate with Formin-2 to drive asymmetric oocyte division.* Curr Biol, 2011. **21**(11): p. 955-60.

225. Francastel, C., et al., *Nuclear compartmentalization and gene activity.* Nat Rev Mol Cell Biol, 2000. **1**(2): p. 137-143.

226. Tan, M., et al., *Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification.* Cell, 2011. **146**(6): p. 1016-28.

227. Bhaumik, S.R., E. Smith, and A. Shilatifard, *Covalent modifications of histones during development and disease pathogenesis.* Nat Struct Mol Biol, 2007. **14**(11): p. 1008-16.

228. Takagi, N. and M. Sasaki, *Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse.* Nature, 1975. **256**(5519): p. 640-2.

229. Wutz, A. and J. Gribnau, *X inactivation Xplained.* Current Opinion in Genetics & Development, 2007. **17**(5): p. 387-393.

230. Starmer, J. and T. Magnuson, *A new model for random X chromosome inactivation.* Development, 2009. **136**(1): p. 1-10.

231. Augui, S., et al., *Sensing X chromosome pairs before X inactivation via a novel X-pairing region of the Xic.* Science, 2007. **318**(5856): p. 1632-6.

232. Xu, N., C.L. Tsai, and J.T. Lee, *Transient homologous chromosome pairing marks the onset of X inactivation.* Science, 2006. **311**(5764): p. 1149-52.

233. Bacher, C.P., et al., *Transient colocalization of X-inactivation centres accompanies the initiation of X inactivation.* Nat Cell Biol, 2006. **8**(3): p. 293-9.

234. Rastan, S. and E.J. Robertson, *X-chromosome deletions in embryo-derived (EK) cell lines associated with lack of X-chromosome inactivation.* J Embryol Exp Morphol, 1985. **90**: p. 379-88.

235. Marahrens, Y., J. Loring, and R. Jaenisch, *Role of the Xist gene in X chromosome choosing.* Cell, 1998. **92**(5): p. 657-64.

236. Matsui, J., Y. Goto, and N. Takagi, *Control of Xist expression for imprinted and random X chromosome inactivation in mice.* Human Molecular Genetics, 2001. **10**(13): p. 1393-1401.

237. Donohoe, M.E., et al., *The pluripotency factor Oct4 interacts with Ctcf and also controls X-chromosome pairing and counting.* Nature, 2009. **460**(7251): p. 128-32.

238. Monkhorst, K., et al., *The probability to initiate X chromosome inactivation is determined by the X to autosomal ratio and X chromosome specific allelic properties.* PLoS One, 2009. **4**(5): p. e5616.

239. Monkhorst, K., et al., *X Inactivation Counting and Choice Is a Stochastic Process: Evidence for Involvement of an X-Linked Activator.* Cell, 2008. **132**(3): p. 410-421.

240. Jonkers, I., et al., *RNF12 is an X-Encoded dose-dependent activator of X chromosome inactivation.* Cell, 2009. **139**(5): p. 999-1011.

241. Barakat, T.S., et al., *RNF12 activates Xist and is essential for X chromosome inactivation.* PLoS Genet, 2011. **7**(1): p. e1002001.

242. Tian, D., S. Sun, and J.T. Lee, *The long noncoding RNA, Jpx, is a molecular switch for X chromosome inactivation.* Cell, 2010. **143**(3): p. 390-403.

243. Barakat, T.S., et al., *X-changing information on X inactivation.* Exp Cell Res, 2010. **316**(5): p. 679-87.

CHAPTER 2

Defective deacetylation of H4K12 in human oocytes is associated with advanced maternal age and chromosome misalignment

I.M. van den Berg, C. Eleveld, M. van der Hoeven, E. Birnie, E.A.P. Steegers, R.-J. Galjaard, J.S.E. Laven, J.H. van Doorninck

Published in *Human Rep. 2011 May; 26(5):1181-90*

Abstract

BACKGROUND: Chromosome segregation errors during human oocyte meiosis are associated with low fertility in humans and the incidence of these errors increases with advancing maternal age. Studies of mitosis and meiosis suggest that defective remodeling of chromatin plays a causative role in aneuploidy. We analyzed the histone acetylation pattern during the final stages of human oocyte maturation to investigate whether defective epigenetic regulation of chromatin remodeling in human oocytes is related to maternal age and leads to segregation errors.

METHODS: Human surplus oocytes of different meiotic maturation stages [germinal vesicle (GV), metaphase (M)I and MII] were collected from standard IVF/ICSI treatments. Oocytes were analyzed for acetylation of different lysines of histone 4 (H4K5, H4K8, H4K12 and H4K16) and for α-tubulin.

RESULTS: Human GV oocytes had an intense staining of the chromatin for all four histone 4 lysine acetylations. MI and MII stage oocytes showed either normal deacetylation or various amounts of defective deacetylation. Residual H4K12 acetylation was more frequently found in oocytes obtained from older women, with a significant correlation between defective deacetylation and maternal age (r = 0.185, P = 0.007). Eighty-eight percent of the oocytes with residual acetylation had misaligned chromosomes, whereas only 33% of the oocytes that showed correct deacetylated chromatin had misaligned chromosomes (P < 0.001).

CONCLUSIONS: We conclude that defective deacetylation during human female meiosis increases with maternal age and is correlated with misaligned chromosomes. As chromosome misalignment predisposes to segregation errors, our data imply that defective regulation of histone deacetylation could be an important factor in age-related aneuploidy.

Key words: histone deacetylation / female meiosis / maternal age / misaligned chromosomes / aneuploidy

Introduction

Aneuploidy is one of the main underlying causes of human infertility and is primarily caused by segregation errors during female meiosis (reviewed in [1,2]) and the frequency of these oocyte errors increases with advancing maternal age [1-3]. Although the occurrence of premature sister chromatid separations and non-disjunction has been described [3-6], the exact mechanisms causing division errors and the specific genes/proteins involved are still unknown. Chromosome structure is of critical importance for faithful chromosome segregation during mitosis as well as meiosis [7-12]. Defective remodeling of chromatin, which determines the structure of chromosomes, is thus a possible cause of abnormal segregation during human oocyte meiosis.

After maturation from a primordial to an antral follicle, oocytes must switch from a transcriptionally active stage to a transcriptionally silent stage characterized by strongly condensed chromosomes, which permits faithful segregation during meiosis. At the final maturation phase, just prior to the meiotic segregations, gene transcription is terminated [13,14], and the chromosomes are arranged for division. For this transition in transcriptional activity, the chromatin of the oocyte needs to be drastically altered from a conformation that favors gene transcription at the germinal vesicle (GV) stage to a structure that allows for two sequential chromosome divisions in a correct and timely manner during metaphase I and II (MI and MII). Changes in chromatin conformation are established through modifications on the basic subunits of the nucleosome, the histone proteins. Histones can be modified through the addition or removal of small molecule groups, such as methyl, phospho and acetyl groups, that are covalently bound to specific residues in the histone tails (reviewed in [15]).

To form a chromatin structure that permits the condensation and segregation of chromosomes, histone modifications that favor gene transcription, such as acetylation of histones H3 and H4, are removed during the final steps of oocyte maturation and meiosis [16,17]. The hypo-acetylated state of chromatin is regulated through a balance of the activity of two groups of enzymes: the histone acetyl transferases (HATs), which are responsible for adding acetyl groups, and the histone deacetylases (HDACs), which remove acetyl groups to permit heterochromatin formation. Inhibition of these HDACs leads to chromosome instability in meiotic and mitotic cells [7-9,11,12,18].

The regulation of chromatin configurations in human oocytes has not yet been investigated, nor is it known whether abnormal chromatin organization is implicated in aneuploidy. To investigate the possible relationship between epigenetic modifications and segregation errors in human oocytes, we studied the deacetylation dynamics of histones during

the last phases of meiotic maturation in combination with an assessment of spindle structure and chromosome alignment in surplus human oocytes. As oocytes of older women are known to have reduced developmental competence and to be extremely prone to aneuploidy [3], we therefore hypothesized that inadequate deacetylation during human oocyte maturation could be involved in the etiology of (age-related) aneuploidy. Based on our experiments, we suggest that segregation errors during human female meiosis are related to the faulty regulation of chromatin and that this regulation becomes more defective in oocytes as maternal age advances.

Materials and Methods

Ovarian stimulation and ovum retrieval

Stimulation schemes were chosen according to patient requirements and generally consisted of ovarian hyperstimulation with recombinant FSH (rFSH) and co-treatment with either GnRH antagonist or GnRH agonist. Ovum retrieval was performed 35 h after hCG injection. In ICSI treatments, oocytes were denuded after ovum retrieval and checked for the maturation stage. In IVF treatments, oocytes were inseminated as cumulus-oocyte complexes. On the following day, the oocytes were checked for fertilization. This study was approved by the local ethics review committee of the Erasmus MC Hospital (MEC 2007-130). To confirm that surplus oocytes could be used for research purposes, couples undergoing an ICSI/IVF treatment were informed of the study and could sign an objection declaration.

Oocyte collection

After ovum retrieval, cumulus-oocytes complexes were collected in G-MOPS culture medium (Vitrolife, Sweden) and cultured in G-IVF medium (Vitrolife, Sweden) under 5% CO_2 until pronuclear assessment at Day 1. Oocytes at the GV and MI stage are considered not suitable for fertilization and are not used in ICSI treatments. GV oocytes were fixed 3–7 h after ovum retrieval. MI and MII oocytes were collected at two time points. One group (Day 0) was obtained from patients whose semen parameters were too low to inject all oocytes or who wanted to fertilize only some of their oocytes for personal reasons. These surplus oocytes were collected 4–8 h after ovum retrieval. The other group (Day 1) was collected 20–24 h after insemination (IVF) or injection (ICSI). These oocytes did not show any signs of fertilization (one polar body and no pronuclei) and were therefore considered surplus. Oocytes from total

fertilization failures were not included in this study. All oocytes were fixed as soon as they became available after donation for research.

Fixation, immunocytochemistry and microscopy

Oocytes were fixed as described previously [19] with some minor modifications. For a detailed description of the methods, see Supplementary data, Table S1. Oocytes and cumulus control cells were analyzed on an epi-fluorescence microscope (Axioplan 2, Zeiss, Germany) equipped with appropriate filter sets and a charge-coupled device video camera, and images were captured with ISIS Imaging System software (Metasystems, Germany). For three-dimensional analysis, we used a confocal microscope with appropriate lasers and software (Zeiss LSM510). Both epifluorescent and confocal images were quantified with Image J [20] through a comparison of the histone modification staining in the 4',6-diamidino-2-phenylindole (DAPI)-positive region with the cytoplasmic background staining. For confocal analysis, settings were kept constant for the laser intensity and the interval of the Z series was kept constant. Bleed through of DAPI was assessed by image acquisition of the acetylated H4K12 (H4K12ac) laser wavelength set to zero. In case of bleed through, the H4K12ac staining was quantified against the DAPI bleed through signal. Cumulus cells were taken as a positive control in each experiment. As each oocyte was fixed individually at the moment of donation, this resulted in a variation in fixation and storage time for which no internal reference standard was available. Meaningful comparison of quantified intensities between individuals was therefore not possible.

Only oocytes that had maternal chromosomes, an indication that they were still in the MII stage, were used. Oocytes that either had a pronucleus or had degenerated DNA were excluded from the analysis.

Oocytes were scored for chromosomal alignment according to previously published classifications [21-23]. Oocytes that had paternal chromatin that was indistinguishable from the maternal chromatin, and oocytes in which the spindle orientation could not be observed, were considered not analyzable. To define the range of the misalignment, we measured the width of the chromosome alignment. A perfect alignment in which all chromosomes were arranged on the equatorial plate was scored as 1 (width of 1 chromosome) and considered normal. Alignments spanning two and three chromosome lengths were still considered normal. Oocytes were considered to have congression failure if the width of the chromosomes around the equatorial plate spanned four chromosome lengths or more. Oocytes with a single chromosome clearly sitting apart from the remaining aligned chromosomes were considered as chromosome lagging. Images were deconvoluted with SVI software (SVI, The Netherlands)

when necessary. Spindle structure and chromosome alignment were scored by two researchers who were unaware of the acetylation status of the chromatin.

Statistics

To analyze the relationship of residual acetylation with maternal age and the association of residual acetylation with chromosome misalignment, we used the λ^2 test and Spearman's correlation. When necessary, correction for small numbers was performed with Fisher's exact test. For the difference in median age between groups of oocyte donors the Mann-Whitney *U*-test was used. Statistical significance was defined as $P < 0.05$ (Statistical Package for the Social Sciences 15.0 USA).

Table I.

	Oocyte acetylation on H4 lysines, +/n (%)			
Maturation stage	**H4K5**	**H4K8**	**H4K12**	**H4K16**
GV	12/12 (100)	12/12 (100)	32/32 (100)	5/5 (100)
MI	2/5 (40)	2/6 (33)	19/41 (46)	4/4 (100)
MII	8/15 (53)	29/48 (81)	96/210 (46)	5/10 (50)

Data (+/n) show the number of oocytes with positive staining/total number of oocytes. GV, germinal vesicle; MI/II, metaphase I/II.

Results

Regulation of histone acetylation during human oocyte maturation

To determine the acetylation pattern of human oocytes during meiotic maturation, we analyzed the acetylation status of four lysine residues on histone 4, H4K5, H4K8, H4K12 and H4K16, at different maturation stages, GV, MI and MII in surplus oocytes from IVF treatments (numbers analyzed shown in Supplementary data, Table S2). We collected both un-inseminated (virgin) MII oocytes at the day of ovarian aspiration (Day 0) and failed fertilized MII oocytes at Day 1 to investigate the acetylation status of MII oocytes that had matured *in vivo* (Supplementary data, Table S2, Table I). In surplus human GV oocytes collected at Day 0 from ICSI treatments, we observed two types of DNA configurations: oocytes with DNA dispersed around the large nucleolus with a number of condensed bodies (non-surrounding nucleolus, NSN, Figure 1A-C), and later-stage oocytes with the DNA completely condensed around the nucleolus (surrounding nucleolus, SN, Figure 1D-F). Both stages of GV oocytes showed intense staining

of all of the four analyzed H4 acetylated lysine residues (Figure 2 and data not shown). As DAPI staining has a preference for AT-rich regions, the DAPI staining does not always overlap with the histone acetylation markers.

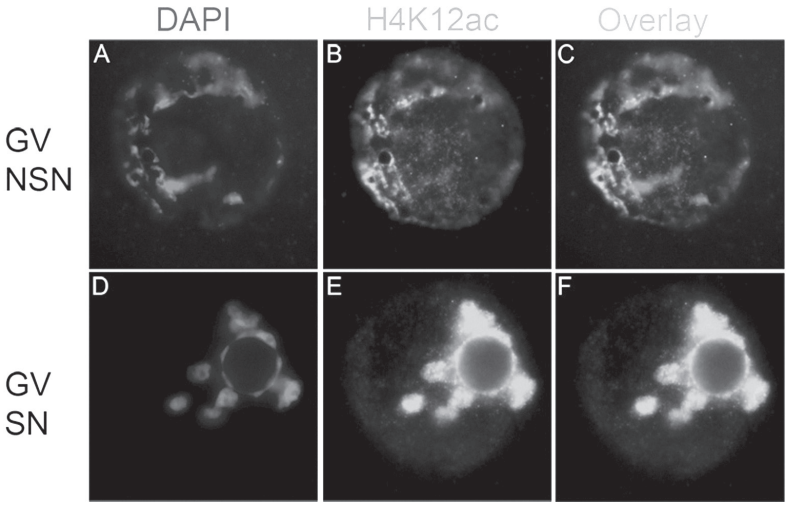


Figure 1. Histone acetylation in human GV oocytes. An early-stage human GV oocyte with a non-surrounding nucleus (NSN)(**A-C**) stained for chromatin (blue, DAPI); (**A**) and histone acetylation (red, anti-H4K12ac) (**B**). Some chromatin regions have intense staining, whereas other (dense) regions showed no acetylation (overlay **C**). A more developed human GV oocyte with a surrounding nucleus stained for chromatin (blue, DAPI; **D**) and histone acetylation (red, anti-H4K12ac; **E**) has chromatin that is far more condensed (**D**) than the NSN GV, but it still has acetylated chromatin (**E**), as shown by the overlay (**F**). Scale bar represents 10 μm (see page 169 for colour figure).

With the progression of meiosis, we found that at the divisional stages MI and MII, chromatin was deacetylated in variable proportions of the oocytes at H4K5, H4K8, H4K12 and H4K16 (Table I, Figure 2). However, a substantial percentage of the oocytes had residual acetylation at the MI and MII stages (Table I, Figure 2) varying from 46% residual acetylation for H4K12 to 81% residual acetylation for H4K8 in MII oocytes. We used oocytes collected from both Day 0 as well as Day 1 and compared the acetylation status between the groups but found no difference in the percentage of residual acetyation (Day 0 39.7% versus Day 1 49.2%; λ^2 $P = 0.743$). We therefore combined all MII oocytes into one group in our further analyses.

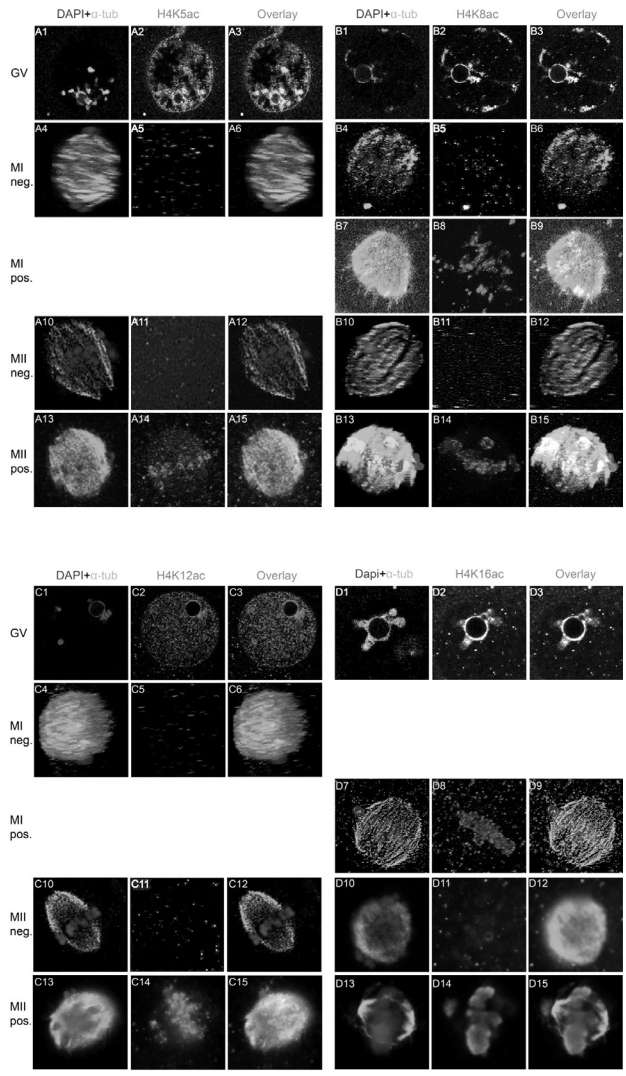


Figure 2. Histone deacetylation during human oocyte maturation. GV, MI and MII oocytes were stained for H4K5ac (**A1–A15**), H4K8ac (**B1–B15**), H4K12ac (**C1–C15**) and H4K16ac (**D1–D15**). Representative images are shown for the indicated groups. GV oocytes showed intense staining for all four lysine residues (**A2, B2**, **C2** and **D2**) and were not analyzed for a-tubulin because no spindle is present at this stage. At the MI stage, the majority of oocytes had no staining for H4K5ac (**A4–6**), H4K8 (**B4–6**) and H4K12ac (**C4–6**). H4K16ac was positive in all tested MI oocytes (**D7–9**). In oocytes of the MII stage, a variable part of the oocytes showed no staining for the four tested lysine acetylations (**A11–D11**). In oocytes with residual acetylation, histone lysine staining was observed (**A14–D14**) overlapping with the DAPI-stained chromosomes (**A13–A15** to **D13–D15**). Scale bar for **A1–A3**, **B1–B3**, **C1–C3** and **D1–D3** represents 30 μm. Scale bar for **A4–A15**, **B4–B15**, **C4–C15** and **D4–D15** represents 10 μm (see page 172 for colour figure).

H4K12ac and maternal age

A factor that is known to strongly influence human oocyte quality and developmental competence is advanced maternal age [2]. A previous study in mouse oocytes showed that maternal age is related to residual acetylation of two lysines on histone 4 (H4K8 and H4K12) [9]. We hypothesized that in human MII oocytes, maternal age might also negatively influence the deacetylation process. Because in mouse oocytes, the difference between the percentage of residual acetylation in old and young mice was most prominent for the acetylation on H4K12 [9] and surplus human oocytes are limited, we focused on the acetylation status of H4K12. Oocytes were grouped according to the following maternal age categories: young maternal age (age 24–30 years), middle maternal age (age 31–35 years) and advanced maternal age (age 36–42 years, Table II). Increasing amounts of acetylated oocytes were found with advancing age. The difference in residual acetylation was significant between the young and intermediate (31.8% versus. 50.0%; λ^2 P = 0.023) and between the youngest and oldest groups (31.8% versus. 55.6%; λ^2 P = 0.009). This indicates that advanced maternal age negatively influenced the deacetylation process of H4K12 during the final stages of human oocyte maturation. A difference in the residual acetylation between Day 1 ICSI and IVF oocytes (42.6% and. 53.8%, respectively) was found, but this was not significant. The difference may be partially explained by the higher maternal age of the IVF oocytes (median age ICSI 32 years versus IVF 34 years; Mann-Whitney U P = 0.009). Thus, advancing maternal age was significantly correlated with increasing residual acetylation in human MII oocytes (r = 0,185, P = 0,007).

Table II.

			H4K12ac oocytes, n (%)	
Maternal age		**N**	**Positive**	**Total**
All ages	(24–42 yrs)	92	96 (46)	210
Young	(24–30 yrs)	22	21 (32)[ab]	66
Middle	(31–35 yrs)	41	45 (50)[a]	90
Advanced	(36–42 yrs)	29	30 (56)[b]	54

N is the number of women and n the number of oocytes. Groups that show a significant difference (λ^2) are indicated with the same superscript [a](P = 0.023) and [b](P = 0.009).

H4K12ac and chromosome misalignment

Previous data on mouse and porcine oocytes have shown that inhibition of HDACs during *in vitro* maturation (IVM) leads to hyperacetylated histones, resulting in a high rate of aneuploidy in oocytes and zygotes [11,24]. To investigate whether residual acetylation following normal *in vivo* maturation is also related to chromosome and spindle abnormalities, we analyzed

human virgin and failed fertilized MII oocytes for H4K12ac and the spindle component α-tubulin. Oocytes were classified according to previously published criteria [21,25]. In total, 55 oocytes had an informative spindle and chromosome structure. We observed different types of chromosome alignment abnormalities, varying from a single chromosome lagging to complete failure of congression of all chromosomes along the metaphase plate (Figure 3 and Supplementary data, Figure S1 (movies)). After scoring for spindle and chromosome alignment, oocytes were categorized according to their acetylation status (residual acetylation or deacetylation). Oocytes that had residual acetylation of H4K12 had significantly more misaligned chromosomes, either with or without an abnormal spindle structure (88.2%, 30/34), than the oocytes with deacetylated chromatin (33.3%, 7/21; λ^2 P = 0.001, Table III). These data show that the risk of chromosome misalignment was 15 times higher in oocytes with residual acetylation compared to oocytes with correct deacetylation (odds ratio = 15; 95% confidence interval: 3.76–59.78, $P < 0.0001$). The Day 0 oocytes, consisting of high-quality Day 0 virgin oocytes, show a similar significant effect of residual acetylation on metaphase abnormalities (Table III, Fisher's exact test for correction on small number P = 0.024). As shown above, our data showed a significant correlation of advancing maternal age with increasing residual acetylation (r = 0.185, P = 0.007, Table II and Supplementary data, Figure S2A) as well as a significant correlation between acetylation and abnormal metaphase alignment (r = 0.568, P = 0.0001, Table III). To control for collection day as a possible confounder, we split the data into groups according to age and collection days. The graph for age and misalignments (Supplementary data, Figure S2B) has a similar pattern as the graph showing age and residual acetylation (Supplementary data, Figure S2A) and there was no difference between the two collection days, indicating that failed fertilized oocytes were not different from Day 0 virgin oocytes.

The observed correlation between acetylation and misalignment may be indirect and caused by factors that increase with advancing maternal age and affect histone acetylation on the one hand and, independently, affect misalignment by a different mechanism. We thus wished to analyze whether advancing age and acetylation have independent effects on chromosomal misalignment in MII oocytes. Splitting the oocytes into three age categories of the donor showed, however, a consistent percentage of misaligned metaphases in acetylated oocytes of, respectively, 86, 88 and 90% in the young, middle and advanced age groups (Supplementary data, Figure S2C). After correction for small numbers, the middle age group still showed a significant difference in the number of abnormal metaphases between acetylated and non-acetylated oocytes (Fisher's exact test, P = 0.004).

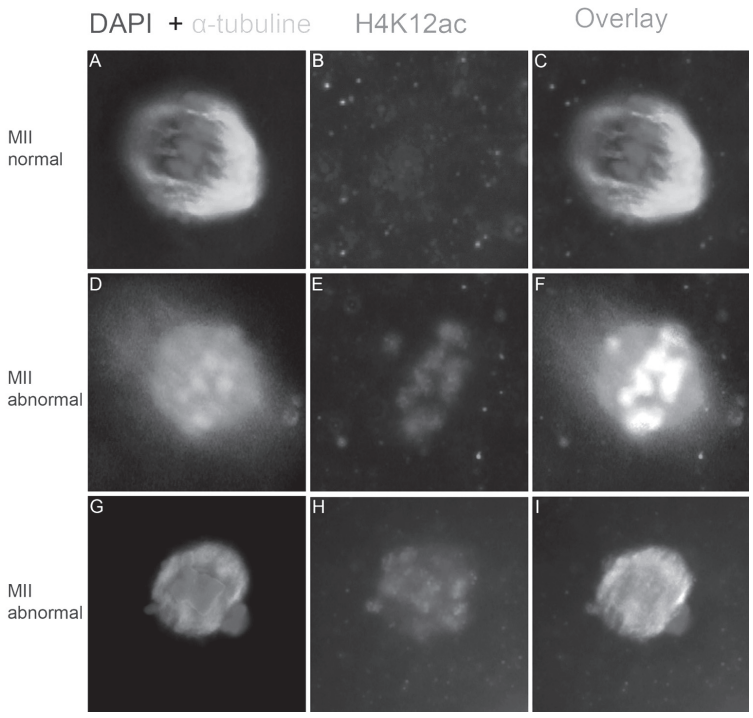


Figure 3. Metaphase alignment and residual acetylation in human MII oocytes. Representative immunofluorescence of an MII oocyte without staining of H4K12ac and with properly aligned chromosomes in the equatorial plane (**A–C**). Residual acetylation of H4K12ac in an MII oocyte is associated with chromosome misalignment of a single chromosome (**D–F**) or congression failure (**G–I**). Scale bar represents 10 µm (see page 173 for colour figure).

Table III.

		Metaphase chromosome conformation		
	H4K12ac staining	**Normal n(%)**	**Abnormal n(%)**	**Total**
D 0 + D 1 oocytes	Ac-	14 (67)	7 (33)[a]	21
	Ac+	4 (12)	30 (88)[a]	34
	Total	18 (23)	37 (67)	55
D 0 oocytes	Ac-	7 (64)	4 (36)[b]	11
	Ac+	1 (9)	10 (91)[b]	11
	Total	8 (36)	14 (64)	22

There were significant differences in the percentage of metaphase misalignment in human oocytes that have residual acetylation compared with those with proper deacetylation, in the subgroup of high-quality Day 0 virgin oocytes (Day 0), in the group of Day 1 oocytes as well as in the group comprising all oocytes (Day 0 + Day 1). Groups that show a significant difference (x2) are indicated with the same superscript [a]$P = 0.024$, [b]$P = 0.002$, [c]$P = 0.001$.

Discussion

In this study, we examined the process of histone deacetylation during the final stages of human oocyte meiosis. Our observations suggest that inadequate histone deacetylation in human is correlated with both advanced maternal age and, independently, with aberrant chromosome alignment. Our results are supported by observational data from mouse oocytes showing a correlation between age and residual acetylation [9,24] as well as experimental data with mouse oocytes that showed a clear causal relationship between induced hyperacetylation and chromosomal misalignment in oocytes, resulting in aneuploidy in zygotes and fetal death [9]. This may indicate that with increasing age, oocytes increasingly fail to deacetylate their chromatin correctly, resulting in a failure to accurately align their chromosomes at metaphase, which predisposes the oocytes to aneuploidy.

Studies in different animal models have shown that mouse, porcine, bovine and sheep oocytes have a general pattern of histone deacetylation during maturation from GV to MII, though not all lysine residues are regulated to the same extent in all studied species [16,17,26-29]. Similar to mouse oocytes, we observed that in human GV oocytes, both NSN and SN configurations had acetylated chromatin, indicating that acetylation of H4 is not always associated with transcriptional activity and that it is more likely related to chromatin remodeling during the final stages of oocyte maturation (Figure 1A-F, Figure 2 and mouse data not shown). Additionally, human oocytes showed deacetylation of H4 lysines during maturation from GV to MII that was similar to other mammals. Only H4K16ac did not show a similar deacetylation at the MI stage. However, the small numbers may not reflect the actual acetylation status of H4K16 at the MI stage in the general population. Although human oocytes showed variable percentages of deacetylation of individual lysine residues (Table I), our data, together with data from animal studies, demonstrate that the process of general deacetylation of chromatin during the final stages of female meiosis is conserved among mammalian species. Because we mainly focused on H4K12ac, the reduced number of oocytes we used to investigate the other lysines may have led to results which do not reflect accurately the actual percentage of residual acetylation. However, because we observed oocytes that were negative for acetylation on all lysine residues at the MII stage, we conclude that deacetylation of chromatin takes place during human oocyte maturation.

H4K12ac and maternal age

The oocytes analyzed for this study consisted of both good-quality Day 0 ‘virgin’ oocytes as well as Day 1 failed fertilized oocytes. We did not see any significant differences with respect to

acetylation or to metaphase alignment in these two groups of oocytes and thus analyzed them as one group. The observation that histone acetylation was not different between oocytes from Day 0 and Day 1 ICSI oocytes (39.7 versus 42.6%) might suggest that histone acetylation does not influence the capacity of the oocyte to be fertilized. In support of this, mouse oocytes treated with the HDAC inhibitor trichostatin A that resulted in hyperacetylation of histones, where not compromised in their fertilization rate, but only in their post-implantation development [9].

Compared with the ICSI oocytes, the IVF oocytes showed a 10% higher, but not significant, remaining acetylation of 53.8%. This may be explained partially by the higher average age in the IVF group compared with the ICSI group (34 versus 32 years). However, it is also possible that IVF oocytes have a higher residual acetylation owing to predominance of a female factor in the IVF subfertility problem, whereas ICSI couples are more often subfertile because of a male factor. Our finding that correct deacetylation of chromatin during the final stage of oocyte maturation is negatively influenced by advanced maternal age may explain the decreased quality and developmental potential of oocytes from women of advanced maternal age [1-3]. Oocytes from young mice completely deacetylate their chromatin during maturation from GV to MII [9,16]. However, in our population, 31.8% of the oocytes obtained from women under 31 years of age had residual acetylation. The average age of menarche is 12 years [30], and the oocytes used in this study were donated by women with an average age of 32 years (24–44 years). Therefore, the studied oocytes may not reflect the complete reproductive lifespan of the general human population, and the percentage of residual acetylation may be lower in adolescent women.

We have attempted to analyze whether advancing age leads to an increase in H4K12 acetylation that subsequentially results in misalignments, or whether these two parameters are independently related to advancing age. It is clear that the percentage of acetylated oocytes is increasing with advancing age (P = 0.023–0.009). Unfortunately, grouping the data according to age, to acetylation status and to misalignment status (Supplementary data, Figure S2C) results in small sample numbers, from which no statistically firm conclusions can be drawn. Supplementary data, Figure S2C suggests that the percentage of misalignments within the acetylated oocyte group is similar in all three age groups, implying that acetylation itself may be a main contributor to misalignment. Thus, it is most likely that oocytes with residual acetylation are inherently more susceptible to mis-segregation. The observation that women of advanced maternal age have a significantly higher percentage of residual acetylation than young women might therefore explain the higher incidence of aneuploidy oocytes in older women. Segregation errors such as premature sister chromatid separation

and non-disjunction of whole chromosomes have been described in human oocytes of women of advanced maternal age [4-6]. However, no molecular pathway has yet been identified that could explain the increased aneuploidy with advanced maternal age [2,3,31]. Studies in older mammalian oocytes have shown that mRNA levels of several genes are diminished, including structural factors that are critical for correct segregation [32-34]. It should be kept in mind that in human oocytes, actual quantification data on protein levels for these factors are limited, and the threshold levels leading to mis-segregation are yet to be determined. Our data that show maternal age to be highly correlated with defective deacetylation point to a molecular pathway affecting genome-wide chromatin remodeling. Thus, catalytically active proteins, such as members of the HDAC family, might be essential for oocyte quality and potential. Unfortunately, detection of histone acetylation in single human oocytes simultaneously with activity measurements of individual HDACs is currently not possible.

Oocyte quality is influenced by interactions between the oocyte and its surrounding cumulus cells and granulosa cells, and is dependent on maternal age, lifestyle and environmental factors. Apart from age, other possible factors that may influence chromatin remodeling are FSH, which is known to affect oocyte quality and embryo aneuploidy [35], a woman's BMI, which influences fecundability in several ways [36] and smoking, which is known to reduce HDAC activity in somatic cells [37]. Unfortunately, we were not able to obtain a sufficiently large dataset to analyze these parameters in our group of oocytes.

Residual acetylation and segregation errors

Apart from maternal age, the only factor that has so far been unequivocally associated with the etiology of aneuploidy in humans is a reduced or altered placement of meiotic recombination [31,38,39], but a mechanism for this is not known. Our finding that inadequate histone deacetylation is associated with aberrant chromosome alignment indicates that, in addition to abnormal meiotic recombination, defective histone deacetylation is a second molecular factor associated with oocyte aneuploidy. However, these two factors do not need to be mutually exclusive. In fact, the 'two-hit' hypothesis proposed by Lamb and Warren [40,41] states that aneuploidy is caused by two independent events that occur at different times during oocyte development. The first 'hit' occurs at the prenatal stage, when ovaries and oocytes are formed and meiotic recombination takes place. Data from trisomic pregnancies provide evidence that recombination sites at specific positions on chromosomes are more susceptible to mis-segregation [31,38,40]. The second 'hit' supposedly happens years later, after the resumption of maturation during the division stages, when these vulnerable oocytes are not able to detect and respond to this recombination failure. Studies in mitotic cells have shown

that an artificially induced hyperacetylated state leads to aberrant localization of many kinds of proteins that are essential during the segregation process, such as the spindle assembly checkpoint proteins MAD2 and BUB1, proteins of the chromosome passenger complex and kinetochore and motor proteins, such as HP1 and CENP E [8,18,26,42-44]. Thus, it is possible that an atypical localization and function of these proteins caused by the disturbed interaction with a scaffold that is formed from inadequately remodeled, acetylated chromatin contributes to inaccurate chromosome alignment and chromosome mis-segregation during human female meiosis. Another factor implicated in age-related aneuploidy is the detoriation of the cohesion complex. The cohesion complex holds the 4 chromatid bivalents together from prophase during fetal development onward, until the oocyte is recruited for meiotic maturation which may happen decades later. The proteolytic activity of Separase will resolve the cohesion complex on the chiasmata in mouse oocytes only during preparation for MI [45,46]. Four mutant mouse strain suggest that cohesions are involved in age-related aneuploidy [47-50]. Human data are not yet conclusive on the role of cohesins in human age-related aneuploidy [51] possibly because only oocytes from younger women and mRNA levels rather than protein levels were studied. The chromatin forms a scaffold on which cohesions are loaded, thus, residual histone acetylation, such as H4K12 acetylation, may interfere with the stepwise cleavage of cohesin proteins. More research is needed to elucidate if the residual acetylation that we found in our study to be associated with maternal age has downstream effects on for example, the cohesion and cohesion-related proteins in human oocytes.

The prospect of IVM of immature oocytes to good quality mature oocytes for certain patient groups has been the attention of many studies. However, up till now, *in vitro* maturation has not been very successful mainly because of the difficulty of developing optimal culture conditions [52]. Our study suggests that histone deacetylation may be an important feature of human oocyte maturation and may be taken into consideration when optimizing culture conditions for IVM. Controlling proper remodeling of chromatin may be important to optimize IVM that allows GV oocytes to mature fully and divide their chromosomes correctly. However, more research is needed to relate the acetylation status of an oocyte to its developmental competence.

In conclusion, our data from human oocytes show that during human female meiosis, residual acetylation is significantly correlated with chromosome misalignment in both young and older women. Advanced maternal age leads to both significantly more oocytes with residual acetylation and more abnormal metaphases. Thus, after the first 'hit' of a susceptible meiotic recombination, defective histone deacetylation is possibly a second hit in the etiology of aneuploidy.

Authors' roles

I.M.v.d.B. collected data and I.M.v.d.B. and J.H.v.D. designed the study, supervised data collection, conducted data analysis and interpretation and wrote the manuscript, C.E. and M.v.d.H. collected data and contributed to the draft version, E.B is a professional statistician who advised on the statistical methods used in this study. E.A.P.S., R.-J.G. and J.S.E.L. obtained funding, interpreted data and revised the draft version of the manuscript.

Acknowledgements

We kindly thank all patients that donated surplus oocytes, the members of the Erasmus MC IVF lab, the fertility centre of the Maasstad hospital, of the Sint Fransiscus Hospital, of the Albert Schweitzer hospital and of the Amphia hospital for their enthusiastic collaboration in this study to include patients and Willy Baarends for sharing antibodies, John Kong-a-San for supporting mouse experiments, Fatima Hammiche for assistance with statistical analysis, and Charène Wever, Arletta Bol, Khatera Eldja, Ton Verkerk and Tom de Vries Lentsch for their technical help.

References

1. Hassold, T. and P. Hunt, *To err (meiotically) is human: the genesis of human aneuploidy.* Nat Rev Genet, 2001. **2**(4): p. 280-91.
2. Jones, K.T., *Meiosis in oocytes: predisposition to aneuploidy and its increased incidence with age.* Hum Reprod Update, 2008. **14**(2): p. 143-58.
3. Pellestor, F., et al., *Maternal aging and chromosomal abnormalities: new data drawn from in vitro unfertilized human oocytes.* Hum Genet, 2003. **112**(2): p. 195-203.
4. Angell, R.R., *Predivision in human oocytes at meiosis I: a mechanism for trisomy formation in man.* Hum Genet, 1991. **86**(4): p. 383-7.
5. Kuliev, A., J. Cieslak, and Y. Verlinsky, *Frequency and distribution of chromosome abnormalities in human oocytes.* Cytogenet Genome Res, 2005. **111**(3-4): p. 193-8.
6. Garcia-Cruz, R., et al., *Cytogenetic analyses of human oocytes provide new data on non-disjunction mechanisms and the origin of trisomy 16.* Hum. Reprod., 2010. **25**(1): p. 179-191.
7. Ekwall, K., et al., *Transient inhibition of histone deacetylation alters the structural and functional imprint at fission yeast centromeres.* Cell, 1997. **91**(7): p. 1021-32.
8. Robbins, A.R., et al., *Inhibitors of histone deacetylases alter kinetochore assembly by disrupting pericentromeric heterochromatin.* Cell Cycle, 2005. **4**(5): p. 717-26.
9. Akiyama, T., M. Nagata, and F. Aoki, *Inadequate histone deacetylation during oocyte meiosis causes aneuploidy and embryo death in mice.* Proc Natl Acad Sci U S A, 2006. **103**(19): p. 7339-44.
10. Ivanovska, I. and T.L. Orr-Weaver, *Histone modifications and the chromatin scaffold for meiotic chromosome architecture.* Cell Cycle, 2006. **5**(18): p. 2064-71.
11. Wang, Q., et al., *Histone deacetylation is required for orderly meiosis.* Cell Cycle, 2006. **5**(7): p. 766-74.
12. Eot-Houllier, G., et al., *Histone deacetylase inhibitors and genomic instability.* Cancer Lett, 2009. **274**(2): p. 169-76.
13. Bouniol-Baly, C., et al., *Differential transcriptional activity associated with chromatin configuration in fully grown mouse germinal vesicle oocytes.* Biol Reprod, 1999. **60**(3): p. 580-7.
14. Miyara, F., et al., *Chromatin configuration and transcriptional control in human and mouse oocytes.* Mol Reprod Dev, 2003. **64**(4): p. 458-70.
15. Fischle, W., Y. Wang, and C.D. Allis, *Histone and chromatin cross-talk.* Current Opinion in Cell Biology, 2003. **15**(2): p. 172-183.
16. Kim, J.M., et al., *Changes in histone acetylation during mouse oocyte meiosis.* J Cell Biol, 2003. **162**(1): p. 37-46.
17. Endo, T., et al., *Changes in histone modifications during in vitro maturation of porcine oocytes.* Mol Reprod Dev, 2005. **71**(1): p. 123-8.
18. Cimini, D., et al., *Histone hyperacetylation in mitosis prevents sister chromatid separation and produces chromosome segregation defects.* Mol Biol Cell, 2003. **14**(9): p. 3821-33.
19. De Santis, L., et al., *Permeability of human oocytes to ethylene glycol and their survival and spindle configurations after slow cooling cryopreservation.* Hum Reprod, 2007. **22**(10): p. 2776-83.
20. Abramoff, M.D., Magalhaes, P.J., Ram, S.J, *Image Processing with ImageJ.* Biophotonics International, 2004. **11**(7): p. 36-42.
21. Battaglia, D.E., et al., *Fertilization and early embryology: Influence of maternal age on meiotic spindle assembly oocytes from naturally cycling women.* Hum. Reprod., 1996. **11**(10): p. 2217-2222.

22. Hodges, C.A., et al., *Experimental evidence that changes in oocyte growth influence meiotic chromosome segregation.* Hum Reprod, 2002. **17**(5): p. 1171-80.

23. Li, Y., et al., *Confocal microscopic analysis of the spindle and chromosome configurations of human oocytes matured in vitro.* Fertil Steril, 2006. **85**(4): p. 827-32.

24. Akiyama, T., et al., *Regulation of histone acetylation during meiotic maturation in mouse oocytes.* Mol Reprod Dev, 2004. **69**(2): p. 222-7.

25. Li, Y., et al., *Confocal microscopic analysis of the spindle and chromosome configurations of human oocytes matured in vitro.* Fertility and Sterility, 2006. **85**(4): p. 827-832.

26. De La Fuente, R., et al., *ATRX, a member of the SNF2 family of helicase/ATPases, is required for chromosome alignment and meiotic spindle organization in metaphase II stage mouse oocytes.* Dev Biol, 2004. **272**(1): p. 1-14.

27. Tang, L.S., et al., *Dynamic changes in histone acetylation during sheep oocyte maturation.* J Reprod Dev, 2007. **53**(3): p. 555-61.

28. Maalouf, W.E., R. Alberio, and K.H. Campbell, *Differential acetylation of histone H4 lysine during development of in vitro fertilized, cloned and parthenogenetically activated bovine embryos.* Epigenetics, 2008. **3**(4): p. 199-209.

29. Manosalva, I. and A. Gonzalez, *Aging alters histone H4 acetylation and CDC2A in mouse germinal vesicle stage oocytes.* Biol Reprod, 2009. **81**(6): p. 1164-71.

30. Miller, J.F., et al., *Fetal Loss after Implantation : A Prospective Study.* The Lancet, 1980. **316**(8194): p. 554-556.

31. Hassold, T. and P. Hunt, *Maternal age and chromosomally abnormal pregnancies: what we know and what we wish we knew.* Curr Opin Pediatr, 2009. **21**(6): p. 703-8.

32. Steuerwald, N., et al., *Association between spindle assembly checkpoint expression and maternal age in human oocytes.* Mol. Hum. Reprod., 2001. **7**(1): p. 49-55.

33. Fragouli, E., et al., *Transcriptomic profiling of human oocytes: association of meiotic aneuploidy and altered oocyte gene expression.* Mol Hum Reprod, 2010.

34. Grondahl, M.L., et al., *Gene expression profiles of single human mature oocytes in relation to age.* Hum. Reprod., 2010. **25**(4): p. 957-968.

35. Baart, E.B., et al., *Milder ovarian stimulation for in-vitro fertilization reduces aneuploidy in the human preimplantation embryo: a randomized controlled trial.* Hum Reprod, 2007. **22**(4): p. 980-8.

36. Robker, R.L., et al., *Obese women exhibit differences in ovarian metabolites, hormones, and gene expression compared with moderate-weight women.* J Clin Endocrinol Metab, 2009. **94**(5): p. 1533-40.

37. Ito, K., et al., *Decreased histone deacetylase activity in chronic obstructive pulmonary disease.* N Engl J Med, 2005. **352**(19): p. 1967-76.

38. Lamb, N.E., S.L. Sherman, and T.J. Hassold, *Effect of meiotic recombination on the production of aneuploid gametes in humans.* Cytogenetic and Genome Research, 2005. **111**(3-4): p. 250-255.

39. Garcia-Cruz, R., et al., *Cytogenetic analyses of human oocytes provide new data on non-disjunction mechanisms and the origin of trisomy 16.* Hum Reprod, 2010. **25**(1): p. 179-91.

40. Lamb, N., et al., *Susceptible chiasmate configurations of chromosome 21 predispose to non-disjunction in both maternal meiosis I and meiosis II.* Nat Genet, 1996. **14**(4): p. 400-405.

41. Warren, W.D. and K.L. Gorringe, *A molecular model for sporadic human aneuploidy.* Trends in Genetics, 2006. **22**(4): p. 218-224.

42. Eot-Houllier, G., et al., *Histone deacetylase 3 is required for centromeric H3K4 deacetylation and sister chromatid cohesion.* Genes Dev, 2008. **22**(19): p. 2639-44.

43. Ma, Y., et al., *Inhibition of protein deacetylation by trichostatin A impairs microtubule-kinetochore attachment.* Cell Mol Life Sci, 2008. **65**(19): p. 3100-9.
44. Vogt, E., et al., *Spindle formation, chromosome segregation and the spindle checkpoint in mammalian oocytes and susceptibility to meiotic error.* Mutat Res, 2008. **651**(1-2): p. 14-29.
45. Kudo, N.R., et al., *Role of cleavage by separase of the Rec8 kleisin subunit of cohesin during mammalian meiosis I.* J Cell Sci, 2009. **122**(Pt 15): p. 2686-98.
46. Kudo, N.R., et al., *Resolution of chiasmata in oocytes requires separase-mediated proteolysis.* Cell, 2006. **126**(1): p. 135-46.
47. Liu, L. and D.L. Keefe, *Defective cohesin is associated with age-dependent misaligned chromosomes in oocytes.* Reprod Biomed Online, 2008. **16**(1): p. 103-12.
48. Hodges, C.A., et al., *SMC1beta-deficient female mice provide evidence that cohesins are a missing link in age-related nondisjunction.* Nat Genet, 2005. **37**(12): p. 1351-5.
49. Revenkova, E., et al., *Oocyte cohesin expression restricted to predictyate stages provides full fertility and prevents aneuploidy.* Curr Biol, 2010. **20**(17): p. 1529-33.
50. Tachibana-Konwalski, K., et al., *Rec8-containing cohesin maintains bivalents without turnover during the growing phase of mouse oocytes.* Genes Dev, 2010. **24**(22): p. 2505-16.
51. Garcia-Cruz, R., et al., *Dynamics of cohesin proteins REC8, STAG3, SMC1 beta and SMC3 are consistent with a role in sister chromatid cohesion during meiosis in human oocytes.* Hum Reprod, 2010. **25**(9): p. 2316-27.
52. Gilchrist, R.B. and J.G. Thompson, *Oocyte maturation: Emerging concepts and technologies to improve developmental potential in vitro.* Theriogenology, 2007. **67**(1): p. 6-15.

Supplementary information

S1 Methods

All fixation, staining and washing steps were done in a 96-well plate to preserve oocyte 3-D morphology. Chemicals were purchased from Sigma (Sigma-Aldrich, Zwijndrecht, The Netherlands) unless stated otherwise. Oocytes were fixed for 30 min at 37°C in microtubule-stabilizing buffer (100 mM PIPES, 5 mM MgCl2, 2.5 mM EGTA, 2% formaldehyde, 2% Triton X-100 and 0.5 mM taxol). Because the spindle is cold-sensitive, care was taken to continuously keep the oocytes at 37°C until the fixation process was finished. After fixation, cells were washed in 0.1% BSA and stored in 1% BSA in 0.04% NaN3 at 4°C. After storage, samples were blocked for 30 min in 1% BSA in PBS-Triton X-100 0.05% (blocking buffer). Incubation with primary antibodies was done overnight at 4°C in blocking buffer. Oocytes were washed three times in 1X PBS and incubated for 1.5–2 hrs at room temperature in blocking buffer with two secondary antibodies. To visualize chromosomes, DNA was counterstained with DAPI (Invitrogen, Breda, The Netherlands) in Vectashield mounting medium (Vector laboratories, Burlingame, UK). Cumulus cells and the polar body were used in each experiment to confirm positive staining of the antibodies

S1 Table. Primary and secondary antibodies used in this study.

	against	label	host	dilution	supplier	Catalog no
primary	H4K5ac	n.a.	rabbit	1 : 500	Abcam	ab51997
	H4K8ac	n.a.	rabbit	1 : 500	Upstate	06-760
	H4K12ac	n.a.	rabbit	1 : 500	Upstate	06-761
	H4K16ac	n.a.	rabbit	1 : 500	Upstate	07-329
	α-tubulin	n.a.	rat	1 : 500	Abcam	ab6160
secondary	rabbit	Alexa 594	goat	1 : 500	Invitrogen	A11012
	rat	Dylight Cy5	donkey	1 : 500	Jackson Laboratory	712-495-153
	mouse	Alexa 488	goat	1:500	Invitrogen	A1101

S2 Table. Number of collected oocytes analyzed for histone lysine acetylation status.

Maturation stage	Number
GV	61
MI	118
MII	321
– MII day 0	78
– MII day 1	170
– MII day 1 ICSI	104
– MII day 1 IVF	66

S3 Movies

Movies can be found at: ttp://humrep.oxfordjournals.org/content/26/5/1181/suppl/DC1

Movie 1 shows a rotating image of a MII oocyte with the chromosome aligned at the equatorial plane with a nicely barrel-shaped spindle (green) and no staining of H4K12ac (red).

Movie 2 shows a rotating image of a MII oocyte with positive staining of H4K12ac and chromosome misalignment (pink overlay of DNA in blue and H4K12ac staining in red). One chromosome is totally detached from the equatorial plane at one pole of the spindle (green) and congression failure of other chromosomes at the other spindle pole.

Legend S2A and B. Analysis of acetylation and misalignments in the three different age groups separated by day of collection. A. The percentage of H4K12 acetylation in MII oocytes increases with advancing maternal age. Groups that differ significantly (chi-square) are indicated with a (P = 0.023) and b (P = 0.009). B. The percentage of misalignment within the age groups did not differ between the days of collection, indicating that day 1 oocytes do not have increased numbers of misaligned oocytes (chi-square P = 0.49). The ascending percentages of misalignment with increasing age is not statistically significant, perhaps due to the small numbers.

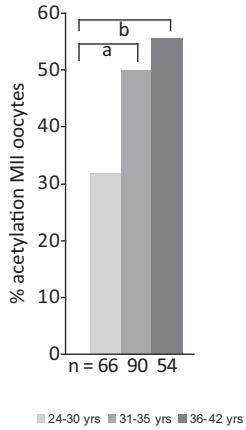


Figure S2a: Acetylation increases with maternal age

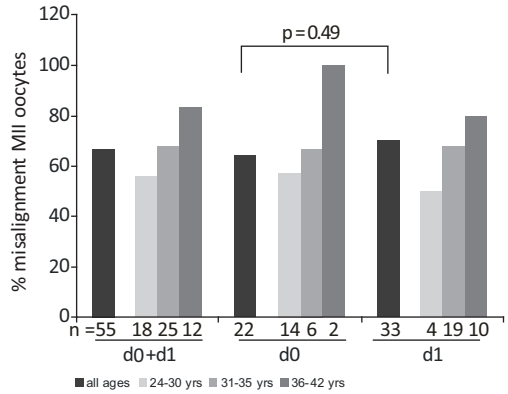
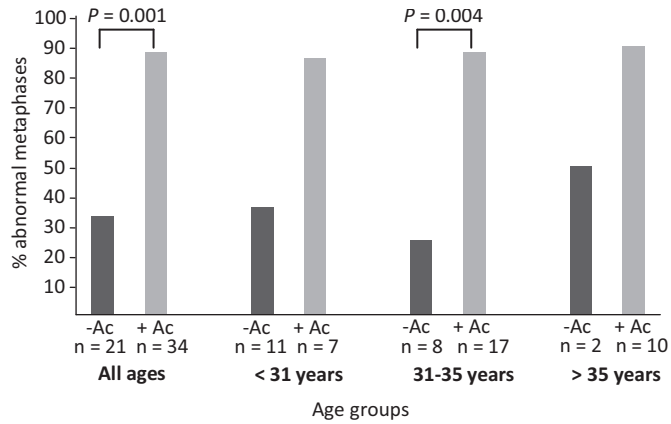
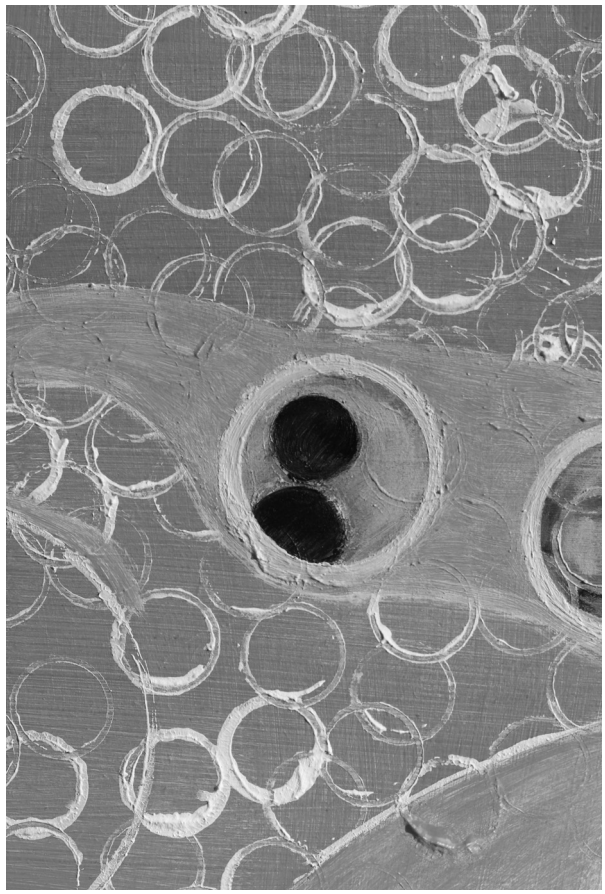


Figure S2b: Misalignment is independent of collection day

Legend S2C. **Acetylation correlates with abnormal metaphases in different age groups**. Histogram showing the percentage of abnormal metaphases in MII oocytes without acetylation of H4K12 (-Ac) and in oocytes with acetylation (+Ac). The combined as well as the three age categories show similar ratios of abnormal metaphases in non- and acetylated oocytes. Numbers of analyzed oocytes are indicated.



CHAPTER 3



Parental origin of chromatin in human mono-pronuclear zygotes revealed by asymmetric histone methylation patterns, differs between IVF and ICSI

I.M. van den Berg*, G.W. van der Heijden*, E.B. Baart, A.A.H.A. Derijck,
E. Martini, P. de Boer
* *These authors contributed equally*

Published in *Mol Reprod Dev. 2009 Jan;76(1):101-8*

Abstract

In mouse zygotes, many post-translational histone modifications are asymmetrically present in male and female pronuclei. We investigated whether this principle could be used to determine the genetic composition of mono-pronuclear human zygotes in conventional IVF and ICSI. First we determined whether male female asymmetry is conserved from mouse to human by staining poly-pronuclear zygotes with antibodies against a subset of histone N-tail post-translational modifications. To analyze human mono-pronuclear zygotes, a modification, H3K9me3, was selected that is present in the maternal chromatin. After IVF, a total of 45 mono-pronuclear zygotes were obtained. In 39 (87%) of the zygotes a non-uniform staining pattern was observed, proof of a bi-parental origin and assumed to result into a diploid conception. Two zygotes showed no staining for the modification, indicating that the single pronucleus was of paternal origin. Four zygotes contained only maternally derived chromatin. ICSI-derived mono-pronuclear zygotes (n = 33) could also be divided into three groups based on the staining pattern of their chromatin: (1) of maternal origin (n = 15), (2) of paternal origin (n = 8) or (3) consisting of two chromatin domains as dominating in IVF (n = 10). Our data show that mono-pronuclear zygotes originating from IVF generally arise through fusion of parental chromatin after sperm penetration. Mono-pronuclear zygotes derived from ICSI in most cases contain uni-parental chromatin. The fact that chromatin was of paternal origin in 24% of ICSI and in 4% of the IVF zygotes confirms earlier results obtained by FISH on cleavage stages. Our findings are of clinical importance in IVF and ICSI practice.

Key words: Mono-pronuclear; chromatin; histone lysine methylation; IVF; ICSI; zygote, human

Introduction

Fertilization entails both cytoplasmic and chromatin fusion of two highly specialized gamete types. Cytoplasmic fusion occurs at sperm penetration. Nuclear fusion follows after syngamy of the two pronuclei when nuclear envelopes dissolve and at the first cleavage division, one mitotic spindle is constructed [1]. In *in vitro* fertilization (IVF), a zygote with two distinct pronuclei is considered to be the result of normal fertilization and is usually encountered. Two types of abnormal fertilization are known to occur after both conventional IVF and intra cytoplasmic sperm injection (ICSI): mono-pronuclear and poly-pronuclear zygotes. Both types of zygotes are able to undergo cleavage divisions and can result in embryos of good morphological quality.

After insemination, 2-6% of the oocytes will display only a single pronucleus after conventional IVF [2] and 5-27% after ICSI [3]. Traditionally and inspired by research into the artificial activation of mouse secondary oocytes [4], mono-pronuclear zygotes are thought to lead to haploid parthenogenetic embryonic development [5]. However, previous studies investigating the chromosomal status of embryos resulting from mono-pronuclear zygotes by fluorescence in situ hybridization (FISH), have shown a diploid chromosome constitution in 49–62% of IVF-derived and 10–30% of ICSI-derived embryos [6,7]. Diploid embryos may be generated from uni-parental mono-pronuclear zygotes and subsequent diploidization at first cleavage [4,8] or by early fusion of paternal and maternal chromatin [9]. Due to imprinting requirements only the latter ones may result in offspring whereas mono-parental diploid embryos of paternal descend can yield complete hydatidiform mole (CHM) pregnancies [10]. Mono-pronuclear zygotes have been reported to lead to progeny in the human [2,11,12], which indicates these to originate from a fusion of male and female chromatin.

Chromosomal FISH analysis of cleavage stage embryos from mono-pronuclear zygotes can differentiate between haploidy and diploidy. For haploid embryos, the Y is a marker for a male chromosome complement. Diploidization can be detected by the presence of two Y chromosomes. Therefore, the frequency assessment of the genetic content of the single pronucleus by FISH is indirect, needing correction for X bearing male pronuclei.

To know the precise fraction of mono-pronuclear zygotes that are precociously fertilized by the untimely fusion of paternal and maternal chromatin (and according to the definition of fertilization are suitable for embryo transfer) it is necessary to determine the origin of the chromatin present in a mono-pronuclear zygote. The fractions of androgenic and gynogenic mono-pronuclear zygotes are simultaneously assessed.

Histones are proteins that together with DNA form the nucleosome, the basic repeat unit of chromatin. The nucleosome protein complex consists of four different histones (H2A, H2B, H3 and H4), each of them present twice [13]. Around this complex approximately 150 base pairs DNA are wrapped. The histones can be modified at specific amino acids by covalent attachment of small molecules (e.g., acetyl, phosphoryl, methyl) or peptides (e.g. ubiquitin, sumo) [14,15]. These post translational modifications are especially numerous at the N-tails of H3 and H4, that extend form the nucleosomal globular domain. Depending on their biological context these modifications influence for instance chromatin compaction, hence gene transcription and DNA repair [14,16]. It has been shown that in the mouse zygote, several H3, H4 lysine methylation marks are absent in the paternal pronucleus but present in the maternal one, a phenomenon denoted as pronuclear asymmetry [17-22].

In mouse zygotes, we observed that the asymmetrical setting of lysine methylation is maintained when parental chromatin is precociously fused, leading to a mono-pronuclear zygote. If the asymmetric setting of parental marks is conserved in the human, it could be used to determine the composition of the chromatin present in mono-pronuclear zygotes. We therefore collected human tri-pronuclear zygotes and determined the presence of methylated lysine residues at positions 4, 9 and 27 of the N-terminal tail of histone H3 by probing with antibodies specific for these modifications. A clear asymmetry was observed for trimethylated histone H3 lysine 9 (H3K9me3) and lysine 27 (H3K27me3). We subsequently stained mono-pronuclear zygotes derived from ICSI or IVF with α-H3K9me3 antibodies, which allowed unambiguous identification of the parental origin. This enabled us to distinguish maternal from paternal chromatin and determine the chromatin composition of mono-pronuclear zygotes on day 1 after conventional IVF or intracytoplasmic sperm injection.

Results

During previous studies in which we determined patterns of post-translational histone modifications and response to double strand DNA breaks in the early mouse zygote, we occasionally observed mono-pronuclear zygotes [17,23]. These were either the result of parthenogenic activation of the secondary oocyte or of fusion of the parental chromatin domains. This conclusion was based on the fact that the partition of chromatin into histone methylation positive and negative domains was maintained (Figure 1). Depending on the modification, the asymmetry existed throughout the first cell cycle (Figure 1h). Experiments done in four different mouse strains showed an incidence ranging from 2.2% to 6.1% in three

and of 11.6% in the C.B17 strain (Table I). Apparently there are strain-specific factors that influence susceptibility for early fusion of the parental chromatin in the mouse.

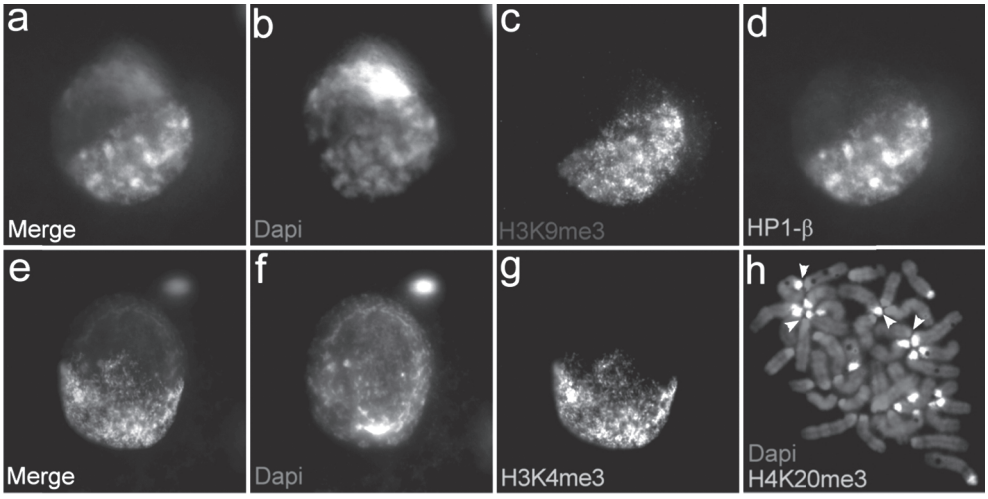


Figure 1. Diploid mouse mono-pronuclear zygotes. Examples of pre S-phase (**A-G**) and mitotic (H) mono-pronuclear zygotes **A-D**: Zygote stained for H3K9me3 (blue) and the H3K9me2,3 binding protein HP1-β [43]. (green) Dapi labels DNA (red). **E-G**) zygote stained for H3K4me3 (green), Dapi labels DNA (red). **H**) zygote stained for H4K20me3 (green), Dapi labels DNA (red). Histone H4K20me3 marks 20 maternal chromosomes at the constitutive heterochromatin. It has been reported that chromosomes in the mouse zygote are interconnected via their α-satellite sequences [38]. This causes some chromosomes to be positioned in a head to head position. Arrowheads indicate head-to-head position of maternal and paternal chromosomes (see page 173 for colour figure).

To determine whether origin-specific chromatin differences are also observed in human zygotes, we studied the distribution of trimethylated histone H3 at lysine 4, 9, and 27 in poly-pronuclear post-S-phase human zygotes derived from IVF and ICSI (Figure 2). We observed a clear staining of both parental chromatin domains for H3K4me3 (Figure 2b, n = 10) whereas trimethylated H3K9 and H3K27 were asymmetrically set (Figure 2c, n = 40; Figure 2d, n = 20). In IVF derived zygotes that originated from polyspermia, we consistently found one positive pronucleus (Figure 2c,d). In the few poly-pronuclear zygotes obtained via ICSI, which arise as a consequence of failure of extrusion of the 2nd polar body, two positive pronuclei were found (Figure 2e). This strongly suggested that, as in mouse and fly, the male pronucleus chromatin lacks certain histone lysine methylations [17,21].

Table I. Frequency of mono-pronuclear zygotes in different mouse strains.

Strain	Mono PN frequency (x/n)	No IVF experiments
B6.CBA hybrid	2.5 (2/79)	6
B6.129 synthetic	2.2 (1/46)	4
C.B17	11.6 (13/112)	6
Scid	6.1 (4/66)	7
Total without C.B17	3.7 (7/191)	
Total	6.6 (20/303)	

Data selected from H3K9me2 and H4K20me3 stained zygotes obtained in [23,44].

We decided to study the constitution of human mono-pronuclear zygotes by staining with an antibody against H3K9me3. A total number of 45 mono-pronuclear zygotes as determined by routine light microscopy (Figure 3a) were collected 16–20 hr after the start of a conventional IVF procedure. In 39 cases we observed one positive and one negative domain after staining. Among the apposed chromatin domains we could distinguish two morphological types: 27 zygotes displayed a clear fusion of the chromatin masses similar to observations in mouse mono-pronuclear zygotes (Figure 3b). In 12 zygotes, however, the two chromatin domains were not fused but in very close proximity of each other as if within the nucleus, a stricter compartmentalization had occurred (Figure 3c). This difference in appearance might be due to chromatin compaction which occurs during zygotic G2 and is more advanced for maternal chromatin. In both cases, one of these domains showed overall staining for the histone modification whereas the other domain was largely negative: although distinct foci could occasionally be observed. A clear distinction in chromatin compaction between the two domains was also noticed (Figure 3c). The maternal H3K9me3 positive domain was always of a smaller size than the paternal counterpart. A total of two zygotes showed a single chromatin domain without staining for the modification although a positive polar body was seen, suggesting that these zygotes only contained male chromatin. In four zygotes only a single, stained chromatin mass was observed, indicating that they contain only maternal chromatin.

A total number of 33 mono-pronuclear zygotes derived from ICSI were collected. We observed three staining patterns in these pronuclei: (1) two asymmetrically stained, fused chromatin domains (n = 10). (2) A single chromatin domain without staining (n = 8); chromatin present in polar bodies stained positive, indicating successful IF. (3) A single, stained chromatin domain and a condensed sperm head (n = 15). The results of the human mono-pronuclear zygotes obtained after IVF and ICSI are summarized in Table II.

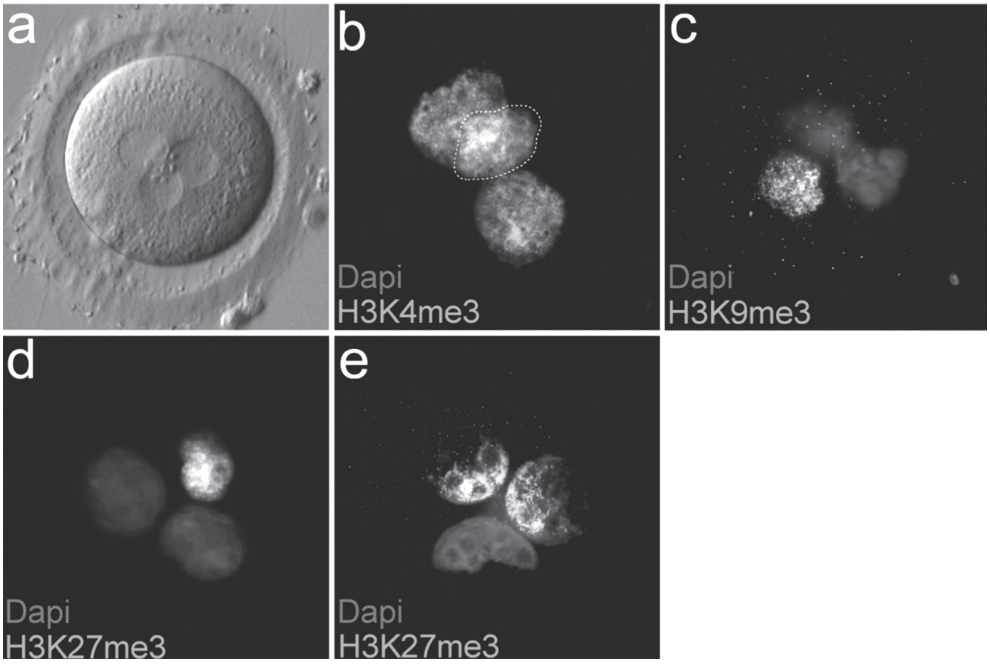


Figure 2. Distribution of H3K4me3, H3K9me3 and H3K27me3 in human tri-pronuclear zygotes. **A**) A light microscopic image of a tri-pronuclear zygote prior to removal of zona pellucida and fixation. **B**) Symmetrical distribution of trimethylated histone H3 lysine 4. Dotted line indicates position of the maternal PN, which can be distinguished from the paternal PN by size. **C**) Asymmetrical localization of histone H3K9me3. A tri-pronuclear zygote obtained after conventional insemination and therefore likely the result of polyspermia. This histone modification is absent from the two larger paternal pronuclei. **D-E**) Absence of histone H3K27me3 from paternal chromatin. In tri-pronuclear zygotes obtained after insemination, H3K27me3 was present in the smaller maternal PN (D). A rare case of a tri-pronuclear zygote after ICSI (E). In these zygotes failure of second polar body extrusion is responsible for the extra PN. Therefore two PNs show this maternal mark (see page 176 for colour figure).

Table II. Origin of Single Pronucleus in Human Mono-pronuclear Zygotes.

Genetic constitution	ART		
	IVF (%)	ICSI (%)	Total
Diploid	39 (86.7)	10 (30.3)	49
Haploid of paternal origin	2 (4.4)	8 (24.2)	10
Haploid of maternal origin	4 (8.9)	15 (45.5)	19
Total	45	33	78

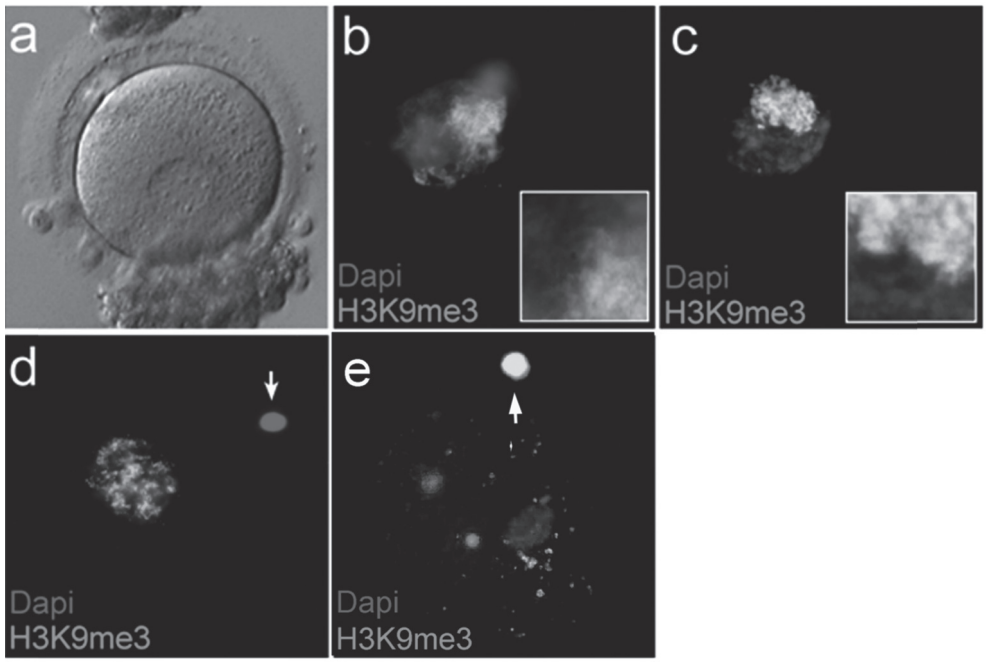


Figure 3. Distribution of H3K9me3 in human mono-pronuclear zygotes **A**) A mono-pronuclear zygote prior to removal of zona pellucida and fixation. **B**) A mono-pronuclear zygote with a clear fusion of the chromatin domains alike the mouse mono-pronuclear zygotes in Figure 1. Two out of focus sperm heads are overlying the pronucleus. Higher magnification shows a diffuse region in between parental chromatin domains. **C**) The mono-pronuclear zygote depicted in 3a after staining. Two not overlapping chromatin domains in close proximity are observed. Higher magnification shows a clear separation of the parental domains. **D**) A mono-pronuclear zygote obtained after ICSI in which the oocyte is activated by the sperm (indicated by arrow) but no further nuclear decondensation of the sperm has occurred. **E**) A mono-pronuclear zygote obtained after ICSI which contains paternally derived chromatin only. The positive domain in proximity of the PN is a polar body (see page 176 for colour figure).

Discussion

The asymmetrical setting of histone lysine methylation for paternal and maternal chromatin in the zygote is observed in a range of species [17,21,24]. Our results demonstrate this to extend to humans as well. Our observations for H3K4me3 and H3K9me3 are identical to the mouse post-S-phase zygote [18,19]. The presence of trimethylated H3K4 in the paternal human PN is intriguing (Figure 2b). This mark is firmly associated with transcriptional active chromatin

[14,15]. In mouse zygotes, it appears in the paternal pronucleus during S-phase and could be linked to the (minor) transcriptional activation of the zygotic genome at this stage [19,25]. In the human early embryo, genetic activation of the embryo occurs between the four- and eight-cell stages, suggesting that the presence of H3K4me3 in zygotic paternal chromatin is not related to transcriptional activation [26]. Trimethylation of paternal histone H3 lysine 27, an important regulatory mark for down regulation of transcription and heterochromatin formation in embryogenesis, occurs during DNA replication in the mouse zygote [18]. In this study human tri-pronuclear zygotes were collected 16–20 hrs after insemination. Most likely, the majority, if not all, of these zygotes were progressing into S-phase or had reached G2. The absence of H3K27me3 in these zygotes might indicate species-specific dynamics of H3K27 methylation, hence heterochromatin formation at first cleavage.

In this study 87% of the mono-pronuclear zygotes obtained by conventional IVF appeared to be bi-parental. In the human oocyte, the sperm nucleus can enter ooplasm at any location whereas in mouse the region overlying the metaphase II spindle is inaccessible for sperm [27]. However, this does not seem to prevent pronuclear fusion in the mouse (Table I). The proximity of the parental chromatin domains early after gamete fusion has an effect on the chance of fusion. This is illustrated by the finding that in the mouse, injection of the sperm close to the maternal complement resulted in 22% mono-pronuclear zygotes [28]. These authors provide evidence for aggregation of chromatin masses before pronucleus formation. In humans, evidence for pronucleus fusion as the way to the mono-pronuclear state, has only been obtained after injection of round spermatids [29]. Bi-parental mono-pronuclear human zygotes most likely are the result of sperm entry close to the metaphase II spindle, suggesting this to be a distinct possibility at natural conception too. The bi-parental mono-pronuclear zygotes obtained after ICSI likely do have the same origin. Although clinical practice aims to puncture the oolemma and deposit the sperm without disturbing the metaphase II spindle, one cannot rule out the possibility that the sperm is close to the maternal complement after injection. This is supported by the fact that the first polar body has proven to be a unreliable marker for the location of the metaphase plate [30]. Imaging the meiotic spindle by using its birefringence has made it possible to analyze the oocyte in more detail, leading to the same insight [31].

The majority of the ICSI-derived mono-pronuclear zygotes (70%) contained an abnormal genomic composition. This high rate of haploid zygotes after ICSI confirms previously published studies [6,32]. A total of 15 zygotes (45%) contained a maternal pronucleus with a condensed sperm head. It has been reported that among low numbers of sperm, the main reason to

revert to ICSI, there are nuclei with a decreased ability to undergo decondensation while still being able to induce oocyte activation [33,34].

In our study one-quarter of the 1 PNs (n = 8) after ICSI and 4% after IVF consisted of paternal chromatin only. Most likely in these cases, the complete maternal chromatin was extruded during formation of the second polar body.

In general, the trends found by us confirm the fractions that can be calculated from the FISH data of Staessen and Van Steirteghem (1997). For instance, despite the relatively low numbers of both studies, assuming an equal ratio of X- and Y-bearing spermatozoa, we found the same proportion of 1 PN embryos with only a paternal contribution. This indicates that complete extrusion of the female chromatin is a frequently occurring mechanism of 1 PN formation.

There are a few case reports [35,36] that imply a relation between mono-pronuclear embryos and a risk of a complete hydatidiform mole (CHM) pregnancy. These likely are male haploid zygotes carrying an X chromosome that undergo diploidization at first cleavage [10].

There have been reports of live births after the transfer of human embryos which had only one pronucleus [2,11,12]. Apparently, the setting of the parent specific histone marks can be correctly executed within one nuclear membrane. The fact that we did not observe a mingling of histone posttranslational states within a mono-pronuclear zygote confirms this.

In the mouse, the implantation success rate of mono-pronuclear zygotes is around 19% (15/79) [28] whereas in humans one study reported a percentage of 8% (3/38) [2]. Although one should be cautious of over interpretation of the relatively small numbers in these studies, human mono-pronuclear zygotes could have a decreased developmental potential. The mouse study on bi-parental mono-pronuclear zygotes indicated that negative effects of a pronuclear fusion in the zygote are limited. No changes in *in vitro* blastocyst development were observed. Also, most offspring derived from mouse mono-pronuclear zygotes were healthy and fertile [28].

Pertinent for clinical practice is the question of a disturbing interaction between paternal and maternal chromatin within one nuclear envelope. For mouse zygotes it has been shown that some proteins, in this case implicated in chromosome condensation, are specifically targeted to the female PN [37]. The fusion of the parental chromatin masses could potentially disrupt such parent specific mechanisms in the zygote. However, an indication for origin specific chromatin condensation in G2 was also obtained for the human mono-pronuclear zygotes (Figure 3c). In chromosome spreads of mono-pronuclear mouse zygotes, trimethylated histone H4 lysine 20 was absent from paternal chromosomes as is the case in normal fertilization (Figure 1h; [22]). Centric heterochromatin domains are intimately

apposed around the nucleolus precursor body at interphase [38]. This indicates that for this modification, fusion of the chromatin sets does not lead to an apparent change in localization of parent of origin specific chromatin marks. Possibly the different origin of the male and female zygotic chromatin may suffice to ensure their separate identities, even when present within one nuclear envelope.

Although this study also has demonstrated that mono-pronuclear zygotes originated after conventional IVF are in most cases fertilized, more research into their epigenetic status and consequences of deviations thereof is needed to determine whether these embryos are suitable for transfer.

Conclusions

Immunofluorescence of histone 3 N-tail post-translational modifications is a reliable way to determine the ancestry of chromatin in human zygote pronuclei. When this tool was used on mono-pronuclear zygotes obtained in classical IVF and ICSI, most PN resulted from early fusion events in IVF whereas for ICSI a mono-parental origin dominated. Androgenic mono-pronuclear zygotes were found both after IVF and ICSI but more so with the latter technique.

Materials and methods

Collection of mono- and multi-pronucleated zygotes

Ovarian stimulation, oocyte retrieval and IVF procedures were performed as described previously [39,40]. The study was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO) and the local ethics review committee of the Erasmus MC hospital. Written consent was obtained from the couples in order to confirm that the zygotes could be used for research purposes. The stage of fertilization was checked 16–20 hours post insemination or sperm injection. Only zygotes that had less or more than two pronuclei were used. Mouse zygotes were collected in parallel to previous projects [17,23].

Fixation and immunofluorescence staining

Prior to fixation the zona pellucida was removed with 0,05% pronase in calcium/magnesium free medium (G-PGD, Vitrolife Sweden).

Thereafter, cells were immobilized in a fibrin clot [41]. Fibrinogen was obtained from Calbiochem (cat. nr. 341573) and thrombin was obtained from Sigma (cat. nr. T-6634, Zwijndrecht, The Netherlands). Cells were fixed in 2% paraformaldehyde (PFA), 0.15% Triton-X-100 for 30 min. Immunofluorescence (IF) was applied as described before [42].

Antibodies

The following antibodies were used: HP1-β (raised in rat, P. Singh; 1:100), Polyclonal rabbit antibodies against H3K9me3; H3K27me3 and H4K20me3 were diluted 1:250 (T.Jenuwein). Rabbit polyclonal antibody for H3K4me3 and H3K9me3 were purchased from Abcam (ab8580, 1:1500 and ab1186, 1:250 respectively).

Secondary antibodies used were goat anti-rabbit IgG (H+L) conjugated with Alexa Fluor 594 (Molecular Probes, Leiden, The Netherlands) and anti-rat IgG, FITC-conjugated (Sigma F6258). All were used in a 1:500 dilution. Negative controls were performed by omitting the first antibody, but non-specific binding was never observed.

Collection of images

Images were collected with a Zeiss Axioplan fluorescence microscope. Pictures were captured by a Zeiss AxioCam MR camera with Axiovision 3.1 software (Carl Zeiss, Oberkochen, Germany). Shown images are either stacks projected into a single image or a single slide of a stack. Whenever necessary, images where deconvoluted with Metamorph software version 6.

Acknowledgements

Dr. T. Jenuwein (IMP, Vienna, Austria), Dr. H. Stunnenberg (Dept. of Molecular Biology, NCMLS, Nijmegen, The Netherlands) and Dr. P. Singh (Research Centre Borstel, Germany) are gratefully acknowledged for the gift of their antibodies. This research was financed by the Dutch ministry of Health, Welfare and Sport.

References

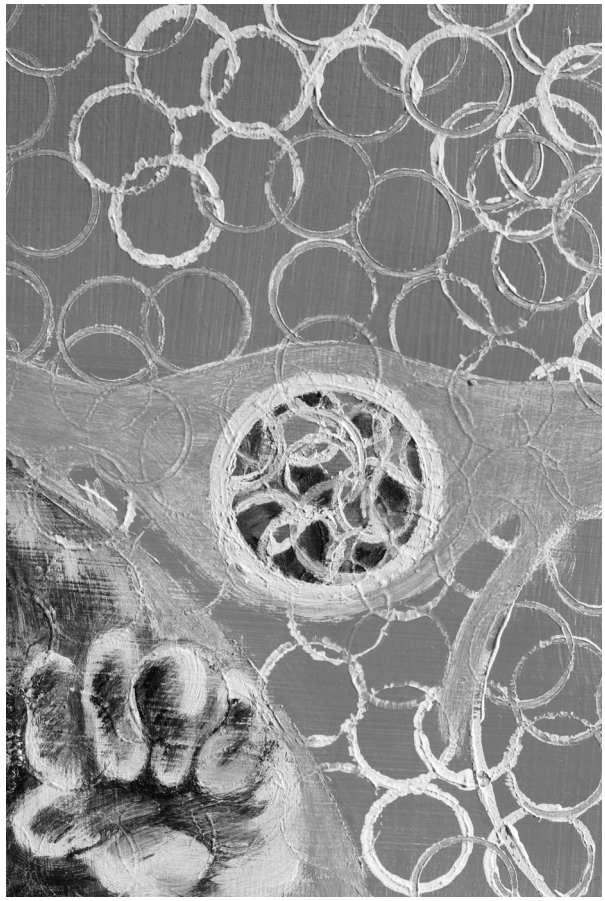
1. Wright, S.J., *Sperm nuclear activation during fertilization.* Curr Top Dev Biol, 1999. **46**: p. 133-78.
2. Staessen, C., et al., *Cytogenetic and morphological observations of single pronucleated human oocytes after in-vitro fertilization.* Hum Reprod, 1993. **8**(2): p. 221-3.
3. Palermo, G., et al., *Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection.* Fertil Steril, 1993. **59**(4): p. 826-35.
4. Kaufman, M.D., *Early Mammalian Development: Parthenogenetic Studies*. 1983: Cambridge Univerity Press.
5. Taylor, A.S. and P.R. Braude, *The early development and DNA content of activated human oocytes and parthenogenetic human embryos.* Hum Reprod, 1994. **9**(12): p. 2389-97.
6. Sultan, K.M., et al., *Chromosomal status of uni-pronuclear human zygotes following in-vitro fertilization and intracytoplasmic sperm injection.* Hum Reprod, 1995. **10**(1): p. 132-6.
7. Staessen, C. and A.C. Van Steirteghem, *The chromosomal constitution of embryos developing from abnormally fertilized oocytes after intracytoplasmic sperm injection and conventional in-vitro fertilization.* Hum Reprod, 1997. **12**(2): p. 321-7.
8. Barton, S.C., et al., *Influence of paternally imprinted genes on development.* Development, 1991. **113**(2): p. 679-87.
9. Longo, F.J., *Fertilization: a comparative ultrastructural review.* Biol Reprod, 1973. **9**(2): p. 149-215.
10. Devriendt, K., *Hydatidiform mole and triploidy: the role of genomic imprinting in placental development.* Hum Reprod Update, 2005. **11**(2): p. 137-42.
11. Gras, L. and A.O. Trounson, *Pregnancy and birth resulting from transfer of a blastocyst observed to have one pronucleus at the time of examination for fertilization.* Hum Reprod, 1999. **14**(7): p. 1869-71.
12. Dasig, D., et al., *Monozygotic twin birth after the transfer of a cleavage stage embryo resulting from a single pronucleated oocyte.* J Assist Reprod Genet, 2004. **21**(12): p. 427-9.
13. Kornberg, R.D. and Y. Lorch, *Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome.* Cell, 1999. **98**(3): p. 285-94.
14. Nightingale, K.P., L.P. O'Neill, and B.M. Turner, *Histone modifications: signalling receptors and potential elements of a heritable epigenetic code.* Curr Opin Genet Dev, 2006. **16**(2): p. 125-36.
15. Fischle, W., Y. Wang, and C.D. Allis, *Histone and chromatin cross-talk.* Curr Opin Cell Biol, 2003. **15**(2): p. 172-83.
16. Kouzarides, T., *Chromatin modifications and their function.* Cell, 2007. **128**(4): p. 693-705.
17. van der Heijden, G.W., et al., *Asymmetry in histone H3 variants and lysine methylation between paternal and maternal chromatin of the early mouse zygote.* Mech Dev, 2005. **122**(9): p. 1008-22.
18. Santos, F., et al., *Dynamic chromatin modifications characterise the first cell cycle in mouse embryos.* Dev Biol, 2005. **280**(1): p. 225-36.
19. Lepikhov, K. and J. Walter, *Differential dynamics of histone H3 methylation at positions K4 and K9 in the mouse zygote.* BMC Dev Biol, 2004. **4**: p. 12.
20. Cowell, I.G., et al., *Heterochromatin, HP1 and methylation at lysine 9 of histone H3 in animals.* Chromosoma, 2002. **111**(1): p. 22-36.
21. Arney, K.L., et al., *Histone methylation defines epigenetic asymmetry in the mouse zygote.* Int J Dev Biol, 2002. **46**(3): p. 317-20.
22. Kourmouli, N., et al., *Heterochromatin and tri-methylated lysine 20 of histone H4 in animals.* J Cell Sci, 2004. **117**(Pt 12): p. 2491-501.

23. Derijck, A.A., et al., *gammaH2AX signalling during sperm chromatin remodelling in the mouse zygote.* DNA Repair (Amst), 2006. **5**(8): p. 959-71.
24. Loppin, B., et al., *The histone H3.3 chaperone HIRA is essential for chromatin assembly in the male pronucleus.* Nature, 2005. **437**(7063): p. 1386-90.
25. Schultz, R.M., *The molecular foundations of the maternal to zygotic transition in the preimplantation embryo.* Hum Reprod Update, 2002. **8**(4): p. 323-31.
26. Braude, P., V. Bolton, and S. Moore, *Human gene expression first occurs between the four- and eight-cell stages of preimplantation development.* Nature, 1988. **332**(6163): p. 459-61.
27. Van Blerkom, J., et al., *Nuclear and cytoplasmic dynamics of sperm penetration, pronuclear formation and microtubule organization during fertilization and early preimplantation development in the human.* Hum Reprod Update, 1995. **1**(5): p. 429-61.
28. Krukowska, A. and A.K. Tarkowski, *Mouse zygotes with one diploid pronucleus formed as a result of ICSI can develop normally beyond birth.* Mol Reprod Dev, 2005. **72**(3): p. 346-53.
29. Tesarik, J. and C. Mendoza, *Spermatid injection into human oocytes. I. Laboratory techniques and special features of zygote development.* Human Reproduction, 1996. **11**(4): p. 772-779.
30. Hardarson, T., K. Lundin, and L. Hamberger, *The position of the metaphase II spindle cannot be predicted by the location of the first polar body in the human oocyte.* Hum Reprod, 2000. **15**(6): p. 1372-6.
31. Rienzi, L., et al., *Relationship between meiotic spindle location with regard to the polar body position and oocyte developmental potential after ICSI.* Hum Reprod, 2003. **18**(6): p. 1289-93.
32. Lim, A.S., et al., *Microscopic assessment of pronuclear embryos is not definitive.* Hum Genet, 2000. **107**(1): p. 62-8.
33. Sakkas, D., et al., *Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection.* Hum Reprod, 1996. **11**(4): p. 837-43.
34. Flaherty, S.P., et al., *Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection.* Hum Reprod, 1995. **10**(10): p. 2623-9.
35. Petignat, P., et al., *Molar pregnancy with a coexistent fetus after intracytoplasmic sperm injection. A case report.* J Reprod Med, 2001. **46**(3): p. 270-4.
36. Edwards, R.G., et al., *Pronuclear, cleavage and blastocyst histories in the attempted preimplantation diagnosis of the human hydatidiform mole.* Hum Reprod, 1992. **7**(7): p. 994-8.
37. Bomar, J., et al., *Differential regulation of maternal and paternal chromosome condensation in mitotic zygotes.* J Cell Sci, 2002. **115**(Pt 14): p. 2931-40.
38. Dozortsev, D., et al., *Nucleoli in a pronuclei-stage mouse embryo are represented by major satellite DNA of interconnecting chromosomes.* Fertil Steril, 2000. **73**(2): p. 366-71.
39. Huisman, G.J., et al., *Implantation rates after in vitro fertilization and transfer of a maximum of two embryos that have undergone three to five days of culture.* Fertil Steril, 2000. **73**(1): p. 117-22.
40. Hohmann, F.P., N.S. Macklon, and B.C. Fauser, *A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist cotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol.* J Clin Endocrinol Metab, 2003. **88**(1): p. 166-73.
41. Hunt, P., et al., *Analysis of chromosome behavior in intact mammalian oocytes: monitoring the segregation of a univalent chromosome during female meiosis.* Hum Mol Genet, 1995. **4**(11): p. 2007-12.
42. Baart, E.B., et al., *Distribution of Atr protein in primary spermatocytes of a mouse chromosomal mutant: a comparison of preparation techniques.* Chromosoma, 2000. **109**(1-2): p. 139-47.

43. Lachner, M., et al., *Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins.* Nature, 2001. **410**(6824): p. 116-20.

44. Derijck, A., et al., *DNA double-strand break repair in parental chromatin of mouse zygotes, the first cell cycle as an origin of de novo mutation.* Hum Mol Genet, 2008. **17**(13): p. 1922-37.

CHAPTER 4



X-chromosome inactivation is initiated in human pre-implantation embryos

I.M. van den Berg, J.S.E. Laven, M. Stevens, I. Jonkers,
R.-J. Galjaard, J. Gribnau and J.H. van Doorninck

Published in *Am. J Hum genet 2009 June 12: 84(6): 771-779*

Abstract

X chromosome inactivation (XCI) is the mammalian mechanism that compensates for the difference in gene dosage between XX females and XY males. Genetic and epigenetic regulatory mechanisms induce transcriptional silencing of one X chromosome in female cells. In mouse embryos, XCI is initiated at the pre-implantation stage following early whole-genome activation. It is widely thought that human embryos do not employ XCI prior to implantation. Here, we show that female pre-implantation embryos have a progressive accumulation of *XIST* RNA on one of the two X chromosomes starting around the 8-cell stage. *XIST* RNA accumulates at the morula and blastocyst stages and is associated with transcriptional silencing of the *XIST*-coated chromosomal region. These findings indicate that XCI is initiated in female human pre-implantation stage embryos, and suggest that pre-implantation dosage compensation is evolutionarily conserved in placental mammals.

Introduction

Mammalian XX females equalize gene dosage relative to XY males by the inactivation of one of their X chromosomes in each cell. The X chromosome inactivation center (XIC) contains several genetic elements essential for the transcription initiation of long noncoding RNAs that are involved in XCI. Initiation of XCI requires the *cis* accumulation of a nontranslated mouse *Xist* RNA or human *XIST* RNA (*Xist/XIST* RNA) (MIM 314670) that coats the X chromosome [1-3]. This is followed by various epigenetic changes on the future inactive X (Xi) chromosome that contribute to chromosome silencing [4]. In somatic cells, the Xi chromosome is visible as a region of dense chromatin called the Barr body [5].

There are two different forms of XCI: random XCI and imprinted XCI. Random XCI of either the maternal or the paternal X chromosome takes place in all somatic cell lineages of eutherian mammals, starting around gastrulation. Random XCI has no specific preference for inactivation of one of the parental X chromosomes [6-8]. In contrast, imprinted XCI results in preferential inactivation of the paternal X chromosome and occurs in female marsupials and mouse placental tissues [9-11]. Although expression of *Xist* RNA and a preferential expression of *Xist* from the paternal allele has long been observed in pre-implantation mouse embryos, the prevailing view has been that actual inactivation of the X chromosome, and thus dosage compensation, begins only after differentiation of the placental precursor cells [12-14]. In recent years, however, it has become apparent that X inactivation of the paternal X chromosome is already present from the 4-cell stage onward in all cells of pre-implantation mouse embryos [15-17]. Imprinted XCI in the mouse persists until the blastocyst stage and continues in the trophectoderm and the primitive endoderm [10,11,18]. However, the inactive paternal X chromosome is reactivated in the inner cell mass (ICM) that forms the embryo proper [15-17] and is followed by random XCI in somatic cell lineages [5,16,19]. It is still unclear exactly how the earlier imprinted XCI in cleavage-stage embryos, trophectoderm cells and primitive endoderm cells is programmed by the parental germline. Evidence exists for a mark on the maternal X chromosome that allows it to remain active [20,21]. On the other hand, there is also evidence for a preference of *Xist*-mediated inactivation of the paternal X chromosome [12,15,22] but these two mechanisms do not need to be mutually exclusive.

Data regarding the mechanism of human XCI are not easy to obtain because of restrictions on the use of human embryos and the generation of human embryonic stem (ES) cell lines. Only a minority of human ES cell lines have two active X chromosomes in their undifferentiated state and will start a process of random XCI upon differentiation, similar to mouse ICM cells and ES cell lines [16,23-25]. The majority of the undifferentiated human ES

cell lines so far examined have already inactivated one X chromosome, evident from the single *XIST* cloud in 20%–70% of the cells and the accumulation of specific chromatin modifications [24-27]. One study showed that differentiation of a human ES cell line resulted in either random XCI or preferential inactivation of a single allele, depending on the differentiated cell type. Only trophoblast cells showed a preferential inactivation of a single X chromosome similar to mouse trophoblast tissue [23]. Although the parental origin of the X chromosome could not be identified in this ES cell line, which was generated from anonymously donated embryos, it does suggest that a form of preferential XCI, such as imprinted XCI, may exist during the first stages of human trophoblast development, similar to mouse extra-embryonic tissues [10,11,18]. Studies of human placental tissue have shown a variety of patterns of XCI. Some reports describe preferential expression from the maternal X chromosome, similar to mice, suggesting conservation of imprinted XCI [28-31]. However, other papers report a random XCI or XCI moderately skewed in favor of an inactive paternal X chromosome [32-36]. If imprinted XCI in humans occurs, it is possible that human trophoblasts gradually lose their imprint and perform random XCI at later stages, as has been demonstrated *in vitro* [37,38]. This may result in an XCI pattern skewed towards an inactivated paternal X, which would explain the mixed results observed in the analysis of placentas [28-36].

Defects in dosage compensation prior to implantation of the embryo lead to abnormal development in a majority of the embryos and is early lethal, as demonstrated by analysis of parthenogenetic mouse embryos that have two maternal genomes [13,39]. Similarly, female mutant embryos that inherit a paternal X chromosome with a deletion of the *Xist* gene are not able to inactivate this X chromosome, resulting in two active X chromosomes and early lethality [40,41].

Previous studies using PCR analysis of human pre-implantation embryos detected *XIST* expression both in female and in male embryos [42,43]. Because female-specific *XIST* expression was not detected, it was concluded that *XIST* RNA was not functional at this stage of development and that dosage compensation was not initiated in human pre-implantation embryos [42,43]. However, in mice, a brief 'pinpoint' expression of *Xist* from both the paternal and maternal X chromosome was later reported in male and female pre-implantation embryos [17,19,44,45]. The initial *Xist* expression on the maternal X chromosome subsequently disappears while *Xist* expression from the paternal X chromosome accumulates in female pre-implantation embryos to coat the future Xi chromosome [17,44-46]. Similar pinpoint signals are also observed during the onset of random X inactivation in male and female ES cells [17,19,46]. Thus, the *XIST* expression previously reported in male human embryos could

be attributable to a brief window of expression of *XIST* from the maternal X chromosome and does not exclude XCI in female embryos.

We therefore decided to reinvestigate XCI in human pre-implantation embryos at the single-cell level to analyse *XIST* RNA localisation and the transcriptional and epigenetic features of XCI.

Material and Methods

Collection of surplus embryos and cryopreserved embryos donated for research

Ovarian stimulation, oocyte retrieval and *in vitro* fertilization (IVF) and/or intracytoplasmic sperm injection (ICSI) procedures were performed as described previously [47]. This study was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO, NL11448) and the local ethics review committee of the Erasmus Medical Center Hospital (MEC 2007–130). Written consent was obtained from the couples in order to confirm that the surplus or cryopreserved embryos could be used for research purposes.

RNA FISH, DNA FISH and Immunofluorescence

Immunofluorescence followed by RNA/DNA fluorescence *in* situ hybridization (FISH) was performed as described previously [17], with some modifications. The zona pellucida of fresh or thawed cryopreserved embryos was removed with 0.05% pronase (Sigma-Aldrich) in calcium- and magnesium-free medium (G-PGD, Vitrolife, Kungsbacka, Sweden). Embryos of the 8-cell and morula stage were incubated in calcium- and magnesium-free medium so that single cells could be obtained. Single cells and blastocysts were washed in phosphate-buffered saline (PBS) and fixed on slides in 1% paraformaldehyde (Sigma-Aldrich) containing 0.5% Triton X-100 for 0,5-1 hr. Slides were washed with PBS and stored in 70% ethanol at -20°C. Cumulus cells and amniocytes were fixed similarly. Primary antibodies used for immunofluorescence were H3K27Me3 (Abcam, 1:50), macroH2A.1 (Upstate, 1:100), H3K9ac (Upstate, 1:100). Secondary antibodies (Invitrogen), used at 1:250 dilutions, were as follows: goat anti-rabbit Alexa 594, goat anti-mouse Alexa 488 and goat anti-mouse Pacific Blue.

For the detection of *XIST* RNA, a 16,4 kb plasmid covering the complete RNA sequence of the *XIST* gene [48] was used on nondenatured cells. *Cot1* RNA detection was performed using labeled Cot1 DNA (Invitrogen) as a probe. For the detection of chromosomes X, Y and 15, the following DNA probes were used: X centromere (pBAMX5), Y chromosome heterochromatin (RPN1305X) and chromosome 15 satellite III region f (D15Z1). The Y probe occasionally

produces a diffuse signal because of the highly polymorphic heterochromatin region [49]. Overlapping *CHIC1* fosmid clones (G248P81074F7 and G248P86549C3) were located using the UCSC genome browser (UC Santa Cruz) and obtained from BACPAC Resources (Oakland). RNA and DNA FISH probes were labeled by nick-translation with fluorochromes Alexa 594 and 555 (Molecular Probes, Invitrogen, Leiden, The Netherlands), diethylaminocoumarin-5-UTP (NEN Life science products, Boston, USA) or Bio-16-dUTP (Roche). Probes were validated in cultured lymphocytes of a normal XY male. A significant distance between the signals of the X centromere probe and *XIST* RNA was often observed, as XIST is located 80 Mb distal to the centromere. *XIST* expression was detected either as a pinpoint signal, a small cloud, or a full cloud where the distinction between the three was usually easy to detect. A pinpoint signal was equivalent in size and intensity to a locus-specific DNA FISH signal, a small cloud was 10–20 times the size of a pinpoint signal, and a full cloud was 100 or more times the size of a pinpoint signal.

Slides were examined with a Zeiss Axioplan 2 epifluorescence microscope, equipped with appropriate filters (Chroma, Rockingham, VT, USA). Images were captured with the ISIS FISH Imaging System (MetaSystems, Altlussheim, Germany) and background correction was applied using Adobe Photoshop CS2 when necessary. For each embryo, the positions of all nuclei were mapped in detail, which allowed an accurate analysis of each nucleus. Chromosomally chaotic embryos (based on X, Y and chromosome 15 analysis) were excluded from analysis and mosaic embryos were included only if less than 50% of the cells were aneuploid for X and Y.

Results

Donated cryopreserved and surplus embryos from *in vitro* fertilization (IVF) treatments were dissociated and fixed at the 8-cell, morula and blastocyst stages. RNA FISH with a human *XIST* probe was performed for the detection of the inactive X-chromosome in single cells, followed by DNA FISH with chromosome X-, Y- and 15 specific probes to identify the sex and diploid status of the embryos (see Figure S1).

XIST expression in early human embryos

Only a fraction of the blastomeres from male embryos showed *XIST* RNA signals. These signals were small 'pinpoint'-like signals reminiscent of expression of unstable *XIST* RNA [44,46]. This form of *XIST* expression was mostly observed at the morula stage, and the pinpoints never accumulated to a 'cloud'-like signal in blastocysts, indicating the absence of XCI in male pre-

implantation embryos (Figure 1A-C, Table I). In contrast, female embryos showed a different *XIST*-staining pattern: the majority of cells had pinpoint signals for *XIST* RNA at the 8-cell stage. The *XIST* signal gradually accumulated to a full cloud on one of the X chromosomes at the late morula and blastocyst stages in female embryos (Figure 1D-F). Distinct patterns of *XIST* expression were observed for different developmental stages (Table I): In 8-cell stage embryos, 65% of the blastomeres displayed one pinpoint signal of *XIST*, 4% of the blastomeres had a small cloud of *XIST* and 19% showed two pinpoints of *XIST RNA*. The remaining 12% of blastomeres had no expression of *XIST*. In morulas, *XIST* expression had progressively accumulated resulting in 49% of the cells displaying a single *XIST* cloud, suggesting that XCI was initiated in these cells and 19% of the cells having a pinpoint of *XIST* RNA. The percentage of cells with two pinpoints was reduced from 19% at the 8-cell stage to 2% in morulas. The rest of the blastomeres either had no *XIST*, a single pinpoint of *XIST*, two small clouds of *XIST*, or a pinpoint together with a cloud signal. Different cells of the same embryo regularly showed variable levels of *XIST* and distinct *XIST* expression patterns (Figure S2) similar to previous observations in mouse embryos [13,44,50,51].

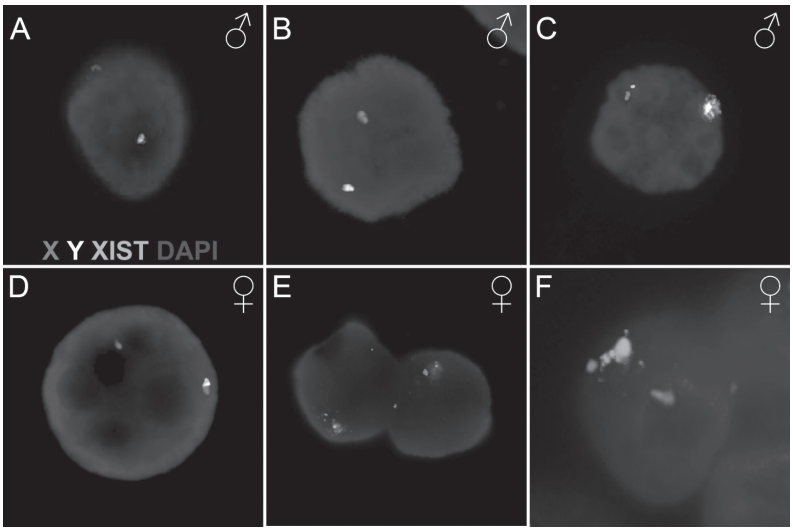


Figure 1. *XIST* expression in male and female human pre-implantation embryos. RNA and DNA FISH staining with probes to detect *XIST* RNA (green) ,the X (red) and Y chromosome (yellow) and DAPI counterstain. Human male embryos (**A-C**) do not generally show *XIST* signals at the 8-cell stage (A) or at the morula stage (B). A minority of male cells at the morula stage show a pinpoint of *XIST* staining (C). Female embryos (**D-F**) show an *XIST* pinpoint in the majority of embryos at the 8-cell stage (D). (E) Two cells at the morula stage each show a beginning cloud of XIST on one of their two X chromosomes. (F) At the blastocyst stage this has further accumulated to a full cloud on one of the two X chromosomes. A third diffuse red signal is an X chromosome from an adjacent cell that is in a different focal plane (see page 177 for colour figure).

Table I. *XIST* patterns in female human embryos at different developmental stage.

	XIST pattern in ♀ embryos							XIST pattern in ♂ embryos		
Embryo stage	No. of ♀ embryos (No. of Cells)	– –	• –	• •	• ●	– ●	● ●	No. of ♀ Embryos (No. of Cells)	–	•
8-cell	5 (26)	12	65	19		4		6 (30)	94	6
Morula	13(89)	19	19	2	6	49	4	9 (91)	86	14
Blastocyst	6 (>100)	5				90	5	5 (98)	93	7

Percentages of analyzable cell that have the indicated pattern of XIST signals. Signals in blastomeres were scored as negative (-). Pinpoint signal (•), small cloud (●), or full cloud (●).

Blastocysts did not disaggregate well during the fixation procedure and were therefore examined as intact embryos. Nuclei that could be visualized showed a full *XIST* cloud in 90% of the cells (Figure 1 and Table I). The remainder of cells either had no *XIST* signal or two small cloud signals. In summary, whereas female embryos showed a clear single pinpoint or small cloud of *XIST* at the 8-cell stage that accumulated to a proper *XIST* cloud at the late morula and blastocyst stage, male embryos showed only brief expression of *XIST* in a minority of the cells. Thus, a clear difference in the timing, duration and the level of *XIST* expression between male and female human embryos suggests the occurrence of XCI in female pre-implantation embryos.

Absence of transcriptional activity on XIST-coated chromosomal region

We analyzed the transcriptional activity together with *XIST* staining since *XIST* RNA accumulation on the X chromosome itself does not automatically imply an inactivated status of the X chromosome. Transcriptional activity was investigated by *Cot1* RNA FISH staining, which highlight areas of ongoing hnRNA transcription; trancriptionally silent nuclear compartments, such as an inactivated X chromosome are devoid of *Cot1* RNA [15, 17]. Human embryos were stained by RNA and DNA FISH with probes for *Cot1* RN*A, XIST* RNA, chromosome 15, and the X and Y chromosomes. Cumulus cells of human follicle complexes were used as positive controls in each experiment (Figure S3A-3C). Cells of human blastocysts showed *XIST* RNA clouds corresponding to areas where *Cot1* RNA (Figure 2A-F) was excluded. Figures 2D-F show a representative cell with an *XIST*-coated X in a *Cot1*-depleted region and the second X chromosome in a *Cot1*-positive area. The *Cot1* exclusion coincided with the *XIST* signal in 89% of the cells (n = 47). Comparable percentages of *Cot1* exclusion from *Xist/XIST* positive areas have been observed in mouse embryos and differentiating human embryonic stem cells

[15,22,24]. The initiation of transcriptional silencing was confirmed with RNA FISH to detect nascent transcripts of the X-linked *CHIC1* gene. Blastocyst cells showed a single spot of *CHIC1* expression close to one X centromere and an *XIST* cloud on the other inactive X chromosome (Figures 2G-J). Bi-allelic expression was not observed in blastocysts and was not assayed in earlier stages. Thus, the X chromosome that is at least partially coated with *XIST* RNA in the human embryo is in a transcriptionally silent area, demonstrating that XCI and dosage compensation commences in pre-implantation female human embryos.

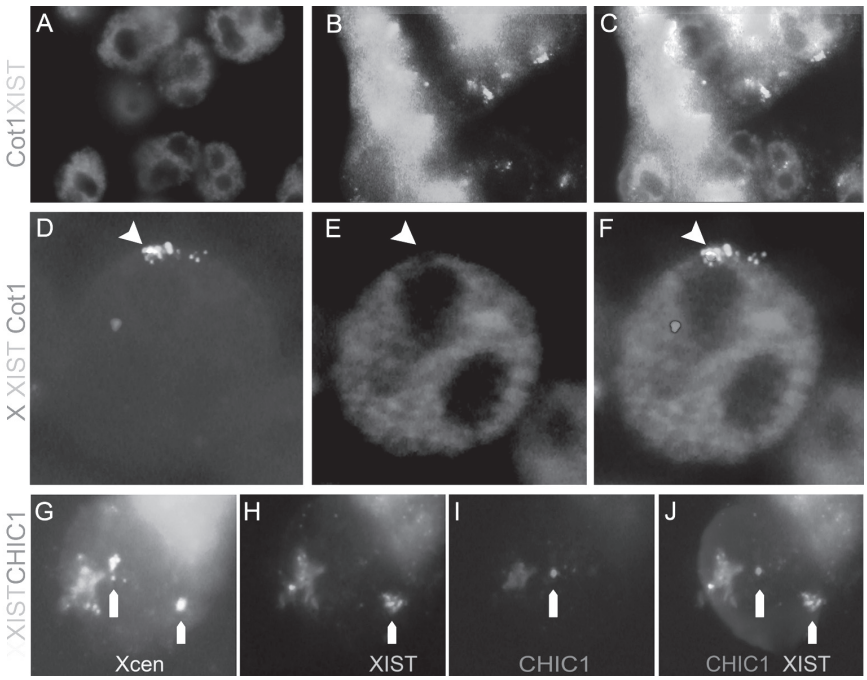


Figure 2. Transcriptional changes on the inactive X chromosome. (**A-F**) *Cot1* exclusion around *XIST*. (A-C) Cells of a female blastocyst embryo with staining for *Cot1* RNA (red in A) and *XIST* RNA (green in B) showing depleted regions of *Cot-1* RNA around the *XIST* signals indicating the position of the inactivated X (merged in C). (**D-F**) Representative cell of a female blastocyst with staining for the X centromeres and *XIST* RNA (D, Xcen in magenta, *XIST* in green) together with *Cot1* (red in E). Transcription of *Cot1* RNA was absent in a region that overlaps with *XIST* RNA staining (F), while the active X without *XIST* staining overlaps with a *Cot1*-positive region. (G-J) Female blastocyst cell with two X centromeres (cyan in G) has a single *XIST* cloud on one X chromosome (green in H) and monoallelic expression of *CHIC1* on the other X chromosome (red in I, merged in J). A dust spot is visible in all colors and is therefore nonspecific staining (see page 177 for colour figure).

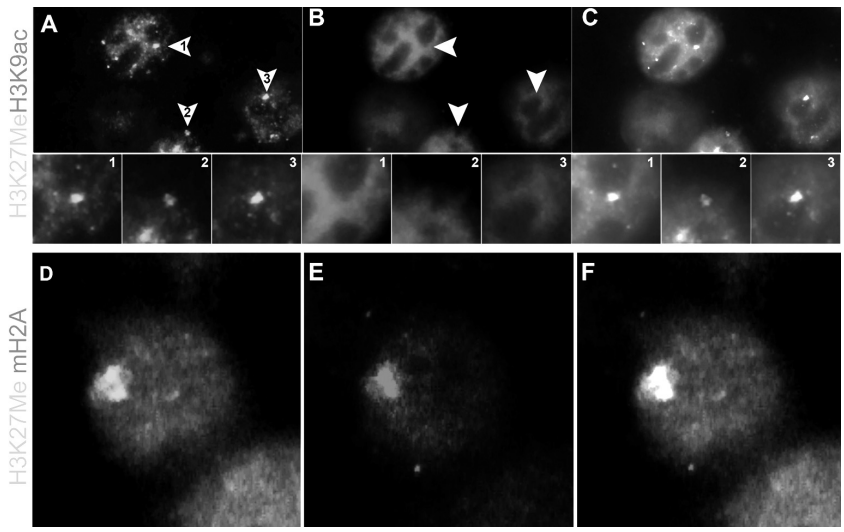


Figure 3. Epigenetic changes on the inactive X chromosome. (**A-C**) Three adjacent blastocyst cells show H3K27Me3 hypermethylation (arrowheads in J and enlarged panels 1-3) and staining for H3K9 acetylation (B, 1-3) shows a H3K9ac-depleted region overlaying the H3K27Me3 accumulation (C, 1-3) indicating the position of the Xi chromosome. (**D-F**) Representative blastocyst cell shows H3K27 hypermethylation (green, D) and enrichment for macroH2A (red, E) with a clear overlap (yellow, F) analogous to the signal around an Xi chromosome (see page 180 for colour figure).

Chromatin conformational changes on the inactive X

To further explore XCI in human pre-implantation embryos, we investigated histone modifications that are established hallmarks of a silenced X chromosome. Specific accumulation of one or more histone modifications form macrochromatin bodies that indicate the position of the inactive X chromosome, as shown for XCI in mouse pre-implantation embryos and in differentiating mouse and human ES cells [1,4,16,17,25, 52-54].

Identification of the chromatin state of X chromosomes was carried out with antibodies to detect hypoacetylation of lysine 9 on histone H3 (H3K9ac), accumulation of trimethylation of lysine 27 on H3 (H3K27Me3), and the enrichment of the histone variant macroH2A [1,4,16,17,52,53] followed by DNA FISH to identify the gender of the embryo. As a control, we used cumulus cells in which the inactivated X can be identified as a chromatin dense Barr body or amniocytes in which an immunostaining together with *XIST* detection is possible. Human cumulus cells showed DAPI-dense Barr bodies that were positively stained for macroH2A and H3K27Me3, marks associated with inactive chromatin. Amniocytes showed

the same accumulation of chromatin markers overlaying the XIST signal (Figure S3H-S3K). In addition, staining with an antibody against H3K9 acetylation, an active chromatin mark, showed exclusion from Barr bodies in cumulus cells or from *XIST*-stained regions in amniocytes (Figure S3P-S3S). These findings confirm the specificity of the antibodies used to indicate a macrochromatin body as the Xi. Staining of cells from pre-implantation blastocysts using the same antibodies showed identical results, i.e., double staining showed a single region where accumulated H3K27Me3 staining exactly overlay the region of H3K9 hypoacetylation (Figure 3A-C), indicating the presence of an Xi. Furthermore, macroH2A enrichment and accumulation of H3K27Me3 (Figure 3D-F) colocalized exactly in blastocyst cells. Up to 30% of the analyzable cells in blastocysts showed a double immunostaining of chromatin marks that are specific for XCI (either H3K27Me3 together with macroH2A or H3K27Me3 in a depleted region of H3K9ac). The other 70% of the cells had no visible accumulation (or depletion in case of H3K9ac) of either antibody, and single accumulations were rarely found (<5%). In contrast, male embryos did not show accumulation of H3K27Me3 or macroH2a, and no specific exclusion of H3k9ac was observed (data not shown).

Taken altogether, these observations show that once *XIST* RNA coats the X chromosome in human embryos, epigenetic changes that are known to lead to XCI are induced, similar to what has been observed in mouse pre-implantation embryos and embryonic stem cells [4,55,56].

Discussion

In contrast to previous suggestions that XCI may not be present in the human pre-implantation embryo, [4,42,43,55,57] our observations of *XIST* RNA accumulation in female embryos, local accumulation of Xi-specific chromatin modifications, and the absence of active transcription in *XIST* RNA-coated areas indicate that XCI occurs in human female pre-implantation embryos starting from the 8-cell stage. However, the extent of the coating by *XIST* and the extent of the transcriptional silencing other than that of the *CHIC1* gene remain unknown.

Conservation of timing of XCI

The developmental stage at which embryonic genome activation occurs in mammalian species varies considerably: at the 1-to-2-cell stage in mice, at the 4-to-8-cell stage in cows and humans, and at the 8-to-16-cell stage in sheep and rabbits [58,59]. The necessity of dosage compensation by XCI is likely to start at the onset of genome activation; thus, around the 2

cell stage in mice and around the 8-cell stage in humans. Initiation of XCI can be detected in mice from the 2-cell stage onwards by *Xist* RNA FISH, showing a small pinpoint of *Xist* in 67% of female cells, and complete X-associated *Xist* accumulation occurs by the 8-cell stage [15,17]. In human embryos, we observed pinpoint *XIST* signals in 65% of female 8-cell blastomeres and *cis* accumulation (cloud-like signals) of *XIST* in late morulas and in blastocysts. We could not analyze embryos before the 6- to 8-cell stage, because such embryos were not available for research. Given that our observations in 6- to 8-cell stage human embryos show *XIST* RNA with exactly the same pinpoint signals and at a similar frequency as in 2-cell stage mouse embryos (67% vs. 65%, Table I and [15]), it is likely that the 8-cell stage represents the actual onset of *XIST* expression in humans. The later stage of XCI in humans relative to mice suggests a correlation with the later transition from maternal to embryonic or zygotic gene expression in humans [60].

XIST patterns were only analyzed in euploid cells, and because similar results were observed in cells derived from normal or mosaic embryos (Table S1), these data were combined. No consistent pattern of *XIST* expression was discernible in the sex aneuploid cells (Table S1). The quality of the embryos did not influence the results; we observed similar patterns of X inactivation in euploid cells of surplus embryos and cryopreserved embryos. It is likely that aneuploidies other than detected with the X, Y or chromosome 15 probes were present in the analyzed cells since human embryos are known to have high aneuploidy rates [61]. However, aneuploidies may only have a subtle effect on skewing of the selected X chromosome [62].

Comparison of several characteristics of X inactivation between mouse and human embryos indicates that although the onset of *XIST* expression may occur later in humans than in mice, the timeline and order of *XIST* expression initiation through actual X inactivation is similar in mice and men (Figure 2 and [63]).

Detection of Xist in male embryos

In contrast to previously reports on the detection of *XIST* in human embryos [42,43], we found a clear difference in *XIST* expression between male and female embryos (Figure 1). The previous data on human male embryos were obtained by nested PCR that, in contrast to our FISH method, detects very low levels of *XIST* RNA. The large number of cycles in this method probably masked the difference in *XIST* levels between male and female embryos, leading to the erroneous conclusion that human embryos do not initiate XCI [42,43]. In general, large differences in levels of *Xist/XIST* expression are found between embryos and between cells of a single embryo in both mice [17,50] and humans (Table I and Figure S2). PCR detection of RNA expression in pooled embryos or even single embryos can mask these variations [50] and

interpretations of these results are therefore unreliable. Thus, although PCR is more sensitive than the FISH method, the ability to localize the transcripts, as in a FISH experiment, is essential for studying the X-inactivation process.

The onset of *Xist/XIST* expression in male embryos, detected as a pinpoint signal by *Xist/XIST* RNA FISH, is slightly later than in human female embryos, both in mice and humans ([17,44,45] and Table I). Although our *XIST* RNA FISH probe did not overlap with the reported transcribed *TSIX* sequence [64] (MIM 300181), which is transcribed in the opposite orientation and may be involved in down regulation of *XIST,* we cannot exclude that the transient male and early female pinpoint signals are (in part) *TSIX* RNA. We therefore tested *TSIX* probes that had been used previously to detect *TSIX* in human fetal cells [64], but failed to obtain specific signals on either embryos or amniocytes from routine amniocentesis. Thus, whether human *TSIX* plays a role in the intitiation of XCI remains to be elucidated.

Similar to mouse embryos, the pinpoint signals in human male embryos never accumulated to a complete cloud, indicating that initial *XIST* and/or *TSIX* expression does not lead to actual inactivation of the X chromosome in male embryos ([17,44,45] and Table I).

What is the mechanism of XCI?

Our data on human embryos show a variety of *XIST*-staining patterns that are similar to mouse *Xist* embryo patterns ([13,15,17,19,44] and Table I, Figure S1). Slight discrepancies between published data can be explained by differences in detection sensitivity, as double *Xist* signals were observed more frequently when larger probes and increased signal amplification were used [13,15,44].

Both random XCI and imprinted XCI can result in patterns of X inactivation that are comparable to the patterns that we have observed in human embryos: Of the different models for random XCI [8], the stochastic model, -in which every X chromosome has a certain probability of being inactivated, resulting in a majority of cells with one Xi, as well as cells with no Xi or two Xi's [7] – best explains our results. Only this model accommodates the variety of *XIST* expression patterns such as we have observed; other random XCI models view this variation as errors of the XCI mechanism. Imprinted XCI has a preferential expression of the paternal *Xist* allele but is only manifested in 70–90% of the cells, whereas the remainder of the cells shows no *Xist* expression. These *Xist* negative cells may at a later stage inactivate the correct number of X chromosomes, akin to random XCI [65]. The human data could thus be explained by both imprinted and random mechanisms of XCI. The majority of blastomeres from our 8-cell human female embryos, showed only one pinpoint *XIST* signal, suggesting a preference for activation of a single *XIST* allele that could be indicative of imprinted XCI, similar

to what is observed in mice. The obvious solution would be parental tracing of the expressed *XIST* gene. Unfortunately, this was not possible with these anonymously donated embryos. Future experiments are necessary in order to determine whether XCI in human embryos is a random process or whether imprinted XCI is fully conserved.

In summary, we find X-associated accumulation of *XIST* RNA in female cleavage-stage and blastocyst embryos, together with transcriptional silencing and Xi-specific histone modifications. These results indicate that (at least part of) the X chromosome is silenced in human pre-implantation embryos. Our findings therefore suggest that X-linked dosage compensation in mammalian pre-implantation embryos is evolutionary conserved.

Acknowledgements

We thank Frank Grosveld and Willy Baarends for valuable comments, Eric Steegers for financial support, Cindy Eleveld, Arletta Bol, Shimaira van Beusekom, Bert Eussen en Erwin Brosens for technical assistance, the Erasmus MC IVF team for support; and the patients for their generous embryo donation.

Web Resources

The URL for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

USSC genome browser, http://genome.ucsc.edu/

References

1. Brockdorff, N., et al., *Conservation of position and exclusive expression of mouse Xist from the inactive X chromosome.* Nature, 1991. **351**: p. 329-331.
2. Brown, C.J., et al., *A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome.* Nature, 1991. **349**: p. 38-44.
3. Penny, G.D., et al., *Requirement for Xist in X chromosome inactivation.* Nature, 1996. **379**: p. 131-137.
4. Heard, E. and C.M. Disteche, *Dosage compensation in mammals: fine-tuning the expression of the X chromosome.* Genes Dev, 2006. **20**: p. 1848-67.
5. Lyon, M.F., *Gene action in the X-chromosome of the mouse (Mus musculus L.).* Nature, 1961. **190**: p. 372-373.
6. Avner, P. and E. Heard, *X-chromosome inactivation: counting, choice and initiation.* Nat Rev Genet, 2001. **2**(1): p. 59-67.
7. Monkhorst, K., et al., *X inactivation counting and choice is a stochastic process: evidence for involvement of an X-linked activator.* Cell, 2008. **132**(3): p. 410-21.
8. Starmer, J. and T. Magnuson, *A new model for random X chromosome inactivation.* Development, 2009. **136**(1): p. 1-10.
9. Graves, J.A., *Mammals that break the rules: genetics of marsupials and monotremes.* Annu Rev Genet, 1996. **30**: p. 233-60.
10. Takagi, N. and M. Sasaki, *Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse.* Nature, 1975. **256**(5519): p. 640-2.
11. West, J.D., et al., *Development of interspecific hybrids of Mus.* J Embryol.Exp.Morphol., 1977. **41**: p. 233-243.
12. Kay, G.F., et al., *Imprinting and X chromosome counting mechanisms determine Xist expression in early mouse development.* Cell, 1994. **77**(5): p. 639-50.
13. Nesterova, T.B., et al., *Loss of Xist imprinting in diploid parthenogenetic preimplantation embryos.* Dev Biol, 2001. **235**: p. 343-350.
14. Zuccotti, M., et al., *Mouse Xist expression begins at zygotic genome activation and is timed by a zygotic clock.* Mol Reprod Dev, 2002. **61**: p. 14-20.
15. Huynh, K.D. and J.T. Lee, *Inheritance of a pre-inactivated paternal X chromosome in early mouse embryos.* Nature, 2003. **426**: p. 857-862.
16. Mak, W., et al., *Reactivation of the paternal X chromosome in early mouse embryos.* Science, 2004. **303**: p. 666-669.
17. Okamoto, I., et al., *Epigenetic dynamics of imprinted X inactivation during early mouse development.* Science, 2004. **303**: p. 644-649.
18. Sugawara, O., N. Takagi, and M. Sasaki, *Correlation between X-chromosome inactivation and cell differentiation in female preimplantation mouse embryos.* Cytogenet.Cell Genet, 1985. **39**: p. 210-219.
19. Matsui, J., Y. Goto, and N. Takagi, *Control of Xist expression for imprinted and random X chromosome inactivation in mice.* Hum Mol Genet, 2001. **10**: p. 1393-1401.
20. Lyon, M.F. and S. Rastan, *Parental source of chromosome imprinting and its relevance for X chromosome inactivation.* Differentiation, 1984. **26**(1): p. 63-7.
21. Tada, T., et al., *Imprint switching for non-random X-chromosome inactivation during mouse oocyte growth.* Development, 2000. **127**: p. 3101-3105.
22. Okamoto, I., et al., *Evidence for de novo imprinted X-chromosome inactivation independent of meiotic inactivation in mice.* Nature, 2005. **438**: p. 369-373.

23. Dhara, S.K. and N. Benvenisty, *Gene trap as a tool for genome annotation and analysis of X chromosome inactivation in human embryonic stem cells.* Nucleic Acids Res, 2004. **32**: p. 3995-4002.
24. Silva, S.S., et al., *X-chromosome inactivation and epigenetic fluidity in human embryonic stem cells.* Proc Natl Acad Sci U S A, 2008. **105**(12): p. 4820-5.
25. Hall, L.L., et al., *X-inactivation reveals epigenetic anomalies in most hESC but identifies sublines that initiate as expected.* J Cell Physiol, 2008. **216**(2): p. 445-52.
26. Hoffman, L.M., et al., *X-inactivation status varies in human embryonic stem cell lines.* Stem Cells, 2005. **23**: p. 1468-1478.
27. Shen, Y., et al., *X-inactivation in female human embryonic stem cells is in a nonrandom pattern and prone to epigenetic alterations.* Proc Natl Acad Sci U S A, 2008. **105**(12): p. 4709-14.
28. Harrison, K.B. and D. Warburton, *Preferential X-chromosome activity in human female placental tissues.* Cytogenet.Cell Genet, 1986. **41**: p. 163-168.
29. Ropers, H.H., G. Wolff, and H.W. Hitzeroth, *Preferential X inactivation in human placenta membranes: is the paternal X inactive in early embryonic development of female mammals?* Hum Genet, 1978. **43**(3): p. 265-73.
30. Harrison, K.B., *X-chromosome inactivation in the human cytotrophoblast.* Cytogenet Cell Genet, 1989. **52**(1-2): p. 37-41.
31. Goto, T., E. Wright, and M. Monk, *Paternal X-chromosome inactivation in human trophoblastic cells.* Mol Hum Reprod, 1997. **3**: p. 77-80.
32. Migeon, B.R., et al., *Incomplete X chromosome dosage compensation in chorionic villi of human placenta.* Proc Natl Acad Sci U S A, 1985. **82**(10): p. 3390-4.
33. Looijenga, L.H., et al., *Heterogeneous X inactivation in trophoblastic cells of human full-term female placentas.* Am J Hum Genet, 1999. **64**: p. 1445-1452.
34. Bamforth, F., G. Machin, and M. Innes, *X-chromosome inactivation is mostly random in placental tissues of female monozygotic twins and triplets.* Am J Med Genet, 1996. **61**: p. 209-215.
35. Uehara, S., et al., *X-chromosome inactivation in the human trophoblast of early pregnancy.* J Hum Genet, 2000. **45**: p. 119-126.
36. Zeng, S.M. and J.-. Yankowitz, *X-inactivation patterns in human embryonic and extra-embryonic tissues.* Placenta, 2003. **24**: p. 270-275.
37. Migeon, B.R., et al., *Complete reactivation of X chromosomes from human chorionic villi with a switch to early DNA replication.* Proc.Natl.Acad Sci U.S.A, 1986. **83**: p. 2182-2186.
38. Migeon, B.R., J. Axelman, and P. Jeppesen, *Differential X reactivation in human placental cells: implications for reversal of X inactivation.* Am J Hum Genet, 2005. **77**(3): p. 355-64.
39. Shao, C. and N. Takagi, *An extra maternally derived X chromosome is deleterious to early mouse development.* Development, 1990. **110**(3): p. 969-75.
40. Lee, J.T., *Disruption of imprinted X inactivation by parent-of-origin effects at Tsix.* Cell, 2000. **103**(1): p. 17-27.
41. Takagi, N., *Imprinted X-chromosome inactivation: enlightenment from embryos in vivo.* Semin.Cell Dev Biol, 2003. **14**: p. 319-329.
42. Daniels, R., et al., *XIST expression in human oocytes and preimplantation embryos.* Am J Hum Genet, 1997. **61**: p. 33-39.
43. Ray, P.F., R.M. Winston, and A.H. Handyside, *XIST expression from the maternal X chromosome in human male preimplantation embryos at the blastocyst stage.* Hum Mol Genet, 1997. **6**: p. 1323-1327.
44. Sheardown, S.A., et al., *Stabilization of Xist RNA mediates initiation of X chromosome inactivation.* Cell, 1997. **91**: p. 99-107.

45. Latham, K.E., et al., *Effects of X chromosome number and parental origin on X-linked gene expression in preimplantation mouse embryos.* Biol Reprod, 2000. **63**(1): p. 64-73.
46. Panning, B., J. Dausman, and R. Jaenisch, *X chromosome inactivation is mediated by Xist RNA stabilization.* Cell, 1997. **90**: p. 907-916.
47. Hohmann, F.P., N.S. Macklon, and B.C. Fauser, *A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist cotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol.* J Clin Endocrinol Metab, 2003. **88**(1): p. 166-73.
48. Plath, K., et al., *Role of histone H3 lysine 27 methylation in X inactivation.* Science, 2003. **300**: p. 131-135.
49. Repping, S., et al., *High mutation rates have driven extensive structural polymorphism among human Y chromosomes.* Nat Genet, 2006. **38**(4): p. 463-7.
50. Hartshorn, C., J.E. Rice, and L.J. Wangh, *Differential pattern of Xist RNA accumulation in single blastomeres isolated from 8-cell stage mouse embryos following laser zona drilling.* Mol Reprod Dev, 2003. **64**: p. 41-51.
51. Hartshorn, C., J.E. Rice, and L.J. Wangh, *Developmentally-regulated changes of Xist RNA levels in single preimplantation mouse embryos, as revealed by quantitative real-time PCR.* Mol Reprod Dev, 2002. **61**: p. 425-436.
52. Costanzi, C. and J.R. Pehrson, *Histone macroH2A1 is concentrated in the inactive X chromosome of female mammals.* Nature, 1998. **393**(6685): p. 599-601.
53. Chadwick, B.P. and H.F. Willard, *Cell cycle-dependent localization of macroH2A in chromatin of the inactive X chromosome.* J Cell Biol, 2002. **157**(7): p. 1113-23.
54. Chaumeil, J., et al., *Integrated kinetics of X chromosome inactivation in differentiating embryonic stem cells.* Cytogenet.Genome Res, 2002. **99**: p. 75-84.
55. Chow, J.C., et al., *Silencing of the mammalian X chromosome.* Annu Rev Genomics Hum Genet, 2005. **6**: p. 69-92.
56. Latham, K.E., *X chromosome imprinting and inactivation in preimplantation mammalian embryos.* Trends Genet, 2005. **21**: p. 120-127.
57. Brown, C.J. and W.P. Robinson, *XIST expression and X-chromosome inactivation in human preimplantation embryos.* Am J Hum Genet, 1997. **61**: p. 5-8.
58. Schultz, G.A. and S. Heyner, *Gene expression in pre-implantation mammalian embryos.* Mutat Res, 1992. **296**(1-2): p. 17-31.
59. Dobson, A.T., et al., *The unique transcriptome through day 3 of human preimplantation development.* Hum Mol Genet, 2004. **13**(14): p. 1461-70.
60. Wells, D., et al., *Expression of genes regulating chromosome segregation, the cell cycle and apoptosis during human preimplantation development.* Hum Reprod, 2005. **20**: p. 1339-1348.
61. Baart, E.B., et al., *Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF.* Hum Reprod, 2006. **21**(1): p. 223-33.
62. Bretherick, K., J. Gair, and W.P.-. Robinson, *The association of skewed X chromosome inactivation with aneuploidy in humans.* Cytogenet.Genome Res, 2005. **111**: p. 260-265.
63. Okamoto, I. and E. Heard, *The dynamics of imprinted X inactivation during preimplantation development in mice.* Cytogenet.Genome Res, 2006. **113**: p. 318-324.
64. Migeon, B.R., et al., *Species differences in TSIX/Tsix reveal the roles of these genes in X-chromosome inactivation.* Am J Hum Genet, 2002. **71**: p. 286-293.
65. Huynh, K.D. and J.T. Lee, *Imprinted X inactivation in eutherians: a model of gametic execution and zygotic relaxation.* Curr.Opin.Cell Biol, 2001. **13**: p. 690-697.

Supplemental Data

X-Chromosome Inactivation is initiated in Human Pre-Implantation Embryos

Table S1. *XIST* patterns in sex euploid and aneuploid human embryonic cells.

Embryos	Genotype	# Cells	"Expected" XIST pattern[a]	(%)	"Unexpected" XIST pattern	Unexpected patterns: more (+) or less (-) XIST spots
XY cleavage stage						
euploid (N = 3)	XY	17	16	(94)	1	+
mosaic (N = 3)	XY	13	12	(92)	1	+
	XX	1	1	(100)		
	XO	3	3	(100)		
	XYY	1	1	(100)		
XY morulas						
euploid (N = 2)	XY	24	24	(100)		
mosaic (N = 7)	XY	67	54	(81)	13	+
	XX	3	0	(0)	3	+
	XO	12	7	(58)	5	+
XY blastocysts						
euploid (N = 3)	XY	52	52	(100)		
mosaic (N = 2)	XY	46	39	(85)	7	+
	XX	1	1	(100)		
	XO,YO	2	2	(100)		
XX cleavage stage						
euploid (N = 2)	XX	11	8	(73)	3	+
mosaic (N = 3)	XX	15	10	(67)	5	2+ and 3-
	XO	1	1	(100)		
	XXXX	1		(0)	1	-
XX morulas						
euploid (N = 4)	XX	30	20	(67)	10	4+ and 6
mosaic (N = 9)	XX	59	41	(69)	18	7+ and 11-
	XO	11	9	(82)	2	+
	XXX	5	3	(60)	2	-
XX blastocysts						
euploid (N = 4)	XX	110	110	(100)		
mosaic (N = 2)	XX	34	32	(88)	2	+
	XO	1	1	(100)		
	XXXX	1		(0)	1	-

Data include only cells with an analysable X/Y, 15 and *XIST*. [a]An ″Expected" pattern is an *XIST* signal for every n1 X chromosome.

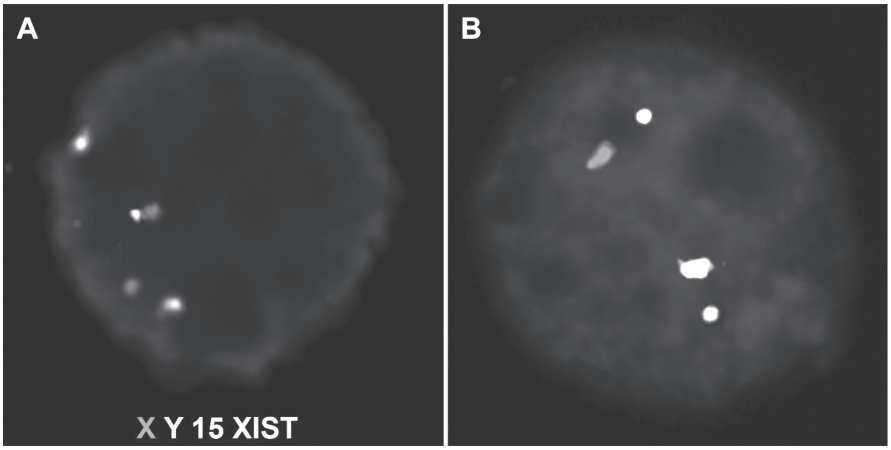


Figure S1. Single cell RNA/DNA FISH analysis of human embryos. (**A**) Diploid blastomere from a female 8-cell embryo displaying two X centromeres (red), two chromosome 15 centromeres (aqua), and an *XIST* pinpoint (green). (**B**) Diploid male blastomere from a 12-cell male embryo with an X chromosome (red), a Y chromosome (yellow) and two chromosomes 15 (aqua). *XIST* RNA was absent (see page 180 for colour figure).

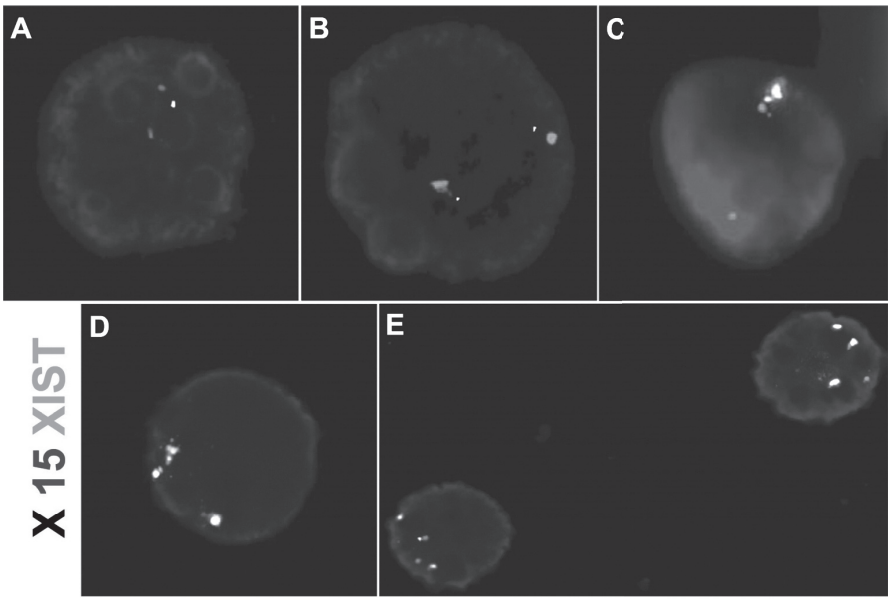


Figure S2. Examples of different patterns of *XIST* RNA signals in female embryos. (**A**) Single pinpoint of *XIST* (green) near the X centromere (red) at the 8-cell stage. (**B**) Two pinpoint signals of *XIST* at morula stage. (**C**) Single cloud signal in blastocyst. (**D**) double cloud signals of *XIST* in late morula embryo. (**E**) Two cells from a morula, one with a pinpoint *XIST* signal and the other with an intermediate cloud of *XIST* RNA (see page 181 for colour figure).

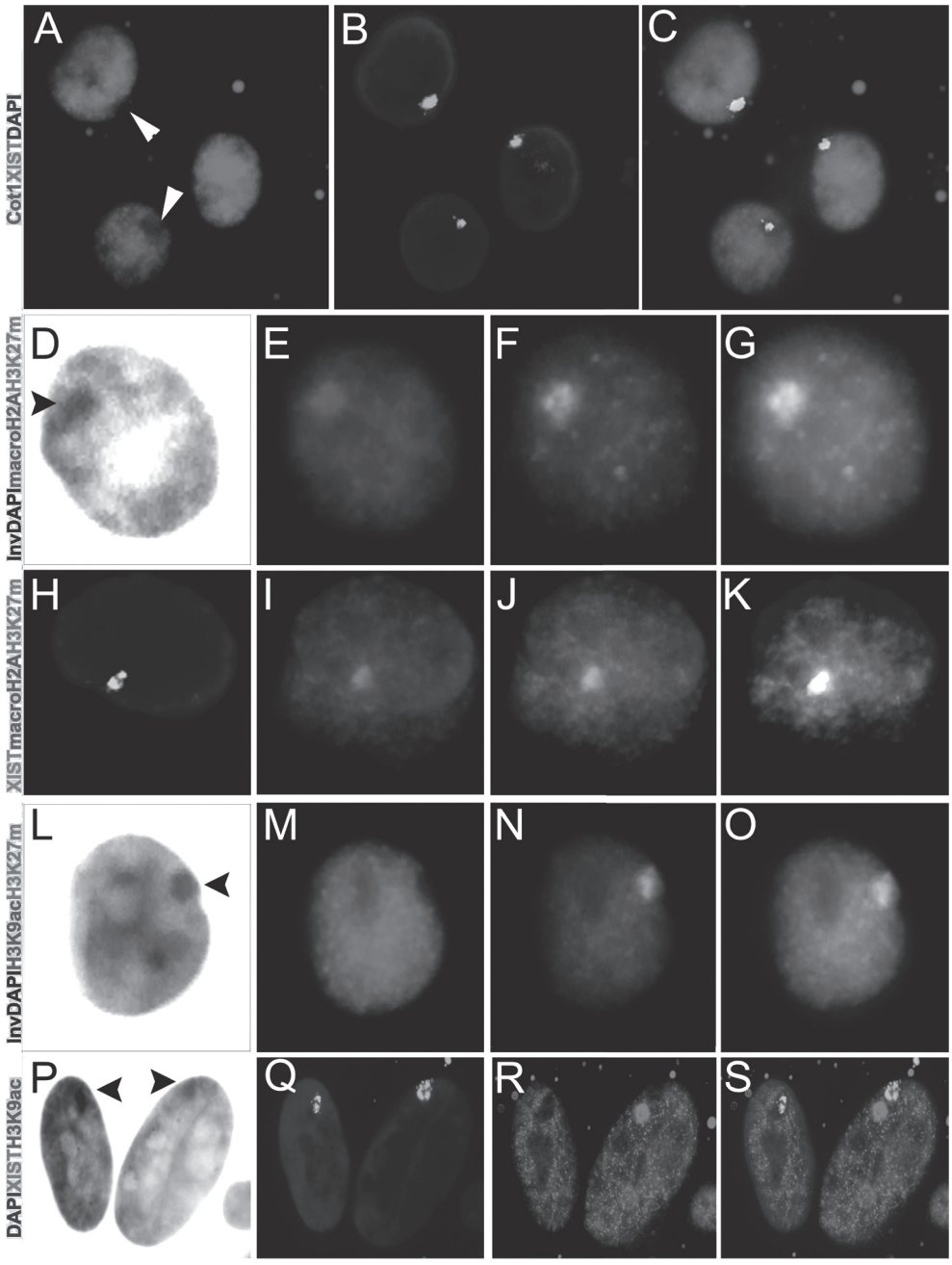
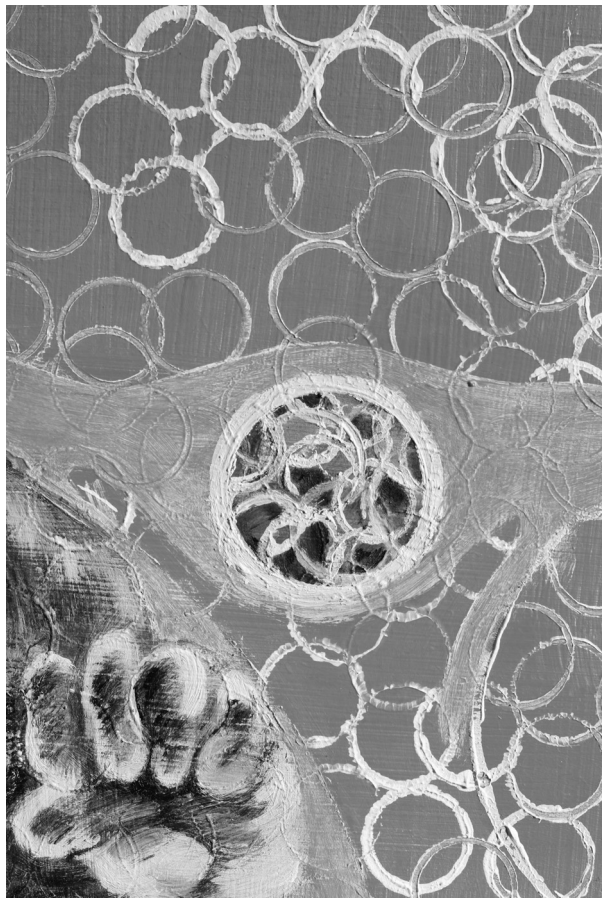

A
B
C
Cot1XISTDAPI
D
E
F
G
InvDAPImacroH2AH3K27m
H
I
J
K
XISTmacroH2AH3K27m
L
M
N
O
InvDAPIH3K9acH3K27m
P
Q
R
S
DAPIXISTH3K9ac

Figure S3. Epigenetic changes on the inactive X chromosome in human cumulus cells (**A-G, L-O**) and female amniocytes (**H-K, P-S**). RNA FISH and immunocytochemistry of human female cumulus cells with probes and antibodies commonly used to characterize the inactive X chromosome. *Cot1* RNA FISH staining of cumulus cells (red in A) shows an excluded area (indicated with arrowheads), that overlaps with *XIST* RNA staining (green in B) as shown in the overlay in (C). The third nucleus is not in focus and the *Cot1* depleted region can thus not be seen. Inverted DAPI staining reveals the position of the inactive X/Barr body (arrowhead in D) in cumulus cells that overlaps with macroH2A staining (red in E) and H3K27Me3 (green in F), merged in G. (**H-K**) In female amniocytes, *XIST* RNA (H) shows a complete overlap with the accumulated nuclear domains of macroH2A (red in I) and H3K27me3 (green in J) as shown in the overlay (merged in K). (L) Cumulus cell with Barr body that is indicated with an arrowhead in the inverted DAPI image. H3K9 acetylation staining shows exclusion of the Barr body area (red in M) and H3K27Me3 gives a strong localized signal staining (green in N) that overlaps exactly with the exclusion of H3K9ac and the position of the Barr body (merged in O). In female amniocytes the Barr body (arrowheads in P) overlaps the *XIST* cloud (green signal in Q) and at this area H3K9ac is depleted (red in R) as shown in the overlay (merged in S) (see page 184 for colour figure).

CHAPTER 4.2



XCI in pre-implantation mouse and human embryos: First there is remodelling…

I.M. van den Berg, R.J. Galjaard, J.S.E. Laven, J.H. van Doorninck

Published in *Hum Genet. 2011 Aug;130(2):203-15*

Abstract

Female eutherians silence one of their X chromosomes to accomplish an equal dose of X-linked gene expression compared with males. The mouse is the most widely used animal model in XCI research and has proven to be of great significance for understanding the complex mechanism of X-linked dosage compensation. Although the basic principles of XCI are similar in mouse and humans, differences exist in the timing of XCI initiation, the genetic elements involved in XCI regulation and the form of XCI in specific tissues. Therefore, the mouse has its limitations as a model to understand early human XCI and analysis of human tissues is required. In this review, we describe these differences with respect to initiation of XCI in human and mouse pre-implantation embryos, the extra-embryonic tissues and the *in vitro* model of the epiblast: the embryonic stem cells.

Introduction

This year, we celebrate the 50th anniversary of Mary Lyon's first publication on X chromosome inactivation. Her first study hypothesized about the basic principle of X chromosome inactivation (XCI) that would result in dosage compensation of X-linked gene expression between male and female mice. In 1991, Brown et al. [1] described a gene that was exclusively expressed from the inactive X, the Xi specific transcript (*Xist*), and it was proposed to be involved in the process of XCI (reviewed in [2]). XCI has become a research field of its own, and many genes have been discovered to be involved in the process of sex-linked dosage compensation. The mechanism of XCI comprises different aspects of molecular biology, cell biology and epigenetics, and it combines many fields of not only basic research, but also translational science. Although the general outcome of the X-inactivation process is similar in somatic cells of mice and men, resulting in dosage compensation of X-linked genes, there are important differences in the genes that take part in the initiation process of XCI, the extent of gene inactivation and how XCI occurs in different tissues. This review focuses on the similarities and the differences between mouse and human tissues with respect to the timing of XCI initiation at the earliest stages of development, namely, (1) the pre-implantation stage, (2) in extra-embryonic tissues and (3) in ES cells.

Brief introduction on some key players of human and mouse XCI

Excellent reviews exist that describe in detail the regulatory elements that are involved in the X inactivation process [3-7]. Most of these data are derived from mouse ES cell differentiation experiments and mutant mouse models. We briefly mention some of the genes here to describe their basic function and describe other genes in the ES cell section. Xist is the most important gene in XCI that codes for *Xist*, a non-coding RNA transcript which wraps itself around the future inactive X chromosome [8,9]. Human XIST shows sequence similarity but has large sequence divergence in the flanking regulatory regions [10,11]. This may have consequences for the initiation and maintenance of XCI. The *Tsix* transcript is a key regulator of Xist in mouse tissues [12,13]. It is expressed in opposite direction of Xist and thereby represses *Xist* expression. The balance between *Xist* and *Tsix* expression ensures that only one X chromosome is inactivated in females and none in males. The human TSIX region is not well conserved, the transcript is truncated and does not overlap the XIST promoter region and lacks other regulatory elements, such as DXPAS34, that are essential for murine *Tsix* function [10,14-16]. Other murine genes that play a role in the XCI process in mouse ES cells such as Rnf12, Jpx, Yy1, CTCF and Eed

[17-21] are conserved in humans although differences exist between mice and humans in the flanking regions of some of the genes [10,11].

XCI during pre-implantation development, the first step

XCI kinetics in mouse embryos

The first initiation of XCI starts at the 2-cell stage of pre-implantation development. Mouse cleavage stage embryos have an imprinted form of XCI in which the inactivated chromosome is always the paternal X chromosome [22,23]. The second step comprises the reactivation of the paternal X in the inner cell mass (ICM) and the maintenance of imprinted XCI in the trophectoderm (TE) and primitive endoderm (PrE) at day 4.5 [24-27]. The third step is the initiation of random XCI in cells of the epiblast between days 5.5-6.3 [28,29], which is recapitulated in *in vitro* ES cell differentiation experiments [3].

Most of our knowledge about XCI in pre-implantation embryos comes from the analysis of mouse embryos (reviewed in [30]). Imprinted XCI is likely caused by opposite marks on the parental X chromosomes in oocytes and spermatozoa [31-34]. Both maternal and paternal imprints ensure the inactive status of Xp and the active status of Xm. It has been hypothesized that the paternal X chromosome enters the oocyte in a pre-inactivated state as a result from the chromatin remodelling and silencing process during spermatogenesis called meiotic sex chromosome inactivation (MSCI) [35]. However, several studies have shown that the paternal X is transcriptionally active at the 2-cell stage [27,36-38]. In addition, in a mouse model in which the murine Xist gene is translocated on an autosome, which is not subjected to MSCI or meiotic silencing of unsynapsed chromosomes (MSUC), *Xist* is still able to induce silencing of neighbouring genes and epigenetic remodelling of the ectopically *Xist* coated region [39]. Thus, it could be that another feature is responsible for paternal X inactivation, for example the presence of paternally specific histones or protamines. Furthermore, as will be discussed below, recent data suggest that initial silencing of X-linked genes may be *Xist* independent.

In a 2-cell stage mouse embryo, only a single pinpoint area of *Xist* RNA is present, but this pinpoint gradually expands to a full cloud at the 4-8 cell stage. At the 4-cell stage, the first signs of transcriptional repression of the Xi appear, including the exclusion of RNA polymerase II and the absence of transcription of Cot1 repetitive elements [27,35]. From the 8-cell stage onwards, epigenetic marks appear such as hypoacetylation of H3K9 and H3K4 hypomethylation [27]. Morula and blastocyst embryos show Eed/Ezh2 association on Xi [27,40], the incorporation of the histone variant macroH2A and the accumulation of H3K27 trimethylation, although

individual embryos are variable in the onset of these marks [25,27,41]. This variability in chromatin modifications might be the consequence of the different levels of *Xist* RNA in the individual blastomeres [42] and may be related to the fate of each cell in the eight cell embryo. As the first cell differentiation takes place at the compaction stage, with the inner cells being predominantly future ICM cells and the outer cells future TE (reviewed in [43,44]), it is possible that this process of cell allocation and specification causes the reactivation of the paternal X in the ICM while the inactive state of Xp is maintained in the TE.

Although the female mouse pre-implantation embryo displays almost all characteristics of XCI, some of the final features, such as DNA methylation of *Xist* on the Xa and Barr body formation, are not established [35]. The lack of final modifications indicates that XCI during pre-implantation development is not as complete as in somatic cells, thereby allowing the reactivation of the Xi in the ICM.

XCI kinetics in human embryos

Studies in human pre-implantation embryos have been hampered by difficulties in obtaining permission for research by ethical committees and in recruiting patients willing to donate their surplus embryos. Another difficulty is that surplus embryos can only be donated after the clinical phase of embryo transfer has been performed, and the remaining good quality embryos are cryopreserved for future clinical use. Embryos of good quality at the earliest cleavage stages are thus even more difficult to obtain. Therefore, very few studies have used human embryos to examine dosage compensation by X-inactivation [45-48].

Human embryos divide somewhat slower than mouse embryos; they consist of 8 cells at day 3 and become morulas at day 4 and blastocysts at day 5, while mouse embryos develop 1–2 days faster. In agreement with the slower development is the later activation of the human embryonic genome at the 4-8 cell stage, while mice activate their genome at the 1–2-cell stage [49,50]. This difference between mice and men is also reflected by the observation that human 8-cell stage embryos have a pinpoint expression of *XIST*, while mouse 8-cell stage embryos have already formed a full cloud of *Xist* expression by that time [25,27,35-37,47]. Initial experiments with RT-PCR analysis showed that both female and male embryos expressed *XIST* [45,46], which at the time led to the conclusion that *XIST* is not functional at these stages in human development. Subsequent experiments challenged these conclusions as single cell analysis of human embryos showed single *XIST* clouds defined as confined areas of *XIST* transcript accumulations, in blastocysts [47]. However, often, loose *XIST* transcripts throughout the nucleus are found which are not observed in human somatic cells ([47] and unpublished observations). Unfortunately, it is not possible to identify the parental origin of the

XIST cloud based on single nucleotide polymorphisms in XIST as both the *XIST* cloud mRNA as well as the loose *XIST* transcripts will be amplified with an RT-PCR (unpublished observations). Importantly, many hallmarks indicative of XCI, such as accumulation of H3K27me3 and macroH2a, depletion of H3K9ac, and the mono-allelic silencing of a gene adjacent to XIST have been observed in single cell analysis of human female embryos [47], demonstrating that the process of XCI is initiated. A recent paper on XCI in rabbit and human embryos reported the presence of two *XIST* clouds in part of the human embryonic cells [48]. We suspect that high XIST probe concentrations detect local accumulations of *XIST* transcripts that are not indicative of an inactivation process [48]. It is clear that XCI in human embryos basically follows the same cascade of events observed in mouse embryos, although the timing differs (Figure 1). Whether human embryos also show imprinted Xi at the pre-implantation stage is not yet known, the predominantly single *XIST* pinpoints in human embryos may suggest that an imprinted mechanism of XCI takes place in human embryos.

Does Xist/XIST coating induces dosage compensation?

Whether the typical hallmarks of XCI lead to actual gene silencing and thus dosage compensation of X-linked coding genes is not yet known. As introduced above, initial silencing of X-linked genes during the first cleavage stages may occur independent of *Xist* expression and coating: a recent study shows that a majority of the X-linked genes have virtually wild-type expression rates in an Xist mutant background as no differences were found in the mono-allelic and bi-allelic expression rates [36]. Interesting exceptions were the genes Rnf12 and Atrx. These genes were affected by the Xist deficiency and since they both play a role in the XCI process this may suggest the presence of feedback mechanisms. Rnf12 has recently been described as an activator of Xist in mouse ES cells [19,51] and Atrx is essential for imprinted XCI in the murine placenta [52,53]. These data suggest that initial silencing of certain regions of the X chromosome is *Xist* independent. Whether *Xist* independent silencing is in fact due to remaining marks of MSCI or protamines [27,35] is not yet known. This form of silencing may be reminiscent of a more ancient form of XCI such as found in marsupials, which is also *Xist* independent [54]. At later stages *Xist* is required to stabilize the imprinted XCI during further embryonic development [36]. It has been postulated that the coating of *Xist* and the modified chromatin configuration creates a territory containing repetitive sequences into which genes can be recruited to be silenced [55,56]. In embryos as well as in ES cells, the actual silencing of X-linked genes and thus dosage compensation does not immediately follow the gradual coating by *Xist* [25,35-37,57]. This indicates that the *Xist* expression and cloud formation alone is not sufficient for silencing [37]. Instead, a gradual conversion of bi-allelic to mono-allelic

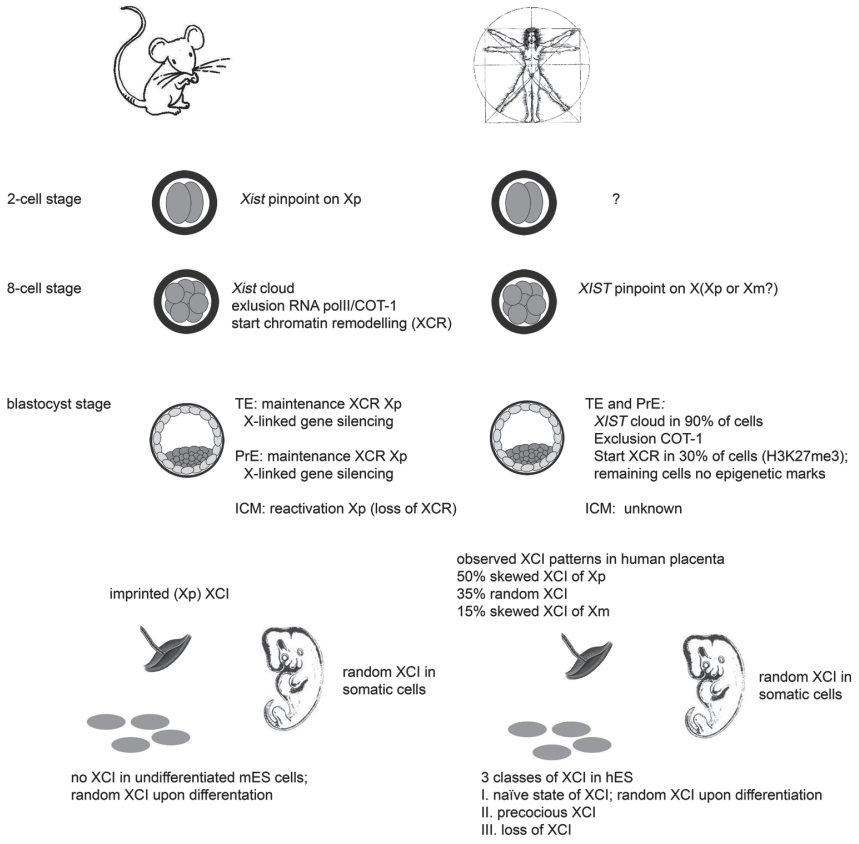


Figure 1. Schematic overview of the three different steps of XCI in mouse and man.

Mouse: In the mouse 2-cell stage embryo, imprinted XCI begins with pinpoint *Xist* expression from the paternal X. At the 8-cell stage the Xp chromosome is remodeled (XCR, see text for details) with *COT-1* exclusion and epigenetic marks; this remodeled X chromosome generally becomes inactivated (XCI) at the blastocyst stage. Mouse blastocyst ICM cells reactivate the paternal X while the TE and PrE retain the imprinted form of XCI (see text for more detailed description). The imprinted form of XCI is maintained in the placenta while the epiblast converts to a random XCI mechanism. **Human**: No data are available for single human 2-cell stage embryos regarding the level and location of *XIST* expression. At the 8-cell stage most cells have a single pinpoint of *XIST* expression but whether this is an imprinted XCI is not yet known. Human blastocysts have a full cloud of *XIST, COT1* exclusion, epigenetic marks and mono-allelic expression of a gene adjacent to XIST in a portion of the cells indicative of XCR and the initiation of XCI [47]. Data on XCI in human placenta point towards a preferential silencing of the paternal allele, although random XCI patterns are often observed. The model hES cell lines to study random XCI are not as good in humans as they are in mice: undifferentiated mouse ES cells have two active X chromosomes and upon differentiation one X is randomly silenced. However, undifferentiated human ES cells are extremely variable in *XIST* expression and so far three classes have been described (see text). ICM = inner cell mass, TE = trophectoderm, PrE = primitive endoderm (see page 185 for colour figure).

expression of X-linked genes is observed. These highly dynamic processes are reflected by the variability in silencing of genes along the X chromosome and the variability in silencing between individual blastomeres of single mouse and human embryos [35-38,47]. It can be postulated that this form of XCI, which starts with the remodelling of the future inactive X by chromatin modifications but does not include Xist DNA methylation may allow for a more dynamic X-linked gene silencing at these early stages.

Effects of in vitro development

Studies in bovine embryos have shown that *in vitro* culture affects X-linked gene expression including the levels of *Xist* RNA [58,59]. Unfortunately, no data exist on the parental origin of the *Xist*-expressing alleles and on whether *in vitro* culture leads to ectopic inactivation in pre-implantation embryos. Additionally, studies in mouse placentas have shown that *in vitro* pre-implantation development can alter the expression pattern of *Xist* and imprinted placental genes [60-63]. Culture conditions such as glucose levels that have no impact on epiblast development, do affect TE differentiation [64]. These findings suggest that the extra-embryonic lineages may be different from the epiblast with respect to epigenetic regulation. This difference indicates that the manipulation of pre-implantation embryos influences imprinted gene expression, and this might be the case for human embryos from assisted reproductive technology (ART) treatments as well. Several studies on children conceived with ART treatments have shown a slightly higher incidence in imprinting disorders, an altered sex ratio shifted towards males, altered birth weight and even skewed XCI [65-69]. A number of reasons may explain the differences between ART outcome and the general population such as the reason for infertility of the parents, the ovarian stimulation procedure, the high amount of multiple pregnancies and the extended time to pregnancy [70]. Although the long-term effects are subtle, influences of the *in vitro* environment must be considered when studying human pre-implantation development.

XCI in extra-embryonic tissues, the second step

Upon differentiation into blastocysts, the inactivation of the paternal X is maintained in the mouse trophectoderm and will remain silenced all throughout placenta development. Overexpression of X-linked genes due to a lack of dosage compensation is lethal, primarily due to placental defects [71]. Whether a mouse embryo can survive with an extra X chromosome depends on the parental origin of the X chromosomes. Having two maternal X chromosomes is lethal (XmXmY) whereas a single X or two paternal X chromosomes can be tolerated and corrected [72-74]. This distinction suggests that the paternal imprint is much easier to reverse

than a maternal imprint. In humans, the difference in embryo lethality caused by the parental origin of the X chromosome aberration is less pronounced. Whether a missing or extra chromosomes are paternally or maternally derived does not seem to be of great influence as both XmXmY and XmXpY Klinefelter syndrome genotypes as well as triple X women (XmXmXp or XmXpXp) are viable. Also in Turner patients (either XpO or XmO) the parental origin of the X chromosomes does not seem to influence the pregnancy outcome [75]. However, up to 90% of the human embryos with sex chromosome aneuploidies die *in utero* because humans have many pseudoautosomal and other escape genes on the X chromosome that are normally not silenced and are thus required at double dosage [76].These data suggest that in human placental development, the parental origin is of less importance in dosage compensation.

Maintenance of imprinting in mice

How the mouse paternal X retains the inactive mark is still unclear, but knockout experiments have identified genes that are essential for maintenance of imprinted XCI in the mouse placenta, namely Eed and Tsix [12,77-80]. *Tsix*, the antisense transcript of *Xist*, represses *Xist* expression on the future active X. *Tsix* thereby ensures that only one X is inactivated in random XCI and protects the maternal X from inactivation in the placenta. The parental origin of the mutated Tsix allele therefore has a strong effect on future mouse placenta development. While the inheritance of a maternal Tsix deletion results in embryonic lethality during post implantation development around d9.5, inheritance of a paternal Tsix mutation has no effect in female embryos [13,81]. In humans, TSIX does not seem to play a role in the process of XCI, as the sequence is truncated compared with its murine counterpart and is, unexpectedly, transcribed from the same allele as *XIST* [15,82]. Thus, it is not likely that human XCI is regulated by *TSIX* expression, neither in the embryo nor in the extra-embryonic lineages.

The polycomb protein EED has been of interest, as it is required to keep the paternal X inactive in the extra-embryonic tissue [79]. Other signals that may be important for the maintenance of the imprinted state in trophoblast cells could be present in the blastocoel fluid, which has direct contact with both the trophoblast as well as the primitive endoderm cells, the two cell types that contain the imprinted XCI mark. A study on imprinted XCI started out using differentiation of mouse ES cell towards the extra-embryonic lineages and found that imprinted XCI was not initiated this *in vitro* system. Subsequent cloning of the nucleus of this ES cell line into an oocyte resulted in aberrant random XCI in the trophectoderm of the resulting blastocyst [83]. This study indicates that ICM cells lose the memory of the X imprint during the reactivation process and that TE cells can only maintain and actively secure the inactive Xp when they directly originate from the pre-implantation embryo.

Do humans prefer to inactivate the paternal X chromosome during placenta development?

Many studies have analysed whether XCI is imprinted in human extra-embryonic tissues similar to mouse trophectoderm, but the results have been contradictory. Preferential imprinted XCI such as is found in mouse embryos describes a situation in which part of the cells shows XCI and those cells always inactivate the paternal X, whereas other cells do not show signs of XCI. On the other hand, a completely imprinted pattern describes the situation as found in the mouse trophectoderm in which all cells show paternal XCI. Skewing on the other hand is defined as a bias of XCI towards the paternal or maternal allele, usually of 70–75% instead of the normal 50:50 ratio. About half of the human placental tissues and isolated trophoblast and cytotrophoblast cells showed skewing towards the paternal X chromosome, but the remainder of the samples showed no skewing or skewing towards the maternal allele [84-93]. No consistency exists between studies that investigated the same tissue or gene, and it is difficult to clarify the discrepancies, as studies with different outcomes used essentially the same methodology.

Two studies investigated XCI after *in vitro* differentiation of trophectoderm from ES cells. In one study using human ES cells, it was suggested that imprinted XCI can be recapitulated upon differentiation towards the extra-embryonic lineages [94]. However, as the parental origin of the X chromosome was not determined, imprinted XCI of the paternal X in human TE cells is not yet conclusive. In addition, whether ES cell lines are an appropriate model to study *in vitro* trophectoderm differentiation is questionable based on the observations from the recent publication discussed above: the initiation of imprinted XCI was not possible *in vitro* but required *in vivo* differentiation [83].

What is the underlying mechanism to explain the different observations from placental studies? If preferential silencing of the paternal allele occurs at the human blastocyst stage, perhaps implied by the prevalence of single pinpoints at the cleavage stage [47], it would explain the skewing of XCI towards the paternal allele that has mostly been found in human placental material [84,85,89,95,96]. It could be hypothesized that during human placental development an initial preferential silencing of the paternal allele that is not as rigid and stable as in the mouse, allows for the reactivation of the paternal X followed by random XCI. Indeed, reactivation of the Xi has been observed in human placental cells of spontaneous abortion material [96], and in somatic cells containing a transgene of Xist, XCI can also be reversed *in vitro* [97]. Such mechanisms of reversible XCI could explain the variable results of independent studies on human extra-embryonic tissues. A slightly different explanation can be made from the observation that only a subset of the cells in human blastocysts carries the

visible epigenetic mark of XCI [47]. If these cells start with a paternal XCI, and the other cells convert to random XCI at a later time point, it will result in a mixture of skewed XCI and random XCI within the studied placentas. It is clear that the final choice of XCI in the placenta occurs fairly early, as large patches of placental villi demonstrate the same choice of XCI [87,93].

If, in contrast, there is no preferential imprinted XCI but random XCI in human blastocysts, it is not easy to explain the predominance of the preferential silencing of the paternal X found in human placentas. Perhaps, the skewed patterns of XCI found at later stages of placental development could be attributable to a selective advantage, only favourable in the placenta, of paternally silenced X-linked genes [71]. Alternatively, the single gene analysis used in most placental X-inactivation studies may have inadvertently created a bias towards imprinted Xp results. If this is true the actual random X-inactivation pattern may be better represented by the analysis of more genes along the X chromosome [88].

Initiation of random XCI in the epiblast and embryonic stem cells, the third step

The third step of XCI takes place in the ICM cells of the blastocyst that will become epiblast cells. It follows the reactivation of the paternal X chromosome and results in random XCI in the epiblast [25,27]. Mouse embryos at day 3.5 have already formed an ICM, but these cells still contain an inactive X as shown by *Xist* clouds. These cells show high levels of pluripotency proteins such as OCT4, SOX2 and NANOG. In the mature blastocyst at d4.5, only the ICM cells that are NANOG positive will reactivate the paternal X chromosome. The other ICM cells that are expressing GATA4/GATA6 will maintain the silenced paternal X and differentiate into PrE. Thus, both the TE and PrE maintain the same imprinted form of XCI [25,26]. At d5.5-d6, epiblast cells will initiate random XCI [25,29], and this process can be recapitulated in ES cells. Therefore, one of the main characteristics of an undifferentiated state of female ES cell lines is the active state of both X chromosomes [3,98].

Mouse models of random XCI initiation

Because ES cell lines were first derived from mice, initiation of random XCI has been extensively studied in this *in vitro* model. Upon differentiation, both X chromosomes begin to express *Xist* at a low level, but only one X chromosome will upregulate *Xist* expression. At day 7 of ES cell differentiation, 80% of the cells will have formed a single *Xist* cloud [99]. The random choice of the X chromosome that becomes inactivated has been a subject of study ever since Mary Lyon's first paper, and different theories have been postulated as to how this random inactivation is regulated [7]. Although studies using mouse ES cells have provided key insights

in the process, important questions still remain, and novel genes are continuously being discovered (reviewed in [5,100-102]).

Xist regulatory sequences contain binding sites for NANOG, OCT4 and SOX2 [103]. Furthermore, downregulation of *Nanog* expression results in upregulation of *Xist*, independent of *Tsix* [104]. Several candidate genes for Xist regulation have been described in mouse ES cells, for example Rnf12 and Jpx. Both are X-linked genes and located distal from Xist. Rnf12 is implied to function as an activator of Xist in both imprinted and random XCI although observations and conclusions do not completely agree [19,51,105]. Double mutant Rnf12 female ES cells do not upregulate *Xist* expression upon differentiation and only sporadic XCI occurs. However, in heterozygous mutant female ES cells which have the same protein level as male ES cells, XCI occurs in a delayed fashion upon differentiation indicating that there are more Xist-activating factors involved that either operate at different time points or in different doses. Ectopic expression of additional copies of human RNF12 in mES cells has the same effect indicating that the function of RNF12 is likely conserved [19,51] however, experiments in hES cells are needed to support this.

The Jpx gene is a likely candidate co-activator of Xist. Deletion of Jpx has no effect in male ES cells, heterozygous female mutant cells however, showed impaired EB formation after differentiation and whereas wild-type female ES cells contained a single cloud of Xist in 75% of the cells at d8, female heterozygous ΔJpx mutant cells only had a single cloud of *Xist* in 6.35% of the cells. The effect of ΔJpx/+ was rescued by placing it into a Tsix mutant background indicating the opposite action of both genes [17]. Although conserved, human JPX differs from murine Jpx as the distance between the JPX CpG island and the first exon is 9 times larger and no sequence conservation of this region has been found [11]. More research is needed to find out if human XCI is under the same genetic regulation as mouse XCI in ES cell differentiation.

As has been mentioned before, TSIX structure and function are not conserved in humans [10,15,16], therefore, other factors are expected to negatively regulate *XIST* expression from the future active X. Candidate genes for this function are the members of the polycomb repressive complex 2 (PRC2) namely Eed, EZH2 and Suz12 as they are responsible for the recruitment of repressive histone modifications such as H3K9 and H3K27 methylation [18]. Especially Eed has been of interest as it is needed to keep the paternal X inactive in the extra-embryonic tissue [77,79] and lack of Eed in the absence of *Tsix* results in elevated levels of *Xist* in differentiated male ES cells [106]. This resulted in partial XCI and retarded outgrowth of embryonic bodies (EB). Even at the undifferentiated state higher expression levels in the double mutant ES cells were found. However, this aberrant expression of Xist was rescued by ectopic expression of Eed. Thus, either Eed or Tsix is sufficient to repress Xist during random

XCI. As humans lack a functional TSIX, EED might be the negative regulator of Xist in human cells. Indeed, PRC2 expression has been found in human pre-implantation embryos at the 4-cell stage [107]. However, functional data on human PRC2 proteins in human pre-implantation embryos and hES cells are lacking.

The specific epigenetic hallmarks that are found on the inactive X in somatic cells [108] which are used to prove the initiation of XCI in pre-implantation embryos, are thought to silence the transcription machinery by recruiting specific silencing factors and forming a heterochromatic region. Candidate genes for recruiting modifying complexes or incorporating histone modifications on the Xi are the genes CTCF, Yy1 and members of the polycomb repressive complexes 1 and 2 (PRC1 and PRC2) which are known to function as histone modifiers [109,110]. CTCF and Yy1 affect *Xist* expression [20] by changing the epigenetic status of the promoter. The human XIST promoter contains a much smaller CTCF binding site compared to the mouse Xist promoter. It is however still functional as a single nucleotide polymorphism (SNP) in this site can cause skewing of XCI [111]. The effect of the polymorphism is moderate as males do not seem to be affected.

The current challenge is to embed all these proteins in a complete network that combines time and space during epiblast and extra-embryonic development.

Why we know little about human ES cells

The regulation of TE and ICM differentiation in human blastocysts has not been well studied. This lack of research is surprising, because many hES cell lines have been characterised without the knowledge of the ground state of pluripotency in human ICM cells. For instance, it is unknown whether human ICM have the same expression profile of pluripotency genes and epigenetic make-up as mouse ICM cells. This lack of knowledge hampers the proper evaluation of the quality of existing human ES cell lines. A reason for this lack of knowledge may be the ethical issues and scarcity of surplus human embryos. More research on human ICM cells would contribute to establishing high quality hES and iPS cell lines both for basic research on XCI kinetics as well as regenerative medicine.

Human models of random XCI initiation

How the process of XCI initiation is regulated in human ES cells is much less understood, and how it is regulated *in vivo* is not possible to investigate. A number of studies have investigated the XCI state in different hES cell lines and it is clear that human ES cells are quite different in several aspects, including XCI, from mouse ES cells [94,98,112-118]. Human ES cell lines that are characterized as being undifferentiated based on morphologic features and the presence

of pluripotency markers show a highly variable pattern of XCI. In general, three patterns of XCI have been observed in hES cell lines: (a) cell lines that showed no signs of XCI but initiate XCI upon differentiation, (b) cell lines in which 20-80% of the cells have an *XIST* cloud or an epigenetic hallmark of XCI such as H3K27me3, macroH2A or H4K20me1, indicative of precocious XCI at d0 of differentiation and (c) cell lines without *XIST* either at d0 or upon differentiation [114]. It has been postulated that the long-term culture and frequent passaging of the cell lines may introduce artefacts, such as loss of *XIST* and expression of the Xi. This may be possible but precocious XCI is also already present in very early passages of some newly established cell lines [112] and has further been observed in human iPS cells [119]. Further, it is possible that human ES cells are derived from ICM cells that have not yet reactivated their Xi and that the observed XCI in the hES cell lines is in fact a remnant from the XCI at the pre-implantation stage. Indeed, some hES cell lines have a non-random precocious XC and reversal to a random pattern of X-linked gene silencing has been observed [112,117]. It has also been proposed that the hES cell lines currently available are actually epiblast stem cells lines and thus one step further differentiated than cells from the ICM [120-122]. Lastly, it has been proposed that specific culture conditions during the derivation process may influence the pluripotency state of human ES cells [123], and these conditions may also have consequences for XCI regulation. Indeed, a recent study showed that human ES cells, when cultured under physiological low O_2-tension, reactivate the Xi and more closely resemble mES cells than cells that were cultured under atmospheric conditions [124]. Still, this finding indicates that the process of XCI is differently regulated in human compared with mouse ES cells. The reason for this difference in sensitivity between human and mouse may originate in the genetic elements that are involved in XCI [10,11]. Importantly, only a few mouse strains allow the derivation of pluripotent ES cell lines [125], indicating that genetic variations such as SNPs and CNVs are of influence on the successful isolation of undifferentiated ES cell lines. As human embryos are far more genetically diverse than most commonly used mouse strains, the derivation of human ES and iPS cell lines may require embryo tailored culture conditions. Recent progression in ES cell derivation using small molecule inhibitors allow the derivation of ES cells from virtual all mouse strains and possibley from humans as well [126].

Discussion

In the past 50 years, much research has been carried out to unravel the phenomenon of mammalian X-linked dosage compensation. Although we now understand the basic principles

of X chromosome inactivation, it is still remarkable that two chromosomes that are genetically the same are epigenetically complete opposites. Monotremes and marsupials have solved the dosage compensation problem by always inactivating the paternal allele. This imprinted form of XCI is also present in the pre-implantation embryo and extra-embryonic tissues of the mouse. Whether human pre-implantation embryos and early placenta also prefer to inactive the paternal X has yet to be determined. Human cleavage stage embryos have 26,5% cells with double staining for *XIST* signals [47] that may be reminiscent of a counting and choice mechanism such as found in differentiating ES cells. However, the predominant single *XIST* pinpoints in 68% of the cells at this stage [47] more resemble the single *Xist* pinpoints in imprinted XCI in mouse embryos. Up till now, the placenta data are variable, but most evidence points towards an initial preference for a paternal Xi [84-93]. As mentioned before, two pathways could explain the observed preferential silencing of the paternal X in human placentas. Either a paternal imprint in the trophectoderm is diluted or reversed with random XCI during further development. However, an argument against an imprinted form of XCI is the equal birth rate of children with an extra maternal or paternal X chromosomes, such as in Klinefelter (XXY) syndrome [127,128] or the relatively healthy status of triple X females (XXX). There are no indications that embryos with extra maternal chromosomes (XmXmXp or XmXmY) have more *in utero* demise than embryos with an extra paternal X chromosome (XmXpXp or XmXpY) [75]. Thus, in contrast to female mice where an extra maternal X is lethal for placenta development, the equal birth-rates of children with an extra Xm or an extra Xp in humans suggests that the parental origin of the sex chromosome aberration is of less importance for embryonic survival. How can these clinical data pointing at random XCI correspond to the molecular data from pre-implantation embryos and placental tissues that more resemble an imprinted XCI mechanism?

Difference between X-linked remodelling and X-linked inactivation?

Although various hallmarks of somatic XCI are found in the mouse pre-implantation embryo, a disturbance of the process, such as a mutation in an XCI essential gene, does not immediately interfere with mouse development at the pre-implantation stage; rather, such a disturbance only affects post-implantation development from d6 onwards leading to death at d7.5-9. In embryos that carry a deletion of the paternal Xist gene or parthenogenetic embryos, which don't have a paternal genome, imprinted XCI is recued by maternal *Xist* expression in a subset of the cells. However, these embryos arrest during post-implantation most likely due to poor development of the extra-embryonic tissues [81,129,130]. This late death suggests that dosage compensation is not immediately necessary, and that there is a window to establish silencing.

Both mouse embryos as well as ES cell differentiation experiments have shown that hallmarks for X-inactivation such as the presence of clouds of *Xist*, exclusion of RNA pol II and several epigenetic remodelling characteristics does not necessarily indicate that the X chromosome is completely silenced [36-38]. Thus, *Xist* clouds and epigenetic remodelling in pre-implantation embryos may suggest the initiation of a dosage compensation mechanism but do not tell us to what extent the X chromosome is actually silenced at this stage. This epigenetic X chromosome remodelling (XCR) modifies the X chromosome into a repressive state that allows future XCI. XCR only creates the possibility for individual genes recruited to be silenced [55,56] rather than that it automatically leads to complete dosage compensation. It is thus possible that only in the extra-embryonic trophectoderm the chromosome wide XCI with definite silencing is completed.

Necessity of dosage compensation

It may be that early remodelling of the Xp in mouse embryos, 3 days before implantation, is not essential for the early embryo itself but prepares for immediate dosage compensation in the extra-embryonic tissues from the moment of implantation onwards. If dosage compensation is only necessary from this point onwards, the late lethality of mouse embryos that carry XCI knockout genes can be better understood. Thus, also in human embryos, the initiation of XCI in blastocysts may not indicate actual inactivation and a widespread dosage compensation mechanism but only remodelling of the X chromosome. So far, only one gene shows mono-allelic expression in human blastocysts [47]. A possible effect of the XCI process itself in human pre-implantation embryos may be the observed retarded growth of female blastocysts [65, 131], however, it is not known whether this is linked to X-inactivation, imprinted genes on the X or an effect of the Y chromosome.

The necessity for immediate dosage compensation in the placenta could be more acute in mice than in humans. Two reasons may account for the divergence between mouse and men: first, the mouse has a large amount of X-linked placental genes that are essential for proper placental development [71]. Second, as human embryos develop along a different, slower time line than mouse embryos, the lack of dosage compensation may be tolerated for a longer period. However, actual data on this are not known and many embryos may be lost as most studies on human birth rates do not include the possible death of an embryo before a recognized pregnancy [132]. Whether failures in X-inactivation play a role in these first trimester deaths is not known.

As science progresses to unravel the mechanism of mammalian dosage compensation, it has become clear that with the in-depth knowledge gained of this process in the mouse,

the challenge remains to extrapolate these findings to the human. The differences in pre-implantation and post-implantation development, in the genetic elements of the XIC and the heterogeneity among humans require that human XCI should be studied from its own point of view. With this review on the three steps of XCI initiation, we hope to provide novel insight regarding the differences that exist between humans and mice.

References

1. Brown, C.J., et al., *A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome.* Nature, 1991. **349**(6304): p. 38-44.
2. Plath, K., et al., *Xist RNA and the mechanism of X chromosome inactivation.* Annu Rev Genet, 2002. **36**: p. 233-78.
3. Barakat, T.S. and J. Gribnau, *X chromosome inactivation and embryonic stem cells.* Adv Exp Med Biol, 2010. **695**: p. 132-54.
4. Chow, J. and E. Heard, *X inactivation and the complexities of silencing a sex chromosome.* Curr Opin Cell Biol, 2009. **21**(3): p. 359-66.
5. Navarro, P. and P. Avner, *When X-inactivation meets pluripotency: an intimate rendezvous.* FEBS Lett, 2009. **583**(11): p. 1721-7.
6. Thorvaldsen, J.L., R.I. Verona, and M.S. Bartolomei, *X-tra! X-tra! News from the mouse X chromosome.* Dev Biol, 2006. **298**(2): p. 344-53.
7. Starmer, J. and T. Magnuson, *A new model for random X chromosome inactivation.* Development, 2009. **136**(1): p. 1-10.
8. Clemson, C.M., et al., *XIST RNA paints the inactive X chromosome at interphase: evidence for a novel RNA involved in nuclear/chromosome structure.* J Cell Biol, 1996. **132**(3): p. 259-75.
9. Penny, G.D., et al., *Requirement for Xist in X chromosome inactivation.* Nature, 1996. **379**(6561): p. 131-7.
10. Chureau, C., et al., *Comparative sequence analysis of the X-inactivation center region in mouse, human, and bovine.* Genome Res, 2002. **12**(6): p. 894-908.
11. Chang, S.C. and C.J. Brown, *Identification of regulatory elements flanking human XIST reveals species differences.* BMC Mol Biol, 2010. **11**: p. 20.
12. Lee, J.T., L.S. Davidow, and D. Warshawsky, *Tsix, a gene antisense to Xist at the X-inactivation centre.* Nat Genet, 1999. **21**(4): p. 400-4.
13. Sado, T., et al., *Regulation of imprinted X-chromosome inactivation in mice by Tsix.* Development, 2001. **128**(8): p. 1275-86.
14. Debrand, E., et al., *Functional analysis of the DXPas34 locus, a 3' regulator of Xist expression.* Mol Cell Biol, 1999. **19**(12): p. 8513-25.
15. Migeon, B.R., et al., *Species differences in TSIX/Tsix reveal the roles of these genes in X-chromosome inactivation.* Am J Hum Genet, 2002. **71**(2): p. 286-93.
16. Cohen, D.E., et al., *The DXPas34 repeat regulates random and imprinted X inactivation.* Dev Cell, 2007. **12**(1): p. 57-71.
17. Tian, D., S. Sun, and J.T. Lee, *The long noncoding RNA, Jpx, is a molecular switch for X chromosome inactivation.* Cell, 2010. **143**(3): p. 390-403.
18. Silva, J., et al., *Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes.* Dev Cell, 2003. **4**(4): p. 481-95.
19. Jonkers, I., et al., *RNF12 is an X-Encoded dose-dependent activator of X chromos ome inactivation.* Cell, 2009. **139**(5): p. 999-1011.
20. Donohoe, M.E., et al., *Identification of a Ctcf cofactor, Yy1, for the X chromosome binary switch.* Mol Cell, 2007. **25**(1): p. 43-56.
21. Chao, W., et al., *CTCF, a candidate trans-acting factor for X-inactivation choice.* Science, 2002. **295**(5553): p. 345-7.

22. Kay, G.F., et al., *Expression of Xist during mouse development suggests a role in the initiation of X chromosome inactivation.* Cell, 1993. **72**(2): p. 171-82.
23. Zuccotti, M., et al., *Mouse Xist expression begins at zygotic genome activation and is timed by a zygotic clock.* Mol Reprod Dev, 2002. **61**(1): p. 14-20.
24. Sugawara, O., N. Takagi, and M. Sasaki, *Correlation between X-chromosome inactivation and cell differentiation in female preimplantation mouse embryos.* Cytogenet Cell Genet, 1985. **39**(3): p. 210-9.
25. Mak, W., et al., *Reactivation of the paternal X chromosome in early mouse embryos.* Science, 2004. **303**(5658): p. 666-9.
26. Silva, J., et al., *Nanog is the gateway to the pluripotent ground state.* Cell, 2009. **138**(4): p. 722-37.
27. Okamoto, I., et al., *Epigenetic dynamics of imprinted X inactivation during early mouse development.* Science, 2004. **303**(5658): p. 644-9.
28. Rastan, S., *Timing of X-chromosome inactivation in postimplantation mouse embryos.* J Embryol Exp Morphol, 1982. **71**: p. 11-24.
29. Takagi, N., O. Sugawara, and M. Sasaki, *Regional and temporal changes in the pattern of X-chromosome replication during the early post-implantation development of the female mouse.* Chromosoma, 1982. **85**(2): p. 275-86.
30. Okamoto, I. and E. Heard, *The dynamics of imprinted X inactivation during preimplantation development in mice.* Cytogenet Genome Res, 2006. **113**(1-4): p. 318-24.
31. Ariel, M., et al., *Gamete-specific methylation correlates with imprinting of the murine Xist gene.* Nat Genet, 1995. **9**(3): p. 312-5.
32. Tada, T., et al., *Imprint switching for non-random X-chromosome inactivation during mouse oocyte growth.* Development, 2000. **127**(14): p. 3101-5.
33. Norris, D.P., et al., *Evidence that random and imprinted Xist expression is controlled by preemptive methylation.* Cell, 1994. **77**(1): p. 41-51.
34. Zuccotti, M. and M. Monk, *Methylation of the mouse Xist gene in sperm and eggs correlates with imprinted Xist expression and paternal X-inactivation.* Nat Genet, 1995. **9**(3): p. 316-20.
35. Huynh, K.D. and J.T. Lee, *Inheritance of a pre-inactivated paternal X chromosome in early mouse embryos.* Nature, 2003. **426**(6968): p. 857-62.
36. Kalantry, S., et al., *Evidence of Xist RNA-independent initiation of mouse imprinted X-chromosome inactivation.* Nature, 2009. **460**(7255): p. 647-51.
37. Namekawa, S.H., et al., *Two-step imprinted X inactivation: repeat versus genic silencing in the mouse.* Mol Cell Biol, 2010. **30**(13): p. 3187-205.
38. Patrat, C., et al., *Dynamic changes in paternal X-chromosome activity during imprinted X-chromosome inactivation in mice.* Proc Natl Acad Sci U S A, 2009. **106**(13): p. 5198-203.
39. Okamoto, I., et al., *Evidence for de novo imprinted X-chromosome inactivation independent of meiotic inactivation in mice.* Nature, 2005. **438**(7066): p. 369-73.
40. Erhardt, S., et al., *Consequences of the depletion of zygotic and embryonic enhancer of zeste 2 during preimplantation mouse development.* Development, 2003. **130**(18): p. 4235-4248.
41. Costanzi, C., et al., *Histone macroH2A1 is concentrated in the inactive X chromosome of female preimplantation mouse embryos.* Development, 2000. **127**(11): p. 2283-9.
42. Hartshorn, C., J.E. Rice, and L.J. Wangh, *Differential pattern of Xist RNA accumulation in single blastomeres isolated from 8-cell stage mouse embryos following laser zona drilling.* Molecular Reproduction and Development, 2003. **64**(1): p. 41-51.

43. Johnson, M.H. and J.M.L. McConnell, *Lineage allocation and cell polarity during mouse embryogenesis.* Seminars in Cell & Developmental Biology, 2004. **15**(5): p. 583-597.
44. Zernicka-Goetz, M., *Patterning of the embryo: the first spatial decisions in the life of a mouse.* Development, 2002. **129**(4): p. 815-29.
45. Daniels, R., et al., *XIST expression in human oocytes and preimplantation embryos.* Am J Hum Genet, 1997. **61**(1): p. 33-9.
46. Ray, P.F., R.M. Winston, and A.H. Handyside, *XIST expression from the maternal X chromosome in human male preimplantation embryos at the blastocyst stage.* Hum Mol Genet, 1997. **6**(8): p. 1323-7.
47. van den Berg, I.M., et al., *X chromosome inactivation is initiated in human preimplantation embryos.* Am J Hum Genet, 2009. **84**(6): p. 771-9.
48. Okamoto, I., et al., *Eutherian mammals use diverse strategies to initiate X-chromosome inactivation during development.* Nature, 2011. **472**(7343): p. 370-4.
49. Schultz, R.M., *Regulation of zygotic gene activation in the mouse.* Bioessays, 1993. **15**(8): p. 531-8.
50. Wells, D., et al., *Expression of genes regulating chromosome segregation, the cell cycle and apoptosis during human preimplantation development.* Hum Reprod, 2005. **20**(5): p. 1339-48.
51. Barakat, T.S., et al., *RNF12 activates Xist and is essential for X chromosome inactivation.* PLoS Genet, 2011. **7**(1): p. e1002001.
52. Garrick, D., et al., *Loss of Atrx affects trophoblast development and the pattern of X-inactivation in extraembryonic tissues.* PLoS Genet, 2006. **2**(4): p. e58.
53. Baumann, C. and R. De La Fuente, *ATRX marks the inactive X chromosome (Xi) in somatic cells and during imprinted X chromosome inactivation in trophoblast stem cells.* Chromosoma, 2009. **118**(2): p. 209-22.
54. Chaumeil, J., et al., *Evolution from XIST-Independent to XIST-Controlled X-Chromosome Inactivation: Epigenetic Modifications in Distantly Related Mammals.* PLoS One, 2011. **6**(4): p. e19040.
55. Chaumeil, J., et al., *A novel role for Xist RNA in the formation of a repressive nuclear compartment into which genes are recruited when silenced.* Genes Dev, 2006. **20**(16): p. 2223-37.
56. Clemson, C.M., et al., *The X chromosome is organized into a gene-rich outer rim and an internal core containing silenced nongenic sequences.* Proc Natl Acad Sci U S A, 2006. **103**(20): p. 7688-93.
57. Lin, H., et al., *Dosage compensation in the mouse balances up-regulation and silencing of X-linked genes.* PLoS Biol, 2007. **5**(12): p. e326.
58. Nino-Soto, M.I., P.K. Basrur, and W.A. King, *Impact of in vitro production techniques on the expression of X-linked genes in bovine (bos taurus) oocytes and pre-attachment embryos.* Mol Reprod Dev, 2007. **74**(2): p. 144-53.
59. Peippo, J., et al., *Sex-chromosome linked gene expression in in-vitro produced bovine embryos.* Mol Hum Reprod, 2002. **8**(10): p. 923-9.
60. Doherty, A.S., et al., *Differential Effects of Culture on Imprinted H19 Expression in the Preimplantation Mouse Embryo.* Biology of Reproduction, 2000. **62**(6): p. 1526-1535.
61. Mann, M.R.W., et al., *Selective loss of imprinting in the placenta following preimplantation development in culture.* Development, 2004. **131**(15): p. 3727-3735.
62. Market-Velker, B.A., A.D. Fernandes, and M.R. Mann, *Side-by-side comparison of five commercial media systems in a mouse model: suboptimal in vitro culture interferes with imprint maintenance.* Biol Reprod, 2010. **83**(6): p. 938-50.
63. Rivera, R.M., et al., *Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development.* Hum Mol Genet, 2008. **17**(1): p. 1-14.

64. Leunda-Casi, A., R. de Hertogh, and S. Pampfer, *Control of trophectoderm differentiation by inner cell mass-derived fibroblast growth factor-4 in mouse blastocysts and corrective effect of FGF-4 on high glucose-induced trophoblast disruption.* Mol Reprod Dev, 2001. **60**(1): p. 38-46.

65. Chang, H.J., et al., *Impact of blastocyst transfer on offspring sex ratio and the monozygotic twinning rate: a systematic review and meta-analysis.* Fertility and Sterility, 2009. **91**(6): p. 2381-2390.

66. Dumoulin, J.C., et al., *Effect of in vitro culture of human embryos on birthweight of newborns.* Human Reproduction, 2010. **25**(3): p. 605-612.

67. Halliday, J., et al., *Beckwith-Wiedemann syndrome and IVF: a case-control study.* Am J Hum Genet, 2004. **75**(3): p. 526-8.

68. Helmerhorst, F.M., et al., *Perinatal outcome of singletons and twins after assisted conception: a systematic review of controlled studies.* BMJ, 2004. **328**(7434): p. 261.

69. King, J.L., et al., *Skewed X inactivation and IVF-conceived infants.* Reprod Biomed Online, 2010. **20**(5): p. 660-3.

70. Owen, C.M. and J.H. Segars, Jr., *Imprinting disorders and assisted reproductive technology.* Semin Reprod Med, 2009. **27**(5): p. 417-28.

71. Hemberger, M., *The role of the X chromosome in mammalian extra embryonic development.* Cytogenet Genome Res, 2002. **99**(1-4): p. 210-7.

72. Goto, Y. and N. Takagi, *Maternally inherited X chromosome is not inactivated in mouse blastocysts due to parental imprinting.* Chromosome Res, 2000. **8**(2): p. 101-9.

73. Matsui, J., Y. Goto, and N. Takagi, *Control of Xist expression for imprinted and random X chromosome inactivation in mice.* Hum Mol Genet, 2001. **10**(13): p. 1393-401.

74. Burgoyne, P.S., O.A. Ojarikre, and J.M. Turner, *Evidence that postnatal growth retardation in XO mice is due to haploinsufficiency for a non-PAR X gene.* Cytogenet Genome Res, 2002. **99**(1-4): p. 252-6.

75. Jacobs, P.A. and T.J. Hassold, *The origin of numerical chromosome abnormalities.* Adv Genet, 1995. **33**: p. 101-33.

76. Carrel, L. and H.F. Willard, *X-inactivation profile reveals extensive variability in X-linked gene expression in females.* Nature, 2005. **434**(7031): p. 400-4.

77. Wang, J., et al., *Imprinted X inactivation maintained by a mouse Polycomb group gene.* Nat Genet, 2001. **28**(4): p. 371-5.

78. Kalantry, S. and T. Magnuson, *The Polycomb group protein EED is dispensable for the initiation of random X-chromosome inactivation.* PLoS Genet, 2006. **2**(5): p. e66.

79. Kalantry, S., et al., *The Polycomb group protein Eed protects the inactive X-chromosome from differentiation-induced reactivation.* Nat Cell Biol, 2006. **8**(2): p. 195-202.

80. Luikenhuis, S., A. Wutz, and R. Jaenisch, *Antisense transcription through the Xist locus mediates Tsix function in embryonic stem cells.* Mol Cell Biol, 2001. **21**(24): p. 8512-20.

81. Lee, J.T., *Disruption of imprinted X inactivation by parent-of-origin effects at Tsix.* Cell, 2000. **103**(1): p. 17-27.

82. Migeon, B.R., et al., *Identification of TSIX, encoding an RNA antisense to human XIST, reveals differences from its murine counterpart: implications for X inactivation.* Am J Hum Genet, 2001. **69**(5): p. 951-60.

83. Murakami, K., et al., *Choice of random rather than imprinted X inactivation in female embryonic stem cell-derived extra-embryonic cells.* Development, 2011. **138**(2): p. 197-202.

84. Goto, T., E. Wright, and M. Monk, *Paternal X-chromosome inactivation in human trophoblastic cells.* Mol Hum Reprod, 1997. **3**(1): p. 77-80.

85. Harrison, K.B., *X-chromosome inactivation in the human cytotrophoblast.* Cytogenet Cell Genet, 1989. **52**(1-2): p. 37-41.

86. Harrison, K.B. and D. Warburton, *Preferential X-chromosome activity in human female placental tissues.* Cytogenet Cell Genet, 1986. **41**(3): p. 163-8.

87. Looijenga, L.H., et al., *Heterogeneous X inactivation in trophoblastic cells of human full-term female placentas.* Am J Hum Genet, 1999. **64**(5): p. 1445-52.

88. Moreira de Mello, J.C., et al., *Random X inactivation and extensive mosaicism in human placenta revealed by analysis of allele-specific gene expression along the X chromosome.* PLoS One, 2010. **5**(6): p. e10947.

89. Ropers, H.H., G. Wolff, and H.W. Hitzeroth, *Preferential X inactivation in human placenta membranes: is the paternal X inactive in early embryonic development of female mammals?* Hum Genet, 1978. **43**(3): p. 265-73.

90. Zeng, S.M. and J. Yankowitz, *X-inactivation patterns in human embryonic and extra-embryonic tissues.* Placenta, 2003. **24**(2-3): p. 270-5.

91. Migeon, B.R., et al., *Incomplete X chromosome dosage compensation in chorionic villi of human placenta.* Proc Natl Acad Sci U S A, 1985. **82**(10): p. 3390-4.

92. Migeon, B.R. and T.T. Do, *In search of nonrandom X inactivation: studies of the placenta from newborns heterozygous for glucose-6-phosphate dehydrogenase.* Basic Life Sci, 1978. **12**: p. 379-91.

93. Willemsen, R., et al., *Timing of the absence of FMR1 expression in full mutation chorionic villi.* Hum Genet, 2002. **110**(6): p. 601-5.

94. Dhara, S.K. and N. Benvenisty, *Gene trap as a tool for genome annotation and analysis of X chromosome inactivation in human embryonic stem cells.* Nucleic Acids Res, 2004. **32**(13): p. 3995-4002.

95. Mohandas, T.K., et al., *X-chromosome inactivation in cultured cells from human chorionic villi.* Somat Cell Mol Genet, 1989. **15**(2): p. 131-6.

96. Migeon, B.R., J. Axelman, and P. Jeppesen, *Differential X reactivation in human placental cells: implications for reversal of X inactivation.* Am J Hum Genet, 2005. **77**(3): p. 355-64.

97. Chow, J.C., et al., *Inducible XIST-dependent X-chromosome inactivation in human somatic cells is reversible.* Proc Natl Acad Sci U S A, 2007. **104**(24): p. 10104-9.

98. Silva, S.S., et al., *X-chromosome inactivation and epigenetic fluidity in human embryonic stem cells.* Proc Natl Acad Sci U S A, 2008. **105**(12): p. 4820-5.

99. Monkhorst, K., et al., *X Inactivation Counting and Choice Is a Stochastic Process: Evidence for Involvement of an X-Linked Activator.* Cell, 2008. **132**(3): p. 410-421.

100. Navarro, P. and P. Avner, *An embryonic story: analysis of the gene regulative network controlling Xist expression in mouse embryonic stem cells.* Bioessays, 2010. **32**(7): p. 581-8.

101. Chaumeil, J., et al., *Integrated kinetics of X chromosome inactivation in differentiating embryonic stem cells.* Cytogenet Genome Res, 2002. **99**(1-4): p. 75-84.

102. Barakat, T.S., et al., *X-changing information on X inactivation.* Exp Cell Res, 2010. **316**(5): p. 679-87.

103. Navarro, P., et al., *Molecular coupling of Xist regulation and pluripotency.* Science, 2008. **321**(5896): p. 1693-5.

104. Navarro, P., et al., *Molecular coupling of Tsix regulation and pluripotency.* Nature, 2010. **468**(7322): p. 457-60.

105. Shin, J., et al., *Maternal Rnf12/RLIM is required for imprinted X-chromosome inactivation in mice.* Nature, 2010. **467**(7318): p. 977-81.

106. Shibata, S., T. Yokota, and A. Wutz, *Synergy of Eed and Tsix in the repression of Xist gene and X-chromosome inactivation.* EMBO J, 2008. **27**(13): p. 1816-26.

107. Hinkins, M., et al., *Expression of Polycomb-group genes in human ovarian follicles, oocytes and preimplantation embryos.* Reproduction, 2005. **130**(6): p. 883-8.

108. Chadwick, B.P. and H.F. Willard, *Chromatin of the Barr body: histone and non-histone proteins associated with or excluded from the inactive X chromosome.* Hum Mol Genet, 2003. **12**(17): p. 2167-78.

109. Schoeftner, S., et al., *Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing.* EMBO J, 2006. **25**(13): p. 3110-22.

110. Hernandez-Munoz, I., et al., *Stable X chromosome inactivation involves the PRC1 Polycomb complex and requires histone MACROH2A1 and the CULLIN3/SPOP ubiquitin E3 ligase.* Proc Natl Acad Sci U S A, 2005. **102**(21): p. 7635-40.

111. Pugacheva, E.M., et al., *Familial cases of point mutations in the XIST promoter reveal a correlation between CTCF binding and pre-emptive choices of X chromosome inactivation.* Human Molecular Genetics, 2005. **14**(7): p. 953-965.

112. Dvash, T., N. Lavon, and G. Fan, *Variations of X chromosome inactivation occur in early passages of female human embryonic stem cells.* PLoS One, 2010. **5**(6): p. e11330.

113. Enver, T., et al., *Cellular differentiation hierarchies in normal and culture-adapted human embryonic stem cells.* Hum Mol Genet, 2005. **14**(21): p. 3129-40.

114. Hall, L.L., et al., *X-inactivation reveals epigenetic anomalies in most hESC but identifies sublines that initiate as expected.* J Cell Physiol, 2008. **216**(2): p. 445-52.

115. Hoffman, L.M., et al., *X-inactivation status varies in human embryonic stem cell lines.* Stem Cells, 2005. **23**(10): p. 1468-78.

116. Liu, W. and X. Sun, *Skewed X chromosome inactivation in diploid and triploid female human embryonic stem cells.* Hum Reprod, 2009. **24**(8): p. 1834-43.

117. Shen, Y., et al., *X-inactivation in female human embryonic stem cells is in a nonrandom pattern and prone to epigenetic alterations.* Proc Natl Acad Sci U S A, 2008. **105**(12): p. 4709-14.

118. Tanasijevic, B., et al., *Progressive accumulation of epigenetic heterogeneity during human ES cell culture.* Epigenetics, 2009. **4**(5): p. 330-8.

119. Tchieu, J., et al., *Female human iPSCs retain an inactive X chromosome.* Cell Stem Cell, 2010. **7**(3): p. 329-42.

120. Brons, I.G., et al., *Derivation of pluripotent epiblast stem cells from mammalian embryos.* Nature, 2007. **448**(7150): p. 191-5.

121. Lovell-Badge, R., *Many ways to pluripotency.* Nat Biotechnol, 2007. **25**(10): p. 1114-6.

122. Tesar, P.J., et al., *New cell lines from mouse epiblast share defining features with human embryonic stem cells.* Nature, 2007. **448**(7150): p. 196-9.

123. Hanna, J., et al., *Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs.* Proc Natl Acad Sci U S A, 2010. **107**(20): p. 9222-7.

124. Lengner, C.J., et al., *Derivation of pre-X inactivation human embryonic stem cells under physiological oxygen concentrations.* Cell, 2010. **141**(5): p. 872-83.

125. Gardner, R.L. and F.A. Brook, *Reflections on the biology of embryonic stem (ES) cells.* Int J Dev Biol, 1997. **41**(2): p. 235-43.

126. Blair, K., J. Wray, and A. Smith, *The liberation of embryonic stem cells.* PLoS Genet, 2011. **7**(4): p. e1002019.

127. Jacobs, P.A., et al., *Klinefelter's syndrome: an analysis of the origin of the additional sex chromosome using molecular probes.* Ann Hum Genet, 1988. **52**(Pt 2): p. 93-109.

128. Lorda-Sanchez, I., et al., *Reduced recombination and paternal age effect in Klinefelter syndrome.* Hum Genet, 1992. **89**(5): p. 524-30.

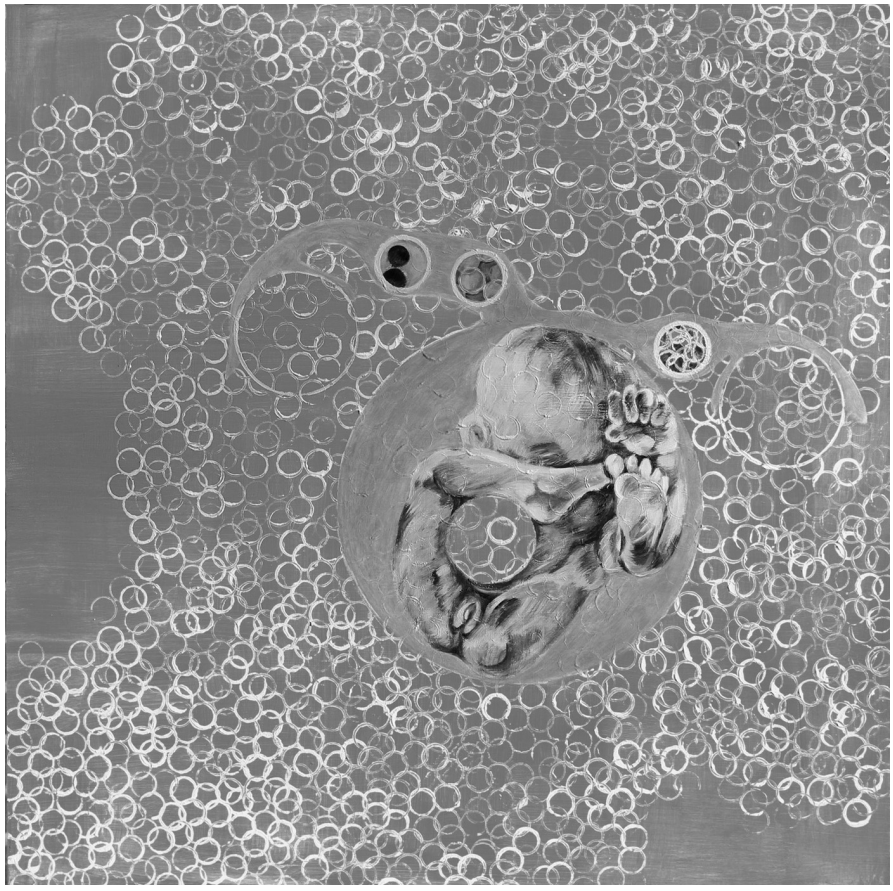
129. Marahrens, Y., et al., *Xist-deficient mice are defective in dosage compensation but not spermatogenesis.* Genes Dev, 1997. **11**(2): p. 156-66.

130. Nesterova, T.B., et al., *Loss of Xist Imprinting in Diploid Parthenogenetic Preimplantation Embryos.* Developmental Biology, 2001. **235**(2): p. 343-350.

131. Alfarawati, S., et al., *The relationship between blastocyst morphology, chromosomal abnormality, and embryo gender.* Fertil Steril, 2011. **95**(2): p. 520-4.

132. Macklon, N.S., J.P. Geraedts, and B.C. Fauser, *Conception to ongoing pregnancy: the 'black box' of early pregnancy loss.* Hum Reprod Update, 2002. **8**(4): p. 333-43.

CHAPTER 5



General discussion

This thesis describes examples of epigenetic regulation during human meiosis and pre-implantation embryo development. Important events such as the meiotic divisions during oocyte meiosis, pronuclei formation in the zygote and dosage compensation between male and female embryos all involve epigenetic regulation, i.e. remodeling of chromatin. Chapter 4.2 reviews the most important steps in the initiation of X chromosome inactivation, and describes the differences between mouse and human findings and will not be further discussed in depth. In this chapter the main findings of the studies in human oocytes and zygotes described in this thesis are summarized and discussed.

Oocyte maturation, from the Germinal Vesicle stage to Metaphase II arrest

Human oocytes have a high frequency of chromosome aberrations and so far, the only factor that has unequivocally been associated with this high error frequency is advanced maternal age. It was previously thought that the majority of segregation errors occur during the first meiotic division, MI, when the homologous chromosomes are separated and that homologous non-disjunction was the predominant error leading to oocyte aneuploidy. This theory was based on data from animal models, data from individuals with Down syndrome and cytogenetic analyses of human oocytes [1-4]. Although Angell et al. proposed that human MII oocytes have a high rate of single chromatids [5-8], which would result from premature sister chromatid separation (PSCS) during MI, these observations were questioned and thought to arise from fixation artefacts. However, recent data on the first and second polar body of human oocytes from ART treatments confirm these observations from Angell et al. and show that it is not homologous chromosome non-disjunction but premature separation of the sister chromatids that is the predominant error of mis-segregation in both MI and MII [9,10]. Studies on mouse oocytes indicate that older mice also suffer from age-related aneuploidy [11-15].

The exact underlying mechanism that causes the mis-segregation in human oocytes, either through homologue non-disjunction or chromatid predivision, is still unknown. However, many proteins and pathways have been identified and found to be essential for correct chromosome segregation in oocytes from animal models. It is reasonable to assume that these are also important for human aneuploidy (reviewed in [16,17]). A pathway that is suggested to be involved in the age-related aneuploidy in mice is the regulation of the cohesion complex which holds the sister chromatids together (Figure 1).

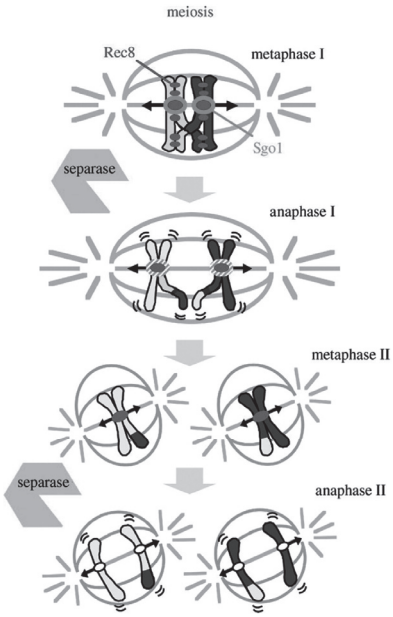


Figure 1. Stepwise cleavage of the cohesion complex in meiosis. The cohesion complex holds the two sister chromatids together. At the transition from MI to AI the REC8 cohesin between the arms of the chromatids is cleaved by Separase to allow the homologues to be separated. Centromeric cohesion is protected by the Shugoshin proteins which prevents untimely cleavage of the sister chromatids at the MI-AI transition. At the MII stage the sister chromatids are now bi-orientated to the spindle poles. This orientation creates tension on the centromeres which relocates Shugoshin from cohesin and allows REC8 to be cleaved by Separase at the MII to AII transition (adapted from [18]) (see page 188 for colour figure).

Two research groups that used genetically modified mice have shown that at least two components of the cohesin complex are not replenished from the fetal meiotic prophase to the adult MII stage indicating that these proteins must last until adult life [19,20]. Oocytes from older mice (12–18 months) have depleted protein levels of the cohesin components REC8, SMC1β and STAG3 [11,13,14,21]. Additionally, oocytes from old mice show loss of the Shugoshin 2 protein that protects the centromeric bound REC8 from untimely cleavage during MI [13]. Thus, maternal aging affects the cohesin complex and associated proteins in mouse oocytes and it likely contributes to the age-related aneuploidy found in mouse oocytes. Furthermore, the same effect of reduced cohesin levels has also been found in aged Drosophila oocytes suggesting that the mechanism may be conserved [22].

Can these findings be extrapolated to humans? A study on human ART oocytes shows that the meiotic cohesin proteins are present in human prophase, MI and MII oocytes. Unfortunately, no protein data were obtained from oocytes of women of advanced maternal age [23]. The high rate of PSCS in human oocytes indicates that the stepwise removal of the cohesin complex during MI and MII may be affected by advanced maternal age. Since women are fertile for more than thirty years after menarche, it is obvious that in human oocytes the cohesin complex must last for decades. This indicates that either the cohesin complex is more stable in humans than in mice or that it is replenished in human oocytes during adult life. Still, other factors that stabilize the cohesin complex during dictyate arrest may also be important in preventing mis-segregation of chromosomes during MI and MII. Reduced function of such factors could lead to depletion of cohesin subunits and subsequently PSCS and thus may be involved in the age-related aneuploidy in both mice and women. It would be interesting to study whether a human- or humanized REC8 protein will rescue older mice from age-related aneuploidy.

Another pathway which has been proposed to be implicated in age-related aneuploidy is the dysregulation of chromatin remodeling during the final stages of oocyte maturation. Studies in mouse and porcine oocytes have shown that defective histone deacetylation, either induced or as consequence of aging, leads to aneuploidy. Our aim was to study whether human oocytes have a similar pattern of histone deacetylation during maturation and also whether there is a relation between histone acetylation and chromosome segregation errors in human oocytes. In our study of human oocytes we analyzed the deacetylation kinetics during the final stages of human oocyte maturation.

Chromatin remodeling during the last stages of oocyte maturation

During maturation from the germinal vesicle stage to the arrest at the metaphase of the second meiotic division, mammalian oocytes prepare for segregation by remodeling their chromatin to enable optimal compaction of the chromosomes. In chapter 2 of this thesis we show that chromatin is deacetylated on lysine residues of histone 4 during maturation of human oocytes. We observed the following pattern in human oocytes: GV oocytes showed staining for all the examined acetylated lysine residues and at the MI and MII stage, oocytes were negative for almost all tested lysine residues. A similar pattern has been observed in other mammalian oocytes, i.e. mouse oocytes, porcine, sheep and bovine oocytes, although some differences exist between individual lysine residues [24-27].

Faithful chromosome division depends on a correct kinetochore-microtubule interaction which is impeded by defective deacetylation or induced hyperacetylation by inhibition of HDACs ([28-35]). Akiyama et al. showed that when mouse oocytes are cultured

in the presence of trichostatin A (TSA), a commonly used HDAC inhibitor, mouse MII oocytes were hyperacetylated for H4 and had an increased percentage of lagging chromosomes. Furthermore they observed that oocytes of older mice (12 months) showed abnormal residual acetylation of two lysine residues, namely H4K8ac and H4K12ac.

One of our major interests is the mechanism that underlies the erroneous division of chromosomes in human oocytes and since defective chromatin remodeling would potentially explain unfaithful chromosome segregation, we chose to study histone acetylation in human oocytes. We analyzed H4K12ac in human MII oocytes, both freshly donated (Day 0) and failed fertilized oocytes (Day 1). We found that residual staining of H4K12ac was more frequently observed in oocytes of women of advanced maternal age indicating that defective deacetylation is associated with advanced maternal age in human oocytes. To study whether residual histone acetylation of H4K12 is also related to chromosome mis-segregation, we analyzed chromosome alignment at the equatorial plate and the microtubule spindle structure together with histone acetylation in human MII oocytes. We observed more lagging chromosomes and complete congression failure in oocytes that were positive for the H4K12ac than in oocytes that had no staining of H4K12. This was observed in oocytes of all age groups indicating that defective deacetylation of chromatin may predispose a human oocyte to aneuploidy independent of maternal age. By calculating the relative risk we found that residual acetylation of H4K12 gives a 15 times higher chance of chromosome mis-alignment. Thus, our data show that in human oocytes defective chromatin remodeling, in this case residual histone acetylation of H4K12, is correlated with chromosome mis-alignment. This suggests that residual acetylation may be one of the major factors that cause age-related aneuploidy. To confirm the causative effect of residual acetylation experimentally, human GV oocytes should be cultured in the presence of HDACi to induce histone hyperacetylation to subsequently study the effect on chromosome segregation. Although surplus human GV oocytes are available as a byproduct after ovarian hyper stimulation, they are known to be of low quality and have less developmental potential when cultured to the MII stage in conventional IVF media (rescue maturation) and are not used for clinical purposes [36-39]. The reason for this might be related to their delayed development. As they have not reached the MII stage after hormonal stimulation they lag behind in maturation for unknown reasons. This delay may possibly affect the chromatin structure and a rescue maturation may thus lead to misinterpretation of normal human developmental patterns. Indeed, we observed that all *in vitro "rescue"* matured human GV to MII oocytes had residual acetylation even without chemical induction. For this reason we were unable to obtain reliable experimental data with human oocytes to verify the causative effect of histone hyperacetylation on chromosome mis-segregation.

How does residual histone acetylation affect chromosome segregation?

The kinetochore is a proteinaceous structure built on centromeric DNA containing numerous proteins whose functions are essential for accurate chromosome segregation ensuring the attachment of spindle microtubules to chromosomes, sister chromatid cohesion regulation and localization of the spindle assembly checkpoint proteins. Correct division of chromosomes not only involves the centromeric chromatin, but also the adjacent region, the pericentomeric chromatin. The constitutive layer of the kinetochore which encompasses the centromeric chromatin is built on the highly repetitive centromeric DNA sequence. The centromeric chromatin contains the histone variant Cenp A (reviewed in [40]) and remains stable and unmodified throughout the whole mitotic cell cycle. The outer kinetochore and the pericentromeric chromatin, on the contrary, are differently modified during the different phases of the cell cycle (reviewed in [41,42]).

Acetylation of histones is considered an “open” chromatin mark and is associated with gene transcription and DNA repair [43]. Deposition of acetyl groups on histone H4 follows DNA replication [44] and deacetylation of chromatin is related to gene silencing, e.g. on the inactive X, with heterochromatin formation and chromosome condensation [29,45,46]. Both the centromeric and the pericentromeric heterochromatin are hypoacetylated during the metaphase of the cell cycle [45,47]. Several studies have shown that inhibition of HDAC’s by either RNAi or chemical agents induces hyperacetylation of chromatin [28-32,34,35,48,49]. A consequence of this hyperacetylated chromatin is that proteins of important regulating complexes such as the spindle assembly checkpoint (SAC), the chromosome passenger complex (CPC) and microtubule motor proteins fail to localize properly, and cannot execute their function (Table I). As mentioned before, data from mouse but also porcine oocytes show that induced hyperacetylation during meiosis leads to aneuploidy [15,50,51]. Because the regulation of the cohesion complex involves many proteins that are affected by induced histone hyperacetylation by chemical agents, defective regulation of histone deacetylation during oocyte maturation may have drastic effects on the fidelity of chromosome segregation. Our data show that also in human oocytes defective remodeling of chromatin, which is implied by the residual histone acetylation, may be an important factor leading to chromosome mis-segregation. New studies should be conducted to see which proteins are affected by induced hyperacetylation or natural residual acetylation in mammalian oocytes.

Table I. Overview of proteins that are affected by HDAC inhibition.

Protein	Function	HDACi phenotype	Cell type used in study	Reference
ATRX	Centromeric binding protein	Loss	Mouse oocytes	[50]
BUB1	Component of SAC	Loss	HeLa, MCF7, PC3	[31,35]
BUBR1	Component of SAC	Diminished localization	HeLa	[48]
Shugoshin	Prevents premature cleavage of cohesin	Diminished levels	HeLa	[30]
MAD2	Component of SAC	Loss/ Centromeric localization failure	HeLa	[31,49]
H3S10Phospo	Entry of mitosis; target of Aurk B	Diminished levels	HeLa, MCF7, PC3	[30,35,48,49]
HP1 (HP1α/Hp1β); Swi6p (yeast)	Heterochromatin binding protein	Localization loss from centromeric chromatin in G2	HeLa, mouse L929, Fission yeast	[28,29,31,34,35]
CENP-E	MT motor protein	Loss	HeLa, MCF7, PC3	[31,35]
Survivin	Component of CPC	Loss	HeLa	[31]
Borealin	Component of CPC	Loss	HeLa	[31]
AURK B	Component of CPC	Centromeric localization failure in G2 and M-phase	HeLa	[31,35]
INCENP	Component of CPC	Centromeric localization failure	HeLa	[31]
CYCLIN B	Target of APC/C	Premature degradation	HeLa	[49]
securin	Target of APC/C	Premature degradation	HeLa	[30,49]
MCAK	Target of CPC; interacts with MT	Loss	HeLa	[31]

The most obvious candidates involved in the dysregulation of deacetylation in human oocytes are the HDAC enzymes. There are four different classes of HDACs (I, II, III and IV) and they differ in their localization and structure. HDAC1, 2, 3 and 8 (class I) localize to the nucleus and are similar to the yeast RPD3 protein. Class II are the HDACs 4, 5, 6, 7, 9, and 10. They are homologous to yeast HDA1 and localize both in the cytoplasm and in the nucleus. Class III HDACs differ from class I and II as they are NAD-dependent similar to the yeast Sirtuin proteins. Class IV only comprises HDAC11 and has features of both class I and II. Mouse MII oocytes have different mRNA levels of class I and II HDACs which are known to be affected by TSA treatment [52]. Most likely, individual HDACs may regulate one or more lysine residues on H3 and H4 and have non-histone targets. This makes it difficult to assess which HDAC or HDACs are involved in the deacetylation of the individual lysine residues during oocyte maturation.

Selective inhibition of the individual HDACs, either by more specific HDAC inhibitors or by RNAi injection in mouse GV oocytes, is needed to determine which HDACs are involved in the age-related aneuploidy. Identification of the responsible HDAC will give new insights and may allow for a more direct approach to study HDACs in human oocytes. Thus, our hypothesis would be that advancing age leads to reduced HDAC activity resulting in defective deacetylation and consequently open chromatin. This open chromatin structure may interfere with several aspects of chromosome segregation, in particular unstable spindle-kinetochore interaction, impaired cohesion protection or a failure of the SAC to monitor and correct unstable microtubule attachment which eventually results in segregation errors [28-32,34,35,48,49].

Chromatin composition in the male and female pronuclei of zygotes

From the moment an oocyte is fertilized, it becomes a zygote, a new life. In ART treatments, fertilization takes place *in vitro* and zygotes are analyzed 16–20 hpi to see whether normal fertilization has occurred. Normally, two pronuclei (PN) are observed: one maternal PN derived from the oocyte and one paternal PN coming from the spermatozoon. Sometimes, however, only one PN or more than two pronuclei can be observed. To study the chromatin and the differences in histone modifications between the maternal and paternal pronuclei only abnormally fertilized human oocytes can be used because of ethical legislations.

Differences in histone modifications between the maternal and paternal pronucleus

The chromatin of a mature spermatozoon exists mainly of protamines and only a small fraction consists of histone proteins. At fertilization, the protamines are replaced by new histones which are subsequently chemically modified by maternal modifying enzymes [53]. Thus, the maternal and paternal chromatin can be distinguished by their histone signature during pronuclei formation (Table II). There is still some controversy whether mono-pronuclear zygotes are indeed fertilized and both gametes have contributed to the single pronucleus. Because little is known about the histone signature of chromatin in human zygotes we decided to analyze histone modifications in human tri-pronuclear zygotes. We studied both euchromatic as well as heterochromatic modifications to analyze whether human zygotes have the same pattern of histone modifications as mouse zygotes and found that also in human zygotes the parental pronuclei are differentially modified (chapter 3). Based on these novel insights we were able to discriminate between the maternal and paternal chromatin and determine the parental chromatin contribution in human mono-pronuclear and tri-pronuclear zygotes.

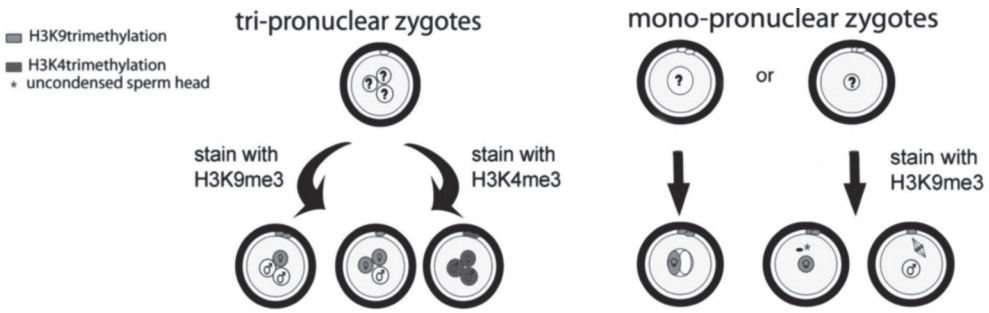


Figure 2. Schematic overview of the experimental approach of our study on human tri-pronuclear and mono-pronuclear zygotes (see page 189 for colour figure).

We observed differentially modified histone modifications in the parental pronuclei of human zygotes similar to what has been found in mouse zygotes (Table II). We found that the euchromatic histone mark H3K4trimethylation is present in both the maternal as well as the paternal pronucleus and thus does not distinguish between maternal and paternal chromatin. As the same has been observed for another euchromatic marker, namely H4R3me3 [54], this suggest that human zygotes follow a similar timeline of euchromatic chromatin modification deposition as mice. In contrast to the euchromatic mark, the heterochromatic histone modifications H3K27trimethylation and H3K9trimethylation were not present in the human paternal pronucleus whereas the maternal pronucleus, that is slightly more compact, was extensively modified for both H3K27me3 as H3K9me3 (Figure 2). This asymmetry between the paternal and maternal pronucleus has also recently been described for the H3K9me2 modification in human tri-pronuclear zygotes [54,55]. Our data for both euchromatic as well as heterochromatic marks suggest that chromatin remodeling is basically conserved between mouse and human zygotes during the pronuclear stages, but some modifications appear to be different. Early pronuclear stage mouse zygotes have an asymmetric staining pattern for the H3K27me3 modification [56,57], which becomes symmetrical at the late pronuclear stage of mouse zygotes [58]. Our observations that the human paternal PN remains unmodified for H3K27me3, may be a reflection of the slower developmental kinetics of human embryos. Whether these differences serve any function or only reflect a different time line remains to be elucidated.

The presence of trimethylated H3K4 in the paternal human PN is intriguing. This mark is usually associated with transcriptional active chromatin [59]. In mouse zygotes, H3K4me3

appears in the paternal pronucleus during S-phase [58] and is possibly linked to the (minor) transcriptional activation of the zygotic genome at this stage [60]. In the early human embryo, however, embryonic genome activation occurs between the four- and eight-cell stages, suggesting that the presence of H3K4me3 in zygotic paternal chromatin is not directly linked to transcriptional activation [61,62]. A similar observation has been made in mammalian germinal vesicle oocytes with a surrounding nuclear configuration. At this stage of oocyte development general transcription is terminated [63]. However, the chromatin carries both inactive as well as active chromatin marks such as the heterochromatin modification H3K9me2/3, the euchromatin modification H3K4me3 [64] as well as acetylation of H3/H4 [24-27]. Furthermore, in our study on human oocytes, we observed that human GV oocytes also contain euchromatic markers. Whether this asynchronous remodeling has a function during these early stages has yet to be clarified.

Table II. Overview of literature on histone modifications in the parental pronuclei in mouse and human zygotes.

Modification	Heterochromatin /euchromatin	Presence in mouse pronuclei	Presence in human pronuclei	Reference
H3K9me1	heterochromatin	Both PN	ND	[54,55,57,91-95]
H3K9me2		♀ Both PN from PN5 stage onwards *	Only ♀	
H3K9me3		Only ♀	Only ♀	
H3K27me1	heterochromatin	Both PN	ND	[57,92,95,96]
H3K27me2		♀ Both PN from PN3 stage onwards *	ND	
H3K27me3		♀ Both PN from PN4 stage onwards *	Only ♀	
H4K20me1	heterochromatin	Both PN	ND	[57]
H4K20me2		Only ♀	ND	
H4K20me3		Only ♀	ND	
H3K4me1	euchromatin	Both PN	ND	[56-58,92]
H3K4me3		♀ Both PN from PN4 stage onwards	Both PN	
H4ac	euchromatin	Both PN	ND	[56,97,98]
H4R3me2	euchromatin	No staining present	ND	[54,56]
H4R3me3		ND	Both PN	

Some conflicting results have been reported on the presence of H3K27me2/3 in the paternal pronucleus. This can be attributed to the examined stage of PN formation because at the PN3 stage the paternal PN is devoid of H3K27me3 whereas at the PN4 and PN5 stage clear staining was observed. For H3K9me2 it likely that the authors of the conflicting studies use different thresholds for the interpretation of the immuno stainingg [58,95].

Parental contribution in mono-pronuclear zygotes after IVF and ICSI

After conventional insemination procedures, around 2-5% of the zygotes have a single pronucleus which is often larger than normal pronuclei. After ICSI, this percentage varies between 2.5%–11.4% (Table III). Studies using FISH have shown that around 31–81% of these mono-pronuclear zygotes and derived embryos originating from conventional insemination procedures have in fact a diploid chromosome constitution. This indicates that fertilization had indeed occurred but that the pronuclei have aberrantly fused. FISH analysis can be used to assess the chromosome composition but an XX or XY genotype does not necessarily mean that both parental genomes are present in a 1 PN zygote (Table III). A female embryo is in most cases the result of a normal fertilization of an oocyte by a single sperm. However, embryos with two X chromosomes can also be the result of parthenogenesis or the fertilization of an empty egg by two spermatozoa. The latter can also result in an XY or YY embryo with a complete paternal diploid genome. We observed that in almost all the human 1 PN zygotes derived from IVF (87%), both parental genomes were present and occupy separate compartments within the single nuclear membrane (Figure 2). The other zygotes contained only paternal chromatin (4%) or maternal chromatin (9%). In this aspect, the human 1 PN IVF zygotes are the same as mouse bi-parental mono-pronuclear zygotes. For 1 PN zygotes after ICSI, we observed that only 10 out of the thirty-three 1 PN zygotes (30%) contained both parental chromatin sets, indicative of fertilization (Figure 2). In the remaining cases, the single PN was of completely maternal (15/33) or paternal origin (8/33). Our data are consistent with other studies that show that 1 PN zygotes from ICSI treatments have much lower rates of diploid embryos than 1 PN IVF zygotes (Table III).

In normally fertilized zygotes, in which the two genomes have their own nuclear envelope, replication occurs within each compartment. Limited data from a mouse study on bi-parental mono-pronuclear zygotes do not suggest a major effect of a pronuclear fusion, however, the pregnancy rates were very low [65]. It has been suggested that during IVF treatments in which only mono-pronuclear zygotes are available after an IVF or ICSI treatment, extended culture to the blastocyst stage may be considered to select a 1 PN embryo suitable for transfer. A previous study showed that around 18% of the IVF mono-pronuclear zygotes can develop to the blastocyst stage, contrary to ICSI 1 PN zygotes, where no survival to the blastocyst stage was observed [66]. In addition, normal hES cell lines have been derived from 1 PN zygotes indicating that extended culture may select against abnormal and haploid embryos [67,68]. However, chemically activated non-inseminated oocytes can also develop to the blastocyst stage [69], which indicates that extended culture does not select against parthenogenetic embryos. To date, mainly case reports have been described of successful

pregnancies after transfer of a mono-pronuclear zygote [70-72] and only one study reported that 2 out of 38 transfers resulted in the birth of a healthy baby [73]. In addition, in our own IVF clinic the ongoing pregnancy rates of embryos derived from 1 PN IVF zygotes are relatively low (15.4% per embryo transfer, unpublished data).

Thus, although mono-pronuclear zygotes are attractive and useful research material that possibly represent relatively normal human zygotes, the transfer of 1PN zygotes does not have additional clinical value. Our policy is that only in the cases where no 2 PN embryos are available for transfer, embryos from 1 PN from IVF but not from ICSI zygotes, are considered for transfer.

Table III. Literature on the chromosome constitution of human mono-pronuclear zygotes.

ART	% 1pn	Day of analysis	% Diploid	% haploid	Method	Reference
IVF	5.5	Day 1	80.5	12.2	Cytogenetic analysis	[73]
IVF	3.6	Day 2/3	55.6 XX: 33.3 XY: 22.2	33.3	Cytogenetic analysis	[99]
IVF	ND	Day 1	31.3	50	FISH	[100]
IVF	4.5	Day 3	71.5 XX: 28.6 XY: 42.9	14.3 XO:14.3 YO: 0	FISH	[101]
ICSI	11.4	Day 3	28.6 XX: 23.8 XY: 4.8	66.7 XO: 61.9 XY: 4.8	FISH	[101]
IVF	7.7	Day 2/3	48.7 XX: 27.0 XY: 21.7 YY: 0	31.2 XO: 26.2 YO: 4.9	FISH	[102]
ICSI	5.0	Day 2/3	27.9 XX: 16.4 XY: 9.8 YY: 1.6	31.2 XO: 26.2 YO: 4.9	FISH	[102]
IVF	ND	Day 2	73.1	23.1	Cytogenetic analysis	[103]
ICSI	ND	Day 2	37.5	58.3	Cytogenetic analysis	[103]
IVF	ND	Day 2	54.3 XX: 21.7 XY: 32.6	23.9 XO: 19.6 YO: 4.3	FISH	[104]
ICSI	ND	Day 2	31.5 XX: 15.1 XY: 16.4	31.5 XO: 24.7 YO: 6.9	FISH	[104]

How do mono-pronuclear zygotes arise?

Different theories have been proposed that would lead to a single pronucleus 16–20 hrs post fertilization or insemination. One is that pronucleus formation occurs asynchronously in these mono-pronuclear zygotes. Indeed, it has been reported that after re-examination, 6%-25% of the mono-pronuclear zygotes showed a second PN [66,73]. However, whether asynchronous pronuclei formation is a normal feature of human zygotes is controversial. Several studies reporting conflicting observations and differences in methodology might underlay the various interpretations [74-76]. The other theory is that fusion of the parental genomes occurs. Although this is not common in mammals, it occurs in other species such as sea urchins [77,78]. The proximity of the parental chromatin domains early after gamete fusion has an effect on the chance of pronuclear syngamy. This is illustrated by the finding that in the mouse, injection of the sperm close to the maternal complement resulted in 22% mono-pronuclear zygotes [65]. The authors provide evidence for the hypothesis that aggregation of chromatin masses within a single nucleus can occur, when the two parental chromatin sets are in close proximity during pronuclei formation. This artificial generation of high rates of mono-pronuclear zygotes has also been observed in humans [79], when round immature spermatids (ROSI) were injected. The authors suggested that pronuclear fusion or syngamy might be a normal but very short phenomenon in human zygotes and injection of immature spermatids would lead to a prolonged state of this syngamy due to yet unknown controlling elements. However, time lapse recording of pronuclear formation after ICSI showed no pronuclear fusion in 2 PN zygotes [76] and it is unlikely that fusion occurs in normal fertilization.

Bi-parental mono-pronuclear human zygotes most likely are the result of sperm entry close to the metaphase II spindle, suggesting this to be a distinct possibility at natural conception, too. Most likely the syngamy is the result of precocious interaction of the maternal and paternal chromatin before nuclear membrane formation. Clinical practice aims to puncture the oolemma and deposit the sperm without disturbing the metaphase II spindle, by keeping the oocyte with the polar body perpendicular to the injection needle. However, one cannot rule out the possibility that the sperm is occasionally close to the maternal complement after injection, especially since the first polar body has proven to be an unreliable marker for the location of the metaphase plate [80,81].

3 PN zygotes: rescue them?

For zygotes that contain three or more pronuclei it is clear that these abnormally fertilized zygotes cannot develop into a genetically normal embryo as they will form molar pregnancies and are therefore not considered for transfer into the uterus. It has been reported that

tri-pronuclear zygotes can be microsurgically rescued into a “normal fertilized” 2 PN zygotes (reviewed in [82]) and can even result in the birth of a healthy child [83]. It should be noted that this procedure is still very experimental and should only be considered when a couple has no other options left. Even then, it remains questionable whether the procedure is safe. One must be sure that after the enucleation procedure, the remaining 2 pronuclei are from maternal and paternal origin and that one did not create a gynogenetic (containing only maternal DNA) or androgenetic (containing only paternal DNA) embryo. The couple must be counseled properly for the risk of having a spontaneous abortion, neonatal death or a child with severe birth defects.

Given these many disadvantages and the limited evidence regarding the safety of the technique, it may presently even be wiser to not consider the microsurgical rescue of tri-pronuclear zygotes for embryo transfer at all. However, as human embryos for research purposes are scarce these rescued 3 PN zygotes could be an extra source for human embryonic stem cells (hES) lines. If this proves to be successful and safe, the step to the ART clinic will be much smaller.

Initiation of XCI in female human pre-implantation embryos

Dosage compensation between males and females is a very important epigenetic process that is initiated in mice during pre-implantation development. To determine if human female embryos initiate the XCI process we analyzed the most important hallmarks of XCI, namely expression and *in cis* coating of *XIST*, the exclusion of the transcription machinery, the exclusion of open chromatin histone modifications on one X chromosome and the accumulation of heterochromatin marks on one X chromosome.

We analyzed human embryos at different stages of pre-implantation development, namely the 8-cell, the morula and the blastocyst stage. At the 8-cell stage we observed that of the cells that express *XIST,* 68% had a single pinpoint at one X chromosome. During development from the morula to the blastocyst stage the pinpoint signal extends to a full cloud of *XIST*. This full cloud is excluded from nascent RNA transcription as we observed that the cloud of *XIST* is located in a hole of *Cot1* RNA staining. Furthermore, we observed accumulation of H3K27me3 in 30% of the nuclei in female blastocysts. This accumulation of a facultative heterochromatic hallmark overlapped another hallmark of XCI namely macroH2A accumulation. Similarly, nuclei that have a single region of accumulation of H3K27me3 showed exclusion of an active chromatin mark, H3K9ac, in this region. These three characteristics were

validated in control cells with DNA FISH where they overlapped the Barr body. Barr bodies are not formed until later stage X inactivation in somatic cells. Lastly, we found that in blastocysts the X-linked CHIC1 gene was mono-allelicly expressed. Based on these data together we concluded that in human female pre-implantation development XCI is initiated. A distinction should be made, however, between the XCI hallmarks observed in pre-implantation human embryos and XCI in somatic cells. Our data show full remodeling of one X chromosome but it may well be possible that chromosome wide gene inactivation on the X chromosome does not occur in human embryos and that only a limited number of genes is inactivated.

In 2011 another group studied the XCI kinetics in human female and male embryos [84]. Their findings were completely different from our observations. One of the differences was that all X chromosomes expressed and accumulated XIST, the single X chromosome in males and both X chromosomes in female embryos. Our new data (manuscript in preparation) with embryos cultured in a different commercial medium, still show the predominance of single XIST clouds, although some female embryos have more double clouds (max 60%). This formation of double clouds may be dependent on the quality of the embryo and the number of X chromosome aberrations. Another difference between the data is that Okamoto et al. did not observe accumulation of H3K27me3 in human female embryos. They did observe this in rabbit and mouse embryos. We performed several immuno-stainings on whole mount human blastocyst embryos and found accumulation of H3K27me3, as well as incorporation of macroH2a which overlapped H3K27me3 and exclusion of the active mark H3K9ac (manuscript in preparation). Furthermore, a DNA FISH confirmed that this single region of accumulation of H3K27me3 with exclusion of H3K9ac overlapped with one of the X chromosomes in female embryos. Additionally, we found that cells of the ICM show accumulation of H3K27me3. Finally, our new data show that TSIX is not expressed in human blastocyst embryos which indicates that during human pre-implantation development *XIST* expression is not regulated by TSIX. These new observations confirm our previous conclusions that XCI is initiated during human pre-implantation development.

Rather than the term XCI, we think that the term X chromosome remodeling (XCR) describes these observations in human embryos more accurately. The contradictory results may be explained by technical aspects or differences in stimulation or culture conditions. It has for example been shown that culture conditions influence XCI in bovine pre-implantation embryos and in human ES cells [85,86]. Thus the IVF culture medium may influence *XIST* RNA expression in human embryos. We have been studying XCI in embryos cultured in two different media and found no differences in the general pattern of single *XIST* clouds in female embryos thus verifying our previous observations. However, in embryos that were cultured

in commercial IVF medium we did observe more double clouds, but not as high numbers as Okamoto et al. [84], as well as *XIST* RNA transcripts throughout the whole nucleus.

The current opinion is that *Xist* accumulation leads to silencing in agreement with our observations. It is therefore difficult to envision that the high rate of double clouds observed by Okamoto et al. would go without consequences. Double clouds can only be tolerated when *Xist* coating and *Xist* mediated silencing are two separate mechanisms. New data from mouse pre-implantation embryos may shed light on this question. Embryos carrying a deletion of the paternal Xist allele have similar levels of X-linked expression as wild type embryos [87], which indicates that imprinted XCI during pre-implantation development may be Xist independent and suggests that *Xist* coating itself at this stage does not lead to chromosome wide X-linked silencing [87-89]. Furthermore, the same group reported that in cells of the ICM, reactivation of the Xp occurs while the X chromosomes are still coated by an *Xist* cloud, which indicates that coating of *Xist* still allows gene transcription during pre-implantation development [90]. It remains a question whether *XIST* independent silencing also exists in human embryos.

Although our data show that all hallmarks for XCI are present, including the silencing of an X-linked gene, they do not exclude an *XIST* independent silencing mechanism. Such a mechanism would explain the observation that human embryos can tolerate double *XIST* clouds. Why the embryos used by Okamoto et al. have such a high percentage of double clouds is unknown and no mechanism is suggested by the authors.

As proposed in chapter 4.2, XCI during murine pre-implantation development may first start with remodeling of the paternal X chromosome, which is subsequently secured in the cells of the TE with chromosome wide gene silencing during post-implantation development. The ICM cells lose the Xp imprint and initiate random XCI in the epiblast during post-implantation development. Whether the paternal X is also preferentially silenced and whether this is maintained in the extra-embryonic lineages in human pre-implantation embryos remains to be answered. New studies on human pre-implantation embryos that will address imprinting and to what extent the X chromosome that accumulates *XIST* and H3K27me3, is actually silenced, may provide more insight in the steps that lead to XCI in humans.

References

1. Abruzzo, M.A. and T.J. Hassold, *Etiology of nondisjunction in humans.* Environ Mol Mutagen, 1995. **25 Suppl 26**: p. 38-47.
2. Pellestor, F., T. Anahory, and S. Hamamah, *Effect of maternal age on the frequency of cytogenetic abnormalities in human oocytes.* Cytogenet Genome Res, 2005. **111**(3-4): p. 206-12.
3. Koehler, K.E., et al., *Spontaneous X chromosome MI and MII nondisjunction events in Drosophila melanogaster oocytes have different recombinational histories.* Nat Genet, 1996. **14**(4): p. 406-14.
4. Antonarakis, S.E., et al., *The meiotic stage of nondisjunction in trisomy 21: determination by using DNA polymorphisms.* Am J Hum Genet, 1992. **50**(3): p. 544-50.
5. Angell, R.R., et al., *Cytogenetic analysis of unfertilized human oocytes.* Hum Reprod, 1991. **6**(4): p. 568-73.
6. Angell, R.R., *Predivision in human oocytes at meiosis I: a mechanism for trisomy formation in man.* Hum Genet, 1991. **86**(4): p. 383-7.
7. Angell, R.R., et al., *First meiotic division abnormalities in human oocytes: mechanism of trisomy formation.* Cytogenet Cell Genet, 1994. **65**(3): p. 194-202.
8. Angell, R.R., J. Xian, and J. Keith, *Chromosome anomalies in human oocytes in relation to age.* Hum Reprod, 1993. **8**(7): p. 1047-54.
9. Fragouli, E., et al., *The cytogenetics of polar bodies: insights into female meiosis and the diagnosis of aneuploidy.* Mol Hum Reprod, 2011.
10. Gabriel, A.S., et al., *Array comparative genomic hybridisation on first polar bodies suggests that non-disjunction is not the predominant mechanism leading to aneuploidy in humans.* J Med Genet, 2011. **48**(7): p. 433-7.
11. Chiang, T., et al., *Evidence that weakened centromere cohesion is a leading cause of age-related aneuploidy in oocytes.* Curr Biol, 2010. **20**(17): p. 1522-8.
12. Chiang, T., R. Schultz, and M. Lampson, *Age-Dependent Susceptibility of Chromosome Cohesion to Premature Separase Activation in Mouse Oocytes.* Biology of Reproduction, 2011.
13. Lister, L.M., et al., *Age-related meiotic segregation errors in mammalian oocytes are preceded by depletion of cohesin and Sgo2.* Curr Biol, 2010. **20**(17): p. 1511-21.
14. Liu, L. and D.L. Keefe, *Defective cohesin is associated with age-dependent misaligned chromosomes in oocytes.* Reprod Biomed Online, 2008. **16**(1): p. 103-12.
15. Akiyama, T., M. Nagata, and F. Aoki, *Inadequate histone deacetylation during oocyte meiosis causes aneuploidy and embryo death in mice.* Proc Natl Acad Sci U S A, 2006. **103**(19): p. 7339-44.
16. Eichenlaub-Ritter, U., N. Staubach, and T. Trapphoff, *Chromosomal and cytoplasmic context determines predisposition to maternal age-related aneuploidy: brief overview and update on MCAK in mammalian oocytes.* Biochem Soc Trans, 2010. **38**(6): p. 1681-6.
17. Jones, K.T., *Meiosis in oocytes: predisposition to aneuploidy and its increased incidence with age.* Hum Reprod Update, 2008. **14**(2): p. 143-58.
18. Watanabe, Y. and T.S. Kitajima, *Shugoshin protects cohesin complexes at centromeres.* Philosophical Transactions of the Royal Society B: Biological Sciences, 2005. **360**(1455): p. 515-521.
19. Revenkova, E., et al., *Oocyte cohesin expression restricted to predictyate stages provides full fertility and prevents aneuploidy.* Curr Biol, 2010. **20**(17): p. 1529-33.
20. Tachibana-Konwalski, K., et al., *Rec8-containing cohesin maintains bivalents without turnover during the growing phase of mouse oocytes.* Genes Dev, 2010. **24**(22): p. 2505-16.
21. Hodges, C.A., et al., *SMC1beta-deficient female mice provide evidence that cohesins are a missing link in age-related nondisjunction.* Nat Genet, 2005. **37**(12): p. 1351-5.

22. Subramanian, V.V. and S.E. Bickel, *Aging predisposes oocytes to meiotic nondisjunction when the cohesin subunit SMC1 is reduced.* PLoS Genet, 2008. **4**(11): p. e1000263.

23. Garcia-Cruz, R., et al., *Dynamics of cohesin proteins REC8, STAG3, SMC1 beta and SMC3 are consistent with a role in sister chromatid cohesion during meiosis in human oocytes.* Hum Reprod, 2010. **25**(9): p. 2316-27.

24. Endo, T., et al., *Changes in histone modifications during in vitro maturation of porcine oocytes.* Mol Reprod Dev, 2005. **71**(1): p. 123-8.

25. Kim, J.M., et al., *Changes in histone acetylation during mouse oocyte meiosis.* J Cell Biol, 2003. **162**(1): p. 37-46.

26. Maalouf, W.E., R. Alberio, and K.H. Campbell, *Differential acetylation of histone H4 lysine during development of in vitro fertilized, cloned and parthenogenetically activated bovine embryos.* Epigenetics, 2008. **3**(4): p. 199-209.

27. Tang, L.S., et al., *Dynamic changes in histone acetylation during sheep oocyte maturation.* J Reprod Dev, 2007. **53**(3): p. 555-61.

28. Cimini, D., et al., *Histone hyperacetylation in mitosis prevents sister chromatid separation and produces chromosome segregation defects.* Mol Biol Cell, 2003. **14**(9): p. 3821-33.

29. Ekwall, K., et al., *Transient inhibition of histone deacetylation alters the structural and functional imprint at fission yeast centromeres.* Cell, 1997. **91**(7): p. 1021-32.

30. Eot-Houllier, G., et al., *Histone deacetylase 3 is required for centromeric H3K4 deacetylation and sister chromatid cohesion.* Genes Dev, 2008. **22**(19): p. 2639-44.

31. Ma, Y., et al., *Inhibition of protein deacetylation by trichostatin A impairs microtubule-kinetochore attachment.* Cell Mol Life Sci, 2008. **65**(19): p. 3100-9.

32. Shin, H.-J., et al., *Inhibition of histone deacetylase activity increases chromosomal instability by the aberrant regulation of mitotic checkpoint activation.* Oncogene, 2003. **22**(25): p. 3853-3858.

33. Stevens, F.E., et al., *Histone deacetylase inhibitors induce mitotic slippage.* Oncogene, 2008. **27**(10): p. 1345-54.

34. Taddei, A., et al., *Reversible disruption of pericentric heterochromatin and centromere function by inhibiting deacetylases.* Nat Cell Biol, 2001. **3**(2): p. 114-20.

35. Robbins, A.R., et al., *Inhibitors of histone deacetylases alter kinetochore assembly by disrupting pericentromeric heterochromatin.* Cell Cycle, 2005. **4**(5): p. 717-26.

36. Chian, R.C. and S.L. Tan, *Maturational and developmental competence of cumulus-free immature human oocytes derived from stimulated and intracytoplasmic sperm injection cycles.* Reprod Biomed Online, 2002. **5**(2): p. 125-32.

37. Haberle, M., et al., *Are cumulus cells necessary for the spontaneous maturation of germinal vesicle-stage oocytes to metaphase II.* J Assist Reprod Genet, 1999. **16**(6): p. 329-31.

38. Goud, P.T., et al., *In-vitro maturation of human germinal vesicle stage oocytes: role of cumulus cells and epidermal growth factor in the culture medium.* Hum Reprod, 1998. **13**(6): p. 1638-44.

39. Janssenswillen, C., Z.P. Nagy, and A. Van Steirteghem, *Maturation of human cumulus-free germinal vesicle-stage oocytes to metaphase II by coculture with monolayer Vero cells.* Hum Reprod, 1995. **10**(2): p. 375-8.

40. Prasad, P. and K. Ekwall, *New insights into how chromatin remodellers direct CENP-A to centromeres.* EMBO J, 2011. **30**(10): p. 1875-6.

41. Pidoux, A.L. and R.C. Allshire, *The role of heterochromatin in centromere function.* Philos Trans R Soc Lond B Biol Sci, 2005. **360**(1455): p. 569-79.

42. Maiato, H., et al., *The dynamic kinetochore-microtubule interface.* J Cell Sci, 2004. **117**(Pt 23): p. 5461-77.

43. Jenuwein, T. and C.D. Allis, *Translating the histone code.* Science, 2001. **293**(5532): p. 1074-80.
44. Sobel, R.E., et al., *Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4.* Proceedings of the National Academy of Sciences, 1995. **92**(4): p. 1237-1241.
45. Jeppesen, P., et al., *Antibodies to defined histone epitopes reveal variations in chromatin conformation and underacetylation of centric heterochromatin in human metaphase chromosomes.* Chromosoma, 1992. **101**(5-6): p. 322-32.
46. Jeppesen, P. and B.M. Turner, *The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression.* Cell, 1993. **74**(2): p. 281-9.
47. Maison, C., et al., *Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component.* Nat Genet, 2002. **30**(3): p. 329-34.
48. Dowling, M., et al., *Mitotic spindle checkpoint inactivation by trichostatin a defines a mechanism for increasing cancer cell killing by microtubule-disrupting agents.* Cancer Biol Ther, 2005. **4**(2): p. 197-206.
49. Magnaghi-Jaulin, L., et al., *Histone deacetylase inhibitors induce premature sister chromatid separation and override the mitotic spindle assembly checkpoint.* Cancer Res, 2007. **67**(13): p. 6360-7.
50. De La Fuente, R., et al., *ATRX, a member of the SNF2 family of helicase/ATPases, is required for chromosome alignment and meiotic spindle organization in metaphase II stage mouse oocytes.* Dev Biol, 2004. **272**(1): p. 1-14.
51. Wang, Q., et al., *Histone deacetylation is required for orderly meiosis.* Cell Cycle, 2006. **5**(7): p. 766-74.
52. Yoshida, N., et al., *Epigenetic discrimination by mouse metaphase II oocytes mediates asymmetric chromatin remodeling independently of meiotic exit.* Developmental Biology, 2007. **301**(2): p. 464-477.
53. Puschendorf, M., et al., *PRC1 and Suv39h specify parental asymmetry at constitutive heterochromatin in early mouse embryos.* Nat Genet, 2008. **40**(4): p. 411-20.
54. Qiao, J., et al., *Changes in histone methylation during human oocyte maturation and IVF- or ICSI-derived embryo development.* Fertil Steril, 2010. **93**(5): p. 1628-36.
55. Chen, X., et al., *Similar DNA methylation and histone H3 lysine 9 dimethylation patterns in tripronuclear and corrected bipronuclear human zygotes.* J Reprod Dev, 2010. **56**(3): p. 324-9.
56. Sarmento, O.F., et al., *Dynamic alterations of specific histone modifications during early murine development.* J Cell Sci, 2004. **117**(Pt 19): p. 4449-59.
57. van der Heijden, G.W., et al., *Asymmetry in histone H3 variants and lysine methylation between paternal and maternal chromatin of the early mouse zygote.* Mech Dev, 2005. **122**(9): p. 1008-22.
58. Lepikhov, K. and J. Walter, *Differential dynamics of histone H3 methylation at positions K4 and K9 in the mouse zygote.* BMC Dev Biol, 2004. **4**: p. 12.
59. Fischle, W., Y. Wang, and C.D. Allis, *Histone and chromatin cross-talk.* Curr Opin Cell Biol, 2003. **15**(2): p. 172-83.
60. Schultz, R.M., *Regulation of zygotic gene activation in the mouse.* Bioessays, 1993. **15**(8): p. 531-8.
61. Braude, P., V. Bolton, and S. Moore, *Human gene expression first occurs between the four- and eight-cell stages of preimplantation development.* Nature, 1988. **332**(6163): p. 459-61.
62. Tesarik, J., et al., *Early morphological signs of embryonic genome expression in human preimplantation development as revealed by quantitative electron microscopy.* Dev Biol, 1988. **128**(1): p. 15-20.
63. De La Fuente, R., et al., *Major chromatin remodeling in the germinal vesicle (GV) of mammalian oocytes is dispensable for global transcriptional silencing but required for centromeric heterochromatin function.* Dev Biol, 2004. **275**(2): p. 447-58.

64. Kageyama, S.-i., et al., *Alterations in epigenetic modifications during oocyte growth in mice.* Reproduction, 2007. **133**(1): p. 85-94.

65. Krukowska, A. and A.K. Tarkowski, *Mouse zygotes with one diploid pronucleus formed as a result of ICSI can develop normally beyond birth.* Mol Reprod Dev, 2005. **72**(3): p. 346-53.

66. Otsu, E., et al., *Developmental potential and chromosomal constitution of embryos derived from larger single pronuclei of human zygotes used in in vitro fertilization.* Fertil Steril, 2004. **81**(3): p. 723-4.

67. Liao, H., et al., *Cytogenetic analysis of human embryos and embryonic stem cells derived from monopronuclear zygotes.* J Assist Reprod Genet, 2009. **26**(11-12): p. 583-9.

68. Suss-Toby, E., et al., *Derivation of a diploid human embryonic stem cell line from a mononuclear zygote.* Hum Reprod, 2004. **19**(3): p. 670-5.

69. Polak de Fried, E., et al., *Human parthenogenetic blastocysts derived from noninseminated cryopreserved human oocytes.* Fertility and sterility, 2008. **89**(4): p. 943-947.

70. Dasig, D., et al., *Monozygotic twin birth after the transfer of a cleavage stage embryo resulting from a single pronucleated oocyte.* J Assist Reprod Genet, 2004. **21**(12): p. 427-9.

71. Gras, L. and A.O. Trounson, *Pregnancy and birth resulting from transfer of a blastocyst observed to have one pronucleus at the time of examination for fertilization.* Hum Reprod, 1999. **14**(7): p. 1869-71.

72. Barak, Y., et al., *Pregnancy and birth after transfer of embryos that developed from single-nucleated zygotes obtained by injection of round spermatids into oocytes.* Fertil Steril, 1998. **70**(1): p. 67-70.

73. Staessen, C., et al., *Cytogenetic and morphological observations of single pronucleated human oocytes after in-vitro fertilization.* Hum Reprod, 1993. **8**(2): p. 221-3.

74. Nagy, Z.P., et al., *Time-course of oocyte activation, pronucleus formation and cleavage in human oocytes fertilized by intracytoplasmic sperm injection.* Hum Reprod, 1994. **9**(9): p. 1743-8.

75. Dozortsev, D., et al., *Timing of sperm and oocyte nuclear progression after intracytoplasmic sperm injection.* Hum Reprod, 1995. **10**(11): p. 3012-7.

76. Payne, D., et al., *Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography.* Hum Reprod, 1997. **12**(3): p. 532-41.

77. Dale, B. and L. Santella, *Sperm-oocyte interaction in the sea-urchin.* Journal of Cell Science, 1985. **74**(1): p. 153-167.

78. Longo, F.J. and E. Anderson, *The fine structure of pronuclear development and fusion in the sea urchin, Arbacia punctulata.* J Cell Biol, 1968. **39**(2): p. 339-68.

79. Tesarik, J. and C. Mendoza, *Spermatid injection into human oocytes. I. Laboratory techniques and special features of zygote development.* Human Reproduction, 1996. **11**(4): p. 772-779.

80. Palermo, G.D., et al., *Intracytoplasmic sperm injection: a novel treatment for all forms of male factor infertility.* Fertil Steril, 1995. **63**(6): p. 1231-40.

81. Rienzi, L., et al., *Relationship between meiotic spindle location with regard to the polar body position and oocyte developmental potential after ICSI.* Hum Reprod, 2003. **18**(6): p. 1289-93.

82. Rosenbusch, B.E., *Selective microsurgical removal of a pronucleus from tripronuclear human oocytes to restore diploidy: disregarded but valuable?* Fertil Steril, 2009. **92**(3): p. 897-903.

83. Kattera, S. and C. Chen, *Normal birth after microsurgical enucleation of tripronuclear human zygotes: Case report.* Human Reproduction, 2003. **18**(6): p. 1319-1322.

84. Okamoto, I., et al., *Eutherian mammals use diverse strategies to initiate X-chromosome inactivation during development.* Nature, 2011. **472**(7343): p. 370-4.

85. Nino-Soto, M.I., P.K. Basrur, and W.A. King, *Impact of in vitro production techniques on the expression of X-linked genes in bovine (bos taurus) oocytes and pre-attachment embryos.* Mol Reprod Dev, 2007. **74**(2): p. 144-53.

86. Lengner, C.J., et al., *Derivation of pre-X inactivation human embryonic stem cells under physiological oxygen concentrations.* Cell, 2010. **141**(5): p. 872-83.

87. Kalantry, S., et al., *Evidence of Xist RNA-independent initiation of mouse imprinted X-chromosome inactivation.* Nature, 2009. **460**(7255): p. 647-51.

88. Namekawa, S.H., et al., *Two-step imprinted X inactivation: repeat versus genic silencing in the mouse.* Mol Cell Biol, 2010. **30**(13): p. 3187-205.

89. Patrat, C., et al., *Dynamic changes in paternal X-chromosome activity during imprinted X-chromosome inactivation in mice.* Proc Natl Acad Sci U S A, 2009. **106**(13): p. 5198-203.

90. Williams, L.H., et al., *Transcription precedes loss of Xist coating and depletion of H3K27me3 during X-chromosome reprogramming in the mouse inner cell mass.* Development, 2011. **138**(10): p. 2049-57.

91. Arney, K.L., et al., *Histone methylation defines epigenetic asymmetry in the mouse zygote.* Int J Dev Biol, 2002. **46**(3): p. 317-20.

92. van den Berg, I.M., et al., *Parental origin of chromatin in human monopronuclear zygotes revealed by asymmetric histone methylation patterns, differs between IVF and ICSI.* Mol Reprod Dev, 2009. **76**(1): p. 101-8.

93. Reik, W., et al., *Epigenetic asymmetry in the mammalian zygote and early embryo: relationship to lineage commitment?* Philos Trans R Soc Lond B Biol Sci, 2003. **358**(1436): p. 1403-9; discussion 1409.

94. Liu, H., J.-M. Kim, and F. Aoki, *Regulation of histone H3 lysine 9 methylation in oocytes and early pre-implantation embryos.* Development, 2004. **131**(10): p. 2269-2280.

95. Santos, F., et al., *Dynamic chromatin modifications characterise the first cell cycle in mouse embryos.* Dev Biol, 2005. **280**(1): p. 225-36.

96. Erhardt, S., et al., *Consequences of the depletion of zygotic and embryonic enhancer of zeste 2 during preimplantation mouse development.* Development, 2003. **130**(18): p. 4235-4248.

97. Yoshida, N., et al., *Epigenetic discrimination by mouse metaphase II oocytes mediates asymmetric chromatin remodeling independently of meiotic exit.* Dev Biol, 2007. **301**(2): p. 464-77.

98. Adenot, P.G., et al., *Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential transcriptional activity in pronuclei of 1-cell mouse embryos.* Development, 1997. **124**(22): p. 4615-25.

99. Jamieson, M.E., J.R. Coutts, and J.M. Connor, *The chromosome constitution of human preimplantation embryos fertilized in vitro.* Hum Reprod, 1994. **9**(4): p. 709-15.

100. Levron, J., et al., *Male and female genomes associated in a single pronucleus in human zygotes.* Biol Reprod, 1995. **52**(3): p. 653-7.

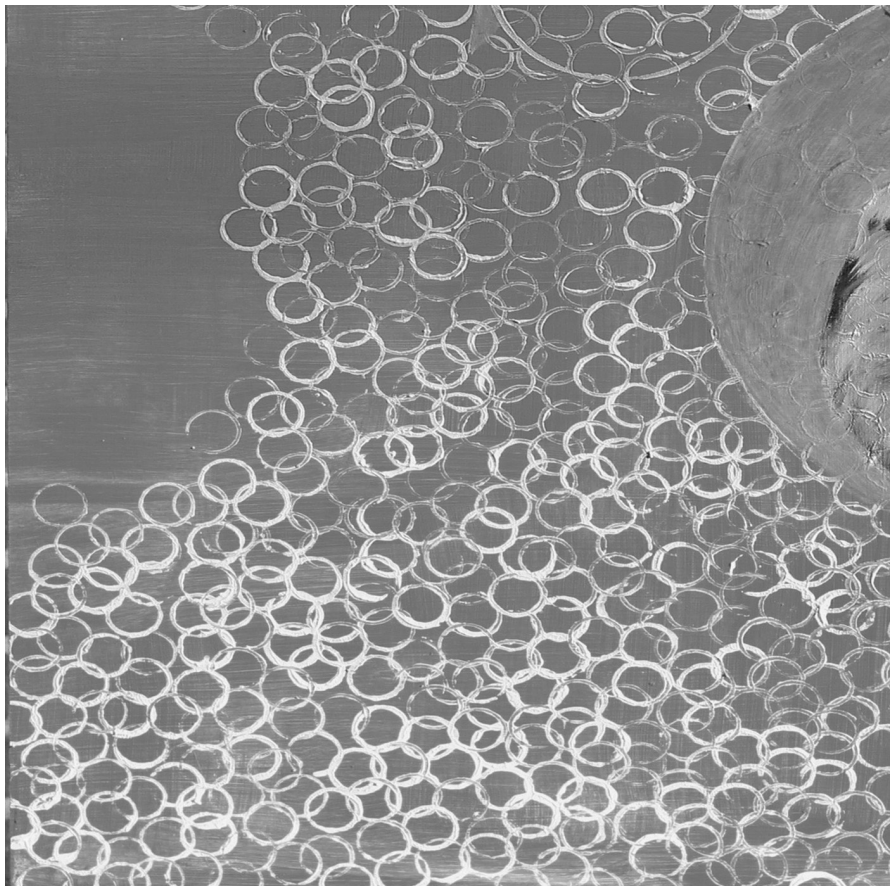
101. Sultan, K.M., et al., *Chromosomal status of uni-pronuclear human zygotes following in-vitro fertilization and intracytoplasmic sperm injection.* Hum Reprod, 1995. **10**(1): p. 132-6.

102. Staessen, C. and A.C. Van Steirteghem, *The chromosomal constitution of embryos developing from abnormally fertilized oocytes after intracytoplasmic sperm injection and conventional in-vitro fertilization.* Hum Reprod, 1997. **12**(2): p. 321-7.

103. Lim, A.S., et al., *Microscopic assessment of pronuclear embryos is not definitive.* Hum Genet, 2000. **107**(1): p. 62-8.

104. Yan, J., et al., *Assessment of sex chromosomes of human embryos arising from monopronucleus zygotes in in vitro fertilization and intracytoplasmic sperm injection cycles of Chinese women.* Gynecol Obstet Invest, 2010. **69**(1): p. 20-3.

CHAPTER 6



Addendum

Summary

This thesis describes several aspects of epigenetic regulation during different phases of gametogenesis and early embryogenesis. This chapter summarizes our main findings.

Chapter 2

It has been postulated that defective remodeling of the chromatin structure and defective deacetylation in particular is associated with chromosome mis-segregation and advanced maternal age in mouse oocytes. Because human oocytes have a high rate of chromosome aberrations we decided to study the acetylation/deacetylation kinetics in human oocytes.

During maturation from the germinal vesicle (GV) stage to the MII stage, deacetylation of lysine residues of histone 4 (H4) takes place. Both immature and mature human GV oocytes were acetylated for H4K5, H4K8, H4K12 and H4K16 which indicates that at this stage acetylation is not directly linked to transcriptional activity. During the process of oocyte maturation, the acetyl groups are removed which results in deacetylated chromosomes at the MII stage. Most likely, a general process of chromatin deacetylation exists in mammalian oocytes, however, some lysines are regulated differently in different species.

Although human oocytes display the same kinetics of deacetylation as mouse oocytes, a substantial percentage of oocytes failed to sufficiently deacetylate the histone acetyl residues. The percentage of human oocytes with residual acetylation of H4K12 significantly increases with advancing maternal age. It has been shown that induced hyperacetylation leads to chromosome mis-segregation in both mitosis and meiosis. We found that human oocytes that had residual acetylated chromosomes for H4K12 also had a high percentage of misaligned chromosomes or complete congression failure at the MII stage whereas oocytes that had no acetylation had a significant lower percentage of chromosome misalignment. Residual histone acetylation gave a 15 times higher risk on misalignment compared to correctly deactylated chromatin. The association of residual acetylation and chromosome misalignment was present equally in oocytes from women of different age groups. These data suggest that advancing maternal age increases the chance of defective histone deacetylation and these acetylated chromosomes subsequently fail to correctly align at the equatorial plate, thus predisposing the oocyte to aneuploidy. It is plausible that defective deacetylation during the final stages of mammalian oocyte maturation is an important factor in the etiology of age-related aneuploidy.

Chapter 3

A zygote contains the two haploid genomes from the parental gametes, the maternal genome from the oocyte and the paternal genome from the spermatozoon. Because little is known about the histone signature of chromatin in human zygotes, we decided to analyze histone modifications in human tri-pronuclear zygotes and with this new knowledge we determined the parental chromatin contribution in human mono-pronuclear zygotes.

We observed that the parental chromatin of human zygotes is differently remodeled for several histone modifications. The maternal chromatin contains both the open chromatin mark H3K4me3 as well as the heterochromatin marks H3K9me3 and H3K27me3. The paternal chromatin, on the contrary, does contain the open chromatin mark H3K4me3 but is devoid of the heterochromatin marks H3K9me3 and H3K27me3. This asymmetry was used to distinguish parental chromatin in mono-pronuclear zygotes. Staining of the chromatin for H3K9me3 showed that almost all 1 PN zygotes after IVF had an asymmetric pattern of chromatin for this histone modification which indicates both a maternal and paternal set of chromatin and that the oocytes were fertilized. On the contrary, ICSI mono-pronuclear zygotes were in most cases aberrantly fertilized as these zygotes contained either only maternal or only paternal chromatin.

Chapter 4.1

Dosage compensation ensures the equal expression of genes between males and females. Mammals achieve this by silencing one of the two X chromosomes in each female somatic cell. In the mouse this process of X chromosome inactivation (XCI) is initiated during pre-implantation development.

Human also use XCI as a means of of dosage compensation and chapter 4.1 describes that, like in the mouse, this process is initiated in human female pre-implantation embryos. The most important hallmarks of XCI are all present in cells of female human pre-implantation embryos. They are:

- coating of one X chromosome by the *XIST* RNA
- exclusion of *Cot1* RNA
- depletion of the open chromatin mark H3K9ac
- accumulation of the facultative heterochromatin mark H3K27me3
- incorporation of the histone variant macroH2A
- monoallelic expression of the X-linked gene CHIC1.

Chapter 4.2

Although the basic principles of XCI are similar in mouse and humans, differences exist in the timing of XCI initiation, the genetic elements involved in XCI regulation and the form of XCI in specific tissues. Chapter 4.2 describes and discusses these differences between human and mouse with respect to initiation of XCI in pre-implantation embryos, in the extra-embryonic tissues and in the embryonic stem cells which are the *in vitro* model of the epiblast. We further propose a different description of the XCI phenomenon, discriminating X chromosome remodeling from actual X chromosomal silencing. This is necessary because mouse and human pre-implantation embryos have a second X chromosome that is not as completely silenced in mouse pre-implantation embryos as it is in somatic cells. This probably allows for the reactivation in the cells of the ICM to prepare for random XCI. Therefore, the first steps towards the silenced state of the X chromosome that take place during pre-implantation development are better described by the term X chromosome remodelling (XCR) rather than by the term X chromosome inactivation (XCI).

Samenvatting

Dit proefschrift beschrijft voorbeelden van epigenetische regulatie tijdens de maturatie van eicellen en vroege embryonale ontwikkeling. Dit hoofdstuk geeft een samenvatting van de belangrijkste bevindingen.

Hoofdstuk 2

Een defecte vorming van de chromatine structuur en in het bijzonder een defecte deacetylatie van histon 3 en 4 tijdens de meiose worden geassocieerd met chromosoom missegregatie en een verhoogde maternale leeftijd bij muizen. Omdat humane eicellen een hoog percentage chromosomale afwijkingen bevatten, besloten wij het acetylatie-deacetylatieproces in humane eicellen te bestuderen. Onze resultaten lieten zien dat zowel mature als immature GV eicellen geacetyleerd waren voor de lysines H4K5, H4K8, H4K12 and H4K16. Omdat er in mature GV eicellen geen transcriptie meer plaatsvindt, is er op dit stadium mogelijk geen relatie tussen geacetyleerd chromatine en gentranscriptie.

Tijdens de laatste fase van het humane eicel-maturatieproces worden de acetylgroepen verwijderd wat uiteindelijk resulteert in gedeacetyleerde chromosomen in het MII stadium. Waarschijnlijk hebben zoogdieren een algemeen patroon van histon deacetylatie tijdens de eicel maturatie, al bestaan er verschillen tussen de individuele lysines en de verschillende diersoorten.

Hoewel humane eicellen een vergelijkbaar patroon van deacetylatie vertonen, heeft een substantieel percentage van de humane MII eicellen nog resterende acetylatie. Het percentage humane MII eicellen met resterende acetylatie neemt significant toe naarmate een vrouw ouder wordt. In diermodellen en somatische cellen is er aangetoond dat geïnduceerde hyperacetylatie leidt tot chromosoom missegregatie, zowel tijdens de mitose als tijdens de meiose. Wij observeerden dat een hoog percentage van de humane eicellen met resterende acetylatie van H4K12, een of meerdere losliggende chromosomen bevatten. Eicellen zonder acetylatie, echter, hadden een significant lager percentage van losliggende chromosomen. Een eicel met resterende acetylatie had een 15x hogere kans om abnormaal te zijn dan een eicel met verwijderde acetylatie. De relatie tussen resterende acetylatie van H4K12 en een verstoorde ordening van chromosomen op het equatoriale vlak, vonden wij in alle leeftijdsgroepen. Waarschijnlijk draagt de resterende histon acetylatie van chromosomen

bij aan de kans op chromosoom missegregatie en is het een belangrijke factor in het ontstaan van leeftijdsgerelateerde aneuploïdie in humane eicellen.

Hoofdstuk 3

Na fertilisatie bevat een zygote twee haploide genomen afkomstig van de parentale gameten; het maternale genoom van de eicel en het paternale genoom van de zaadcel. Omdat er weinig bekend is over de histon code van het chromatine in humane zygoten besloten wij histon modificaties te analyseren van humane tri-pronucleaire zygoten (3 PN) en met deze kennis hebben we de parentale contributie vastgesteld in mono-pronucleaire zygoten (1 PN).
Wij vonden dat er in humane zygoten verschillen zijn tussen de twee parentale chromatine structuren in de aanwezige histon modificaties. Het maternale chromatine bevat zowel heterochromatine als euchromatine modificaties namelijk respectievelijk H3K9me3 en H3K27me3 en de open chromatine marker H3K4me3. Het paternale chromatine daarentegen bevat alleen de open chromatine marker H3K4me3 en niet de heterochromatine markers H3K27me3 en H3K9me3. Dit verschil tussen het maternale en het paternale chromatine hebben wij toegepast om onderscheid te kunnen maken tussen maternaal en paternaal chromatine in mono-pronucleaire zygoten.

Na kleuring van het chromatine voor de asymmetrische marker H3K9me3 observeerden wij dat bijna alle 1 PN IVF zygoten beide parentale chromatine structuren bevatten wat betekent dat de eicellen waren bevrucht. In tegenstelling tot de IVF 1PN zygoten, was de meerderheid van de 1 PN zygoten na ICSI abnormaal bevrucht, aangezien deze zygoten óf alleen maar maternaal óf alleen paternaal chromatine bevatten.

Hoofdstuk 4.1

Dosis compensatie compenseert het verschil in gen-expressie tussen mannen en vrouwen. X chromosoom inactivatie (XCI) is het mechanisme dat zoogdieren gebruiken om de genexpressie van één X chromosoom stil te leggen. Ook mensen gebruiken XCI als dosis compensatie mechanisme en in hoofdstuk 4.1 is beschreven dat, net als in de muis, dit proces geïnitieerd wordt tijdens de pre-implantatie ontwikkeling. Wij vonden dat de meest belangrijke kenmerken van XCI aanwezig zijn in de kernen van humane vrouwelijke embryo's. Deze zijn:

- Coaten van één X chromosoom met het *XIST* RNA
- Exclusie van *COT1* RNA
- Depletie van de open chromatine marker H3K9ac
- Accumulatie van de facultatieve heterochromatine marker H3K27me3
- incorporatie van de histon variant macroH2A
- mono-allelische expressie van het op het X-chromosoom gelegen gen CHIC1

Hoofdstuk 4.2

Hoewel de basale kernmerken hetzelfde zijn tussen muizen en mensen, bestaan er verschillen in het moment van XCI initiatie, de betrokken genetische elementen in de regulatie van XCI en welke vorm van XCI aanwezig is in bepaalde weefsels. Hoofdstuk 4.2 beschrijft deze verschillen ten op zichte van XCI in humane en murine pre-implantatie embryo's, in de extra-embryonale weefsels en in de embryonale stamcellen, het *in vitro* model van de epiblast. Tijdens de murine pre-implantatie ontwikkeling wordt de paternale X preferentieel geïnactiveerd. Deze vorm van XCI wordt imprinted XCI genoemd. Aangezien er nog steeds transcriptionele activiteit is van de paternale X, is XCI nog niet volledig tijdens de pre-implantatie ontwikkeling. Ook wordt de Xp weer geactiveerd in de cellen van de ICM. Wij stellen voor dat de term X chromosoom remodeling (XCR) een betere omschrijving is van de eerste stappen in het dosis compensatie proces tijdens de pre-implantatie ontwikkeling dan de term X chromosoom inactivatie (XCI).

Glossary

Definitions

Parthenogenetic embryo

An embryo that contains only the genetic material derived from the oocyte. The second polar body is not extruded and the embryo is diploid. However, as it lacks the paternal DNA and its epigenetic imprints, parthenogenetic embryos are not viable. Parthenogenesis can occur naturally or by chemical induction.

Androgenetic embryo

An embryo that contains only the genetic material of the spermatozoon. An androgenetic embryo can arise by the fertilization of an empty oocyte by a single spermatozoon or by two spermatozoa. In the first case, the haploid DNA will be duplicated and the embryo is homozygous. In the latter case the embryo is heterozygous. An androgenetic embryo can also be artificially created by removal of the maternal DNA from the oocyte before insemination or sperm injection. Androgenetic embryos are not viable due to imprinting defects and usually result in spontaneous abortions.

Gynogenetic embryo

A gynogenetic embryo contains only maternal DNA but from two different oocytes. These embryos can only be artificially created and are used in imprinting and XCI studies. Gynogenetic embryos are not viable.

List of abbreviations

Ac	acetylation
AI	anaphase I
AII	anaphase II
APC/C	anaphase promoting complex/ cyclosome
ART	artificial reproductive technology
CHM	complete hyadatiform mole
CPC	chromosome passenger complex
DAPI	4',6-diamino-2-phenylindole
DNA	deoxyribonucleic acid
DSB	double strand break
EB	embryonic bodies
ES cells	embryonic stem cells
FISH	fluorescence *in situ* hybridization
rFSH	recombinant Follicle stimulating hormone
GV	germinal vesicle
GVBD	germinal vesicle breakdown
Hpi	hours post insemination
H4K12	lysine 12 of histone 4 (example)
HAT	histone acetyltransferase
HDAC	histone deacetylase
HMT	histone methyltransferase
ICSI	intra cellular sperm injection
ICM	inner cell mass
IF	immune fluorescence
IVF	*In vitro* fertilization
LH	luteinizing hormone
Me	methylation
MI	metaphase I
MII	metaphase II
MSCI	meiotic sex chromosome inactivation
MTOC	microtubule organizing centers
NHEJ	non-homologous end-joining
PAR	pseudo-autosomal region
PGC	primordial germ cell
Phos	phosphorylation
PN	proncleus (singular), pronuclei (plural)
PMSC	post-meiotic sex chromatin
PrE	primitive endoderm
PSCS	premature sister chromatid separation
PTM	post-translational modification
RNA	ribonucleic acid
SAC	spindle assembly checkpoint
TE	trophectoderm
Xa	active X chromosome
XCI	X chromosome inactivation
XCR	X chromosome remodeling
Xi	inactive X chromosome
XIC	X chromosome inactivation center
Xist/ XIST	X chromosome inactive specific transcript
Xm	maternal X chromosome
Xp	paternal X chromosome
ZP	zona pellucida

Curriculum Vitae

Ilse Maria van den Berg was born on the 23rd of December 1982 in Roosendaal and Nispen, The Netherlands. In 2002 she graduated from secondary school at the "Katholieke Scholengemeenschap Etten-Leur". After her graduation she started her study "Biologie en Medisch Laboratorium Onderzoek (HLO)" at the Avans University of applied Science. The internships at the Erasmus MC at the Department of Clinical Genetics, supervised by Dr. Diane Van Opstal, and the Department of Obstetrics & Gynaecology, supervised by Dr. Esther Baart and Dr. Elena Martini, raised her interest in chromosome segregation and human pre-implantation development. After finishing her Bachelor of Applied Science (B AS) in 2004, she started working as a research technician on the project Pre-implantation Genetic Screening (PGS) at the Departments of Clinical Genetics and Obstetrics & Gynaecology at the Erasmus MC. In august 2007, she started her Ph.D. project "Epigenetic reprogramming during human oocyte maturation and early human development" under supervision of Dr. Hikke van Doornink and Dr. Robert-Jan Galjaard. In June 2011, she obtained a travel grant from the KNAW/Ter Meulen Fund and she is currently visiting the lab of Dr. Nobuaki Kudo at the IRDB institute at Imperial College, London to study the first meiotic division in mouse wild type and Dicer deleted oocytes using the techniques of micro-injection and live imaging.

Ilse Maria van den Berg werd geboren op 23 december 1982. In 2002 voltooide zij het VWO aan de Katholieke Scholengemeenschap Etten-Leur. Daarna begon zij de opleiding Biologie en Medisch Laboratorium Onderzoek (HLO) aan de Avans Hogeschool (voorheen Hogeschool Brabant). Tijdens haar stages aan het Erasmus MC op de afdeling Klinische Genetica, onder supervisie van Dr. Diane Van Opstal, en op de afdeling Verloskunde & Gynaecologie, onder supervisie van Dr. Esther Baart en Dr. Elena Martini, werd haar interesse gewekt in de chromosoomsegregatie en de ontwikkeling van humane pre-implantatie embryo's. Na het behalen van haar Bachelor of Applied Science (B AS) diploma in 2004 begon zij als research analist op het project Pre-implantatie Genetische Screening op de afdelingen Klinische Genetica en Verloskunde & Gynaecologie van het Erasmus MC. In augustus 2007 startte zij als onderzoeker in opleiding (o.i.o) haar promotieproject getiteld "Epigenetische reprogrammering gedurende de humane eicel-maturatie en vroege embryonale ontwikkeling" onder supervisie van Dr. Hikke van Doorninck en Dr. Robert-Jan Galjaard. Momenteel bezoekt zij met een reisbeurs van het KNAW/Ter Meulen Fonds de onderzoeksgroep van Dr. Nobuaki Kudo van het IRDB van het Imperial College te Londen, waar zij bezig is met het opzetten van de technieken micro-injectie en live-imaging van oocyten voor het bestuderen van de eerste meiotische deling in wildtype en Dicer knock-out muizen.

PhD Portfolio

Summary of PhD training and teaching

Name PhD student: Ilse M. van den Berg Erasmus MC Department: Obstetrics & Gynaecology, subdivision Reproductive Medicine and Dept of Clinical Genetics Research School: MGC/Molmed	PhD period: 1-8-2007 / 1-3-2012 Promotor(s): Prof. Dr. F. Grosveld and Prof. Dr. J.S.E. Laven Supervisor(s): Dr. J.H. van Doorninck and Dr. R-J Galjaard

1. PhD training

	Year	Workload (Hours/ ECTS)
General courses		
– Reading and Discussing Literature	2008	4 ects
– Molecular Cell Biology	2008	6 ects
– Laboratory animal science	2009	4 ects
– Biomedical English Writing and Communication	2010	4 ects
Specific courses (e.g. Research school, Medical Training)		
– Informatics/Pubmed search	2008	16 hrs
– Veilig werken in het laboratorium (MGC)	2008	8 hrs
– Exam Anatomy and Physiology (tutorial)	2009	200 hrs
– From Development To Disease (MGC)	2009	24 hrs
– Epigenetics and Chromatin (MGC)	2009	6 hrs
– Basic introduction course on SPSS (MolMed)	2009	8 hrs
– Huygens Deconvolution Workshop	2010	8 hrs
– Writing succesful Grant Proposals (MolMed)	2011	10 hrs
– Photoshop & Indesign (MolMed)	2011	8 hrs
Seminars and workshops		
– OIC Hires workshop	2010	8 hrs
– ESHRE Pre congress course	2008	8 hrs
– NWO talent class Moving on in your Career	2011	8 hrs

Presentations and (inter)national conferences		
– MGC Research School annual meeting: oral presentation	2007	12 hrs
– Gordon Research Seminar on Meiosis (poster contribution)	2010	12 hrs
– Annual meeting of the British Society of fertility (invited lecture)	2010	8 hrs
– Annual research meeting of the department of Obstetrics & Gynaecology: oral presentation	2008/2011	24 hrs
– MolMed Research School annual meeting: poster presentation/oral presentation	2009/2011	24 hrs
– VFS meetings: oral presentation (award best presentation 2010)	2007/2010/2011	36 hrs
– ESHRE annual meeting	2008	24 hrs
– SGI annual meeting (oral presentation)	2009	40 hrs
– ESHRE annual meeting (oral presentation)	2009	40 hrs
– EMBO annual meeting (poster contribution(s))	2009	32 hrs
– Gordon Research Conference on Meiosis (poster contribution)	2010	60 hrs
– EMBO conference on Meiosis (poster contribution)	2011	32 hrs

Other activitites		
– Member of PhD committee	2009-2011	1 ects
– Member of the PhD-day organizing committee	2009-2011	1 ects
Scholarships & awards		
– Award for best oral presentation at Alpha meeting, Istanbul, Turkey	2008	1 ects
– Annual BFS/VFS exchange award for best presentation, Antwerp, Belgium	2009	1 ects
– Basic science award at the international ESHRE conference	2009	
– Research Fellowship Department Obstetrics & Gynaecology	2010	
– Simonsfonds travel grant for the attendance of attendance of the GRS/GRC, New London, USA	2010	
– Ter Meulen Fonds KNAW travel grant, for a work visit of 3 months at London Imperial College	2011	
– Trust Fonds travel grant for the attendance of the EMBO conference on Meiosis, Paestum Italy	2011	

2. Teaching		
– Supervision of Junior Science project	2008-2010	12 hrs
– Supervision of HBO laboratory students	2008-2010	2 ects
– Supervision of Medical Student for period of 5 months	2009-2010	1 ects
– Supervision of Junior Science project	2008-2010	12 hrs
– Supervision of HBO laboratory students	2008-2010	2 ects
– Supervision of Medical Student for period of 5 months	2009-2010	1 ects

List of publications

Peer reviewed scientific articles

1. **Van den Berg I.M.**, Galjaard RJ, Laven J.S.E, van Doorninck, J.H. *XCI in early mouse and human embryos: First there is remodelling…*(invited review) Hum Genet. 2011 Aug;130(2):203-15

2. **Van den Berg I.M.**, Eleveld C., van der Hoeven M., Birnie E., Steegers E.A.P, Galjaard R.-J., Laven J.S.E. *Defective deacetylation of histone 4 K12 in human oocytes is associated with advanced maternal age and chromosome misalignment* Hum Reprod. 2011 May;26(5):1181-90.

3. **Van den Berg I.M.**, Laven J.S.E, Stevens M., Jonkers I., Galjaard RJ, Gribnau J., van Doorninck J.H. *X Chromosome Inactivation Is Initiated in Human Preimplantation Embryos.* Am. J Hum genet 2009 June 12: 84(6): 771-779

4. **Van den Berg I.M.**, van der Heijden GW, Baart EB, Derijck A, Martini E, de Boer P. *Parental origin of chromatin in human mono-pronuclear zygotes revealed by asymmetric histone methylation patterns, differs between IVF and ICSI.* Mol Reprod Dev. 2009 Jan;76(1):101-8

5. Van der Heijden G.W., Derijck A., Baart E.B., **van den Berg I.M.**, Martini E., de Boer P. *Sperm-derived histones contribute to zygotic chromatin in humans* BMC Developmental Biology 2008, 8:34

6. Baart E.B., **van den Berg I.M.**, Martini .E, Eussen H.J., Fauser B.C., Van Opstal D. *FISH analysis of 15 chromosomes in human day 4 and 5 preimplantation embryos:the added value of extended aneuploidy detection.* Prenat Diagn. 2007 Jan;27(1):55-63.

7. Baart E.B., Martini E., **van den Berg I.M.**, Macklon N.S., Galjaard R.J., Fauser B.C., Van Opstal D. *Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF.* Hum Reprod. 2006 Jan;21(1):223-33.

Dankwoord

Ik schrijf dit hoofdstuk terwijl ik in Londen ben en dus op redelijke afstand van iedereen die mij geholpen heeft met het bereiken van deze mijlpaal. Maar misschien is dit wel de beste manier om terug te kijken naar mijn periode als onderzoeker in opleiding. In dit hoofdstuk wil ik iedereen bedanken die op zijn of haar manier heeft bijgedragen aan dit proefschrift.

Dr. J.H. van Doorninck, Hikke, bedankt dat je altijd in mij hebt geloofd. In het begin toen niet iedereen er van overtuigd was dat ik promovendus-waardig was en op het eind wanneer ik het zelf niet altijd meer zag zitten. Je hebt me leren onderzoeken (focus!), en dankzij jou ben ik enorm gegroeid (stel nu eens een vraag!). Je was altijd weer geïnteresseerd in mijn nieuwe hersenspinsels en je liet mij zelfs meeschrijven aan een nieuw onderzoeksproject (wat we nog toegewezen kregen ook!). Hoewel ik het niet altijd besef en laat blijken, ik waardeer het enorm hoe wij met elkaar omgaan. Je hebt een enorm gevoel voor humor en daardoor voelde ik mij altijd op me gemak (en soms iets te). Bedankt voor al je tijd, energie en toewijding. Ik hoop dat ik ook in de toekomst met je mag blijven samenwerken.

Dr. Galjaard, Robert-Jan, hoewel we geen regelmatige werkbesprekingen hadden, maakte je toch altijd tijd voor me vrij wanneer ik je weer eens een mailtje stuurde. Bedankt voor het meedenken en schrijven aan de manuscripten, tijd en zorgen.

Beste Prof. Dr. F. Grosveld en Prof. Dr. Laven, hartelijk dank dat jullie mij als HLO-er de kans hebben gegeven om te promoveren. Frank, elke keer als ik bij je langskwam voor advies ging ik weer vol inspiratie verder. Je weet altijd door te dringen tot de kern en zo mij op het juiste onderzoekspad te houden.

Joop, bedankt voor je steun en advies. Hoewel mijn project niet geheel in je vakgebied lag, wist je toch me altijd weer verder te helpen. Je hebt me in zekere zin vrijgelaten tijdens mijn promotie maar toch kon ik altijd langskomen voor advies. Bedankt voor je steun, je complimenten en je vertrouwen in mij.

Dr. Gribnau, Dr. Baarends, Dr. Poot, Joost, Willy en Raymond. Bedankt dat jullie in mijn kleine commissie wilden deelnemen en voor het kritisch doorlezen van mijn proefschrift. Joost, als XCI-expert was je onmisbaar voor mijn proefschrift. Bedankt voor al je tijd en advies. Willy, jouw meiose-kennis is onuitputtelijk. Bedankt voor al je advies en niet te vergeten je aanbevelingsbrieven. Raymond, bedankt voor het kritisch doorlezen van mijn proefschrift en ook voor je mooie woorden (via Hikke).

Prof. Dr. Repping en Prof. Dr. Hofstra, bedankt dat u wilt deelenemen in de grote commissie.

Cindy en Marjan, partners in crime. Mijn labmaatjes! Bedankt voor alle gezelligheid op het lab en op de kamer. Maar natuurlijk ook voor de mooie resultaten die we samen hebben behaald. Marjan, bedankt voor al je steun, ik waardeer je eerlijkheid en oprechtheid enorm. Ik kon altijd bij je terecht wanneer ik er weer helemaal doorheen zat. Cindy, jij was er van het begin bij. Jut en Jul (wie is nu ook alweer wie?). Eerst de PGS en daarna mijn (ons) promotie-onderzoek. Wij hebben heel wat ups en downs meegemaakt! Ik weet dat ik (soms) onuitstaanbaar kan zijn (nogmaals sorry) maar het komt altijd weer goed. Samen in het donker heeft op een lab een hele andere betekenis. Ik hoop dat we nog vaak samen van mijn moeders pasteitjes mogen genieten! Dear Joana, you joined our lab in June and then I left in October. It was a pleasure to work/have fun with you these few months. You made me appreciate my own country again (Bloemencorso in Zundert, jaarmarkt in het Ginnneken). Is Carla Perez still shaking?

Ik wil alle co-auteurs bedanken voor de prettige samenwerking en de mooie publicaties. De meiose-groep uit Nijmegen: Peter, Godfried, Alwin en Maud, bedankt.

Natuurlijk wil ik alle medewerkers van het Voortplantingscentrum bedanken, alle analisten, artsen, medewerkers van het secretariaat, mede-onderzoekers en embryologen. Ik ben bang dat ik hier mensen vergeet te noemen. Dit is geen kwade opzet, jullie zijn gewoon met velen. Het IVF-lab, het lab waar nieuw leven ontstaat. Esther en Wouter, bedankt voor jullie samenwerking/begeleiding. Ik heb veel van jullie geleerd, als stagiaire, analist en O.I.O. Ik wil iedereen bedanken voor de inzet (de roze stickers!!), jullie betrokkenheid en natuurlijk het verzamelen van al het materiaal: Jeroen, Diana, Amy, Sanne, Samantha, Karin en Karin, Nel, Shirley, Lydia, Linda, Hans en Miranda. Ook de vorige medewerkers: Elena, Helineth, Jaqueline, Martin, Heidi, Pieter en Carla. De medewerkers van het secretariaat, alle IVF artsen, alle verpleegkundigen en de stafleden; bedankt voor jullie medewerking. Erwin Berni en Fatima, bedankt voor jullie hulp bij de statistiek. Het wordt langzaam duidelijk.

Alle (vorige) medewerkers van Klinische Genetica, ik werk nu al een aantal jaren met veel plezier op jullie afdeling en ik kan altijd rekenen op jullie hulp. Julie zijn met zovelen dat ik bang ben dat ik iemand vergeet. Daarom bedank ik jullie als een groep, maar mijn dank is zeer groot! Prenatale Cytogenetica, daar waar het allemaal begon. In 2003 begon ik als stagiaire en leerde ik karyotyperen, FISH en CGH. Daarna kwam ik nog regelmatig langs voor “kweekbakjes”. Bedankt voor al jullie hulp en gezelligheid op het FISH-lab. Diane en Petra, nogmaals bedankt voor de begeleiding tijdens mijn stage. Alle medewerkers van de Postnatale en Tumor Cytogenetica, DNA diagnostiek, ook hier kwam ik regelmatig langs en jullie waren altijd bereid mij te helpen. Bedankt hiervoor. (Andere) Ilse, wij kennen elkaar nog van het HLO. Jij, Gaby en ik samen naar Rotterdam. Sindsdien is er veel gebeurd en zien we elkaar wat minder. Laten we toch maar weer eens lunchen!

Research groep Klinische Genetica/Oogheelkunde, Annelies en Bert, Hannie en Jolanda, Liesbeth, Daan, Thomas, Erwin en Anna. Bedankt voor al jullie hulp en de gezellige (afscheid) lunches en het dobbelspel.

Afdeling Voortplanting & Ontwikkeling, ook hier kwam ik regelmatig langs om iets te vragen of te "lenen". Joost en Willy bedankt voor jullie begeleiding langs de "zijlijn". Stefan, Iris, Evelien en Evelyne, Akiko, Sam, Bas, Godfried, Catherine en Maureen. Bedankt voor jullie tijd en hulp.

Niels Galjart en Frank Sleutels, hoewel ons projectje helaas geen succes werd, wil ik jullie toch bedanken voor jullie tijd en enthousiasme (en antilichamen). Misschien in de toekomst toch een bespreking?

Om de een of andere reden trok ons onderzoek alleen maar vrouwelijke studenten. Charlène, Mary, Arletta, Shimaira, Khatera, Marjon en Arewika. Ook jullie hebben bijgedragen aan mijn proefschrift. Bedankt voor jullie inzet. Ook voor mij was het begeleiden een zeer leerzame ervaring. Ook wil ik al mijn mede-o.i.o's bedanken (gedeelde smart is halve smart): Daan, Christine, Margarida, Liesbeth, Thomas, Erwin, Anna en alle promovendi van V&G: Olivier, Sharon, Yvonne, Wendy, John, Fatima, Sam, Bas en Marijana (en iedereen die ik vergeet te noemen, ook jullie zijn met velen).

De mannen van de confocale, Alex en Gert, altijd weer bereid om mij te helpen met mijn onmogelijk onderzoeksmateriaal. Dankzij jullie heb ik heel wat mooie foto's en filmpjes kunnen maken!! Bedankt. Tom en Ruud, jullie hebben mijn microscoop-plaatjes bewerkt tot publicatie-waardige foto's. De 7de verdieping. Ook hier kwam ik regelmatig voor hulp. Mariëtte, Mhernaz, Jeffrey, Ton, Josef, Leo en Mario, Melle , Leo en Koos, bedankt voor al jullie hulp.

John, Iris en Alex, bedankt voor jullie hulp bij de dierexperimenten. Iris, bedankt voor je flexibele instelling (ook op het laatste moment). John, met jou is het nooit saai op het EDC! Bedankt voor al je tips en hulp. Het Biomics-team, Wilfred, Antoine, Zeliha, Christel en Edwin. Bedankt voor jullie hulp.

Dr. Kudo, Kaisa, Ben, Dafni, Gemma, Anne, Ruth and all my other Londen colleagues. Thanks for these last months. Biba en Zoë, thanks for your help with the micro-injection.

Dear prof. Gough and miss Suzuki, David and Erico, I was almost afraid that I couldn't find a reasonable room in London. Thanks for your warm welcome and making me feel at home. Dear David, thanks for your input on my thesis. Next time, I'll beat you with chess!

Meiden van de trein: Jojanneke, Jolanda, Sanne en Wendy. Bedankt voor alle gezelligheid die soms ook na de trein werd voortgezet (Pecannoten-kalua cake/ Sangria!). De reis verliep niet altijd even vlekkeloos (waterbus) en geen onderwerp bleef onbesproken. De lange reistijd werd dankzij jullie een stuk dragelijker.

ECS computers: Eddy, Stefan, Jaap, Gerry, Bjorn, Sjoerd, Heidi, Ingrid en mijn Eddy. Bedankt voor jullie gastvrijheid. Eddy Mouws, bedankt dat ik bij jullie in “afzondering” mocht schrijven.

Jeroen, een hele tijd geleden heb ik je al gevraagd om mijn paranimf te zijn. Jij bent iemand van het bredere perspectief. Bedankt voor alle discussies, boekentips en advies die je me hebt gegeven (heb je dat stuk gelezen in het NRC? Wat zijn hoogopgeleide mensen toch onnozel en kortzichtig!). Ik hoop dat we het nog vaak met elkaar (on)eens gaan zijn! Evelien, bedankt voor het ontwerpen van de omslag. Mijn proefschrift is in ieder geval een artistiek meesterwerk.

Denise en Marjolein, wij gaan ver terug. Hoewel we elkaar wat minder vaak zien dan toen (door drukte en afstand), het voelt altijd weer als vanouds. Of we nu gaan carnavallen (waar is mijn snor?), wadlopen (arme Eddy) of Madrid bezichtigen (heerlijk die Paëlla!), we maken er altijd iets bijzonders van. Ik hoop dat we nog vaak samen erop uit gaan.

Jan en Willy, Wendy en Gremar. Mijn tweede familie. Jullie zorgen voor de juiste afleiding. Of we nu gamen, joggen of kamperen, met jullie is het altijd een gezellig avontuur. Volgend jaar gewoon weer met Sint surprise??

Mijn thuisbasis: Papa, Mama. Bedankt voor al jullie steun, talloze knuffels, heerlijke pasteitjes, luisterende oren en zorgen (zelfs over zee)! Ik weet dat ik altijd bij jullie terecht kan. Saar en Cris, en natuurlijk de jongens, Thijs en Lucas. Bedankt voor jullie steun en begrip. Lieve Saar, deze smurf kan zich geen betere zus wensen! Niet voor niets ben jij mijn paranimf. We hebben al heel wat meegemaakt, weet dat ik er ook altijd voor jou ben!

Lieve, lieve Eddy (TW). Waar zal ik beginnen. Zonder jouw steun was dit boekje er niet geweest. Hoe vaak heb ik je in het weekend niet kunnen verleiden tot het maken van een motorritje naar Rotterdam. Je kent alle smaken van het koffiezetapparaat op de 22ste en was thuis (en soms ook op het werk) mijn ICT-redder-in-nood! Hoewel we beiden zeiden er nooit aan te beginnen is het je toch gelukt een van Opdorp van mij te maken. En dan twee weken daarna weer uit elkaar. Jij in Breda en ik in Londen. Maar: Ik hou van jou, alleen van jou. Ik kan niet leven in een wereld……. (PW).

Colour figures

Chapter 2

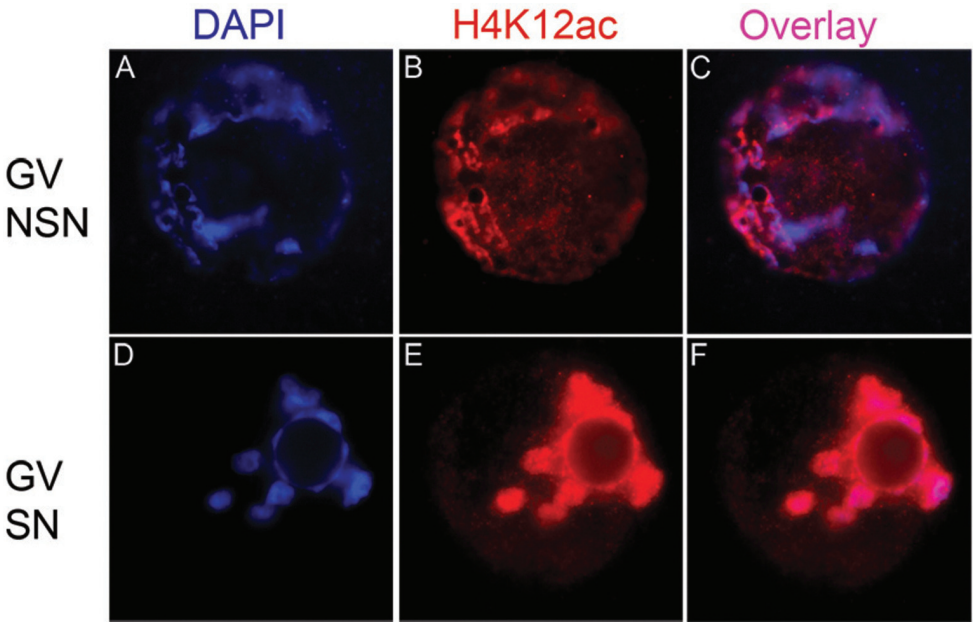


Figure 1. Histone acetylation in human GV oocytes. An early-stage human GV oocyte with a non-surrounding nucleus (NSN)(A-C) stained for chromatin (blue, DAPI); (A) and histone acetylation (red, anti-H4K12ac) (B). Some chromatin regions have intense staining, whereas other (dense) regions showed no acetylation (overlay C). A more developed human GV oocyte with a surrounding nucleus stained for chromatin (blue, DAPI; D) and histone acetylation (red, anti-H4K12ac; E) has chromatin that is far more condensed (D) than the NSN GV, but it still has acetylated chromatin (E), as shown by the overlay (F). Scale bar represents 10 µm (see page 49).

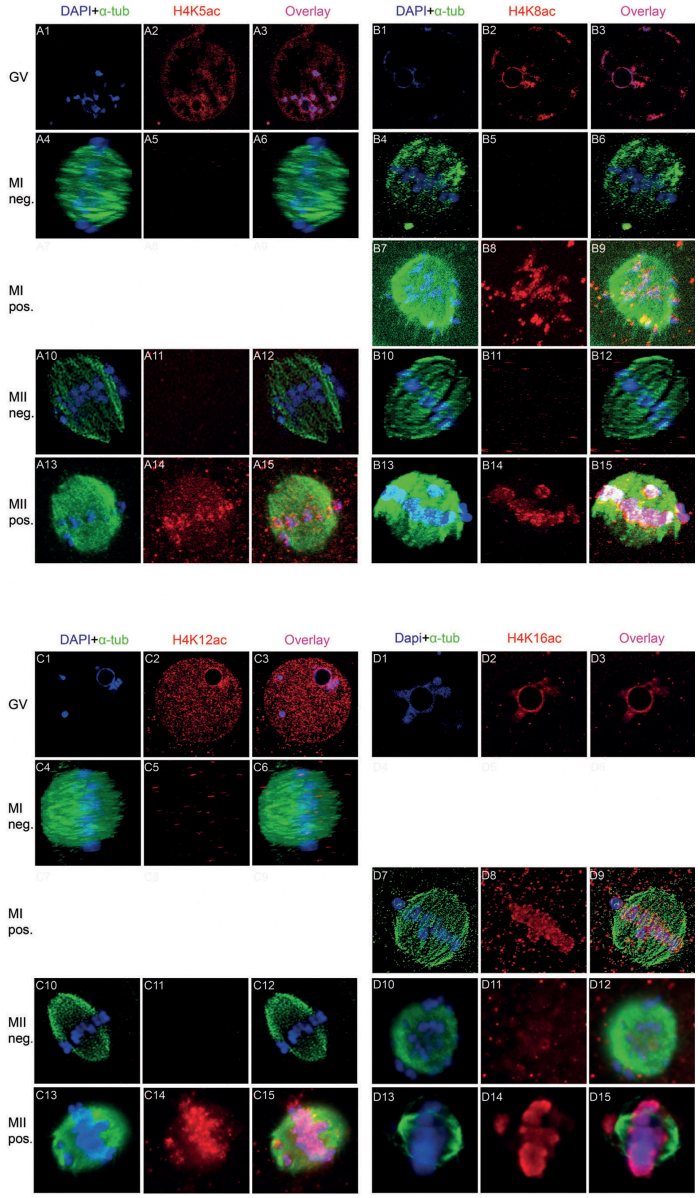


Figure 2. Histone deacetylation during human oocyte maturation. GV, MI and MII oocytes were stained for H4K5ac **(A1–A15)**, H4K8ac **(B1–B15)**, H4K12ac **(C1–C15**) and H4K16ac **(D1–D15)**. Representative images are shown for the indicated groups. GV oocytes showed intense staining for all four lysine residues (A2, B2, C2 and D2) and were not analyzed for a-tubulin because no spindle is present at this stage. At the MI stage, the majority of oocytes had no staining for H4K5ac **(A4–6)**, H4K8 **(B4–6)** and H4K12ac **(C4–6)**. H4K16ac was positive in all tested MI oocytes **(D7–9)**. In oocytes of the MII stage, a variable part of the oocytes showed no staining for the four tested lysine acetylations **(A11–D11)**. In oocytes with residual acetylation, histone lysine staining was observed **(A14–D14)** overlapping with the DAPI-stained chromosomes **(A13–A15 to D13–D15)**. Scale bar for **A1–A3, B1–B3, C1–C3** and **D1–D3** represents 30 µm. Scale bar for **A4–A15, B4–B15, C4–C15** and **D4–D15** represents 10 µm (see page 50).

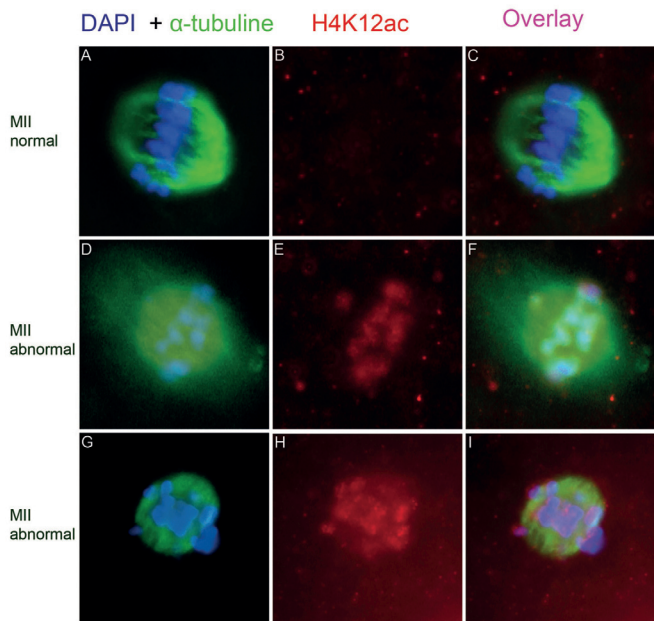


Figure 3. Metaphase alignment and residual acetylation in human MII oocytes. Representative immunofluorescence of an MII oocyte without staining of H4K12ac and with properly aligned chromosomes in the equatorial plane **(A–C)**. Residual acetylation of H4K12ac in an MII oocyte is associated with chromosome misalignment of a single chromosome **(D–F)** or congression failure **(G–I).** Scale bar represents 10 µm (see page 53).

Chapter 3

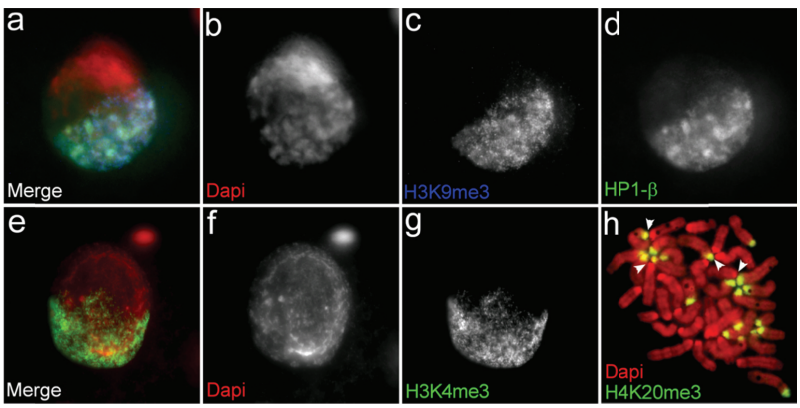


Figure 1. Diploid mouse mono-pronuclear zygotes. Examples of pre S-phase (**A-G**) and mitotic (H) mono-pronuclear zygotes **A-D**: Zygote stained for H3K9me3 (blue) and the H3K9me2,3 binding protein HP1-β [43]. (green) Dapi labels DNA (red). **E-G**) zygote stained for H3K4me3 (green), Dapi labels DNA (red). **H**) zygote stained for H4K20me3 (green), Dapi labels DNA (red). Histone H4K20me3 marks 20 maternal chromosomes at the constitutive heterochromatin. It has been reported that chromosomes in the mouse zygote are interconnected via their α-satellite sequences [38]. This causes some chromosomes to be positioned in a head to head position. Arrowheads indicate head-to-head position of maternal and paternal chromosomes (see page 69).

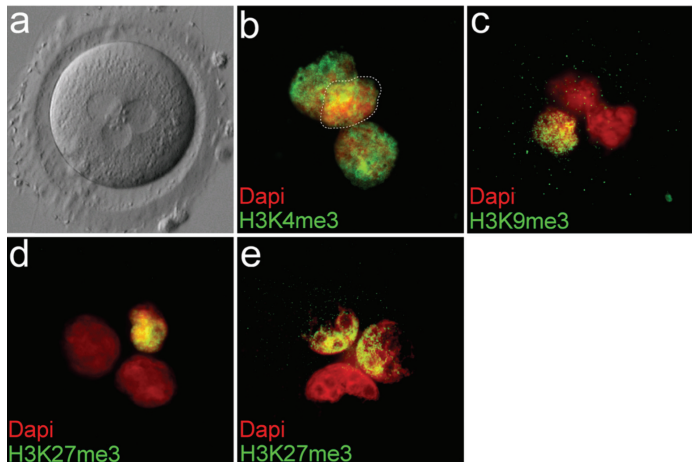


Figure 2. Distribution of H3K4me3, H3K9me3 and H3K27me3 in human tri-pronuclear zygotes. **A**) A light microscopic image of a tri-pronuclear zygote prior to removal of zona pellucida and fixation. **B**) Symmetrical distribution of trimethylated histone H3 lysine 4. Dotted line indicates position of the maternal PN, which can be distinguished from the paternal PN by size. **C**) Asymmetrical localization of histone H3K9me3. A tri-pronuclear zygote obtained after conventional insemination and therefore likely the result of polyspermia. This histone modification is absent from the two larger paternal pronuclei. **D-E**) Absence of histone H3K27me3 from paternal chromatin. In tri-pronuclear zygotes obtained after insemination, H3K27me3 was present in the smaller maternal PN (D). A rare case of a tri-pronuclear zygote after ICSI (E). In these zygotes failure of second polar body extrusion is responsible for the extra PN. Therefore two PNs show this maternal mark (see page 71).

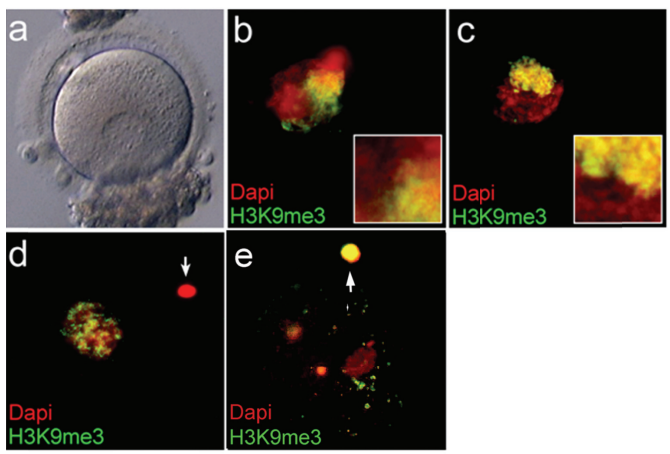


Figure 3. Distribution of H3K9me3 in human mono-pronuclear zygotes **A**) A mono-pronuclear zygote prior to removal of zona pellucida and fixation. **B**) A mono-pronuclear zygote with a clear fusion of the chromatin domains alike the mouse mono-pronuclear zygotes in Figure 1. Two out of focus sperm heads are overlying the pronucleus. Higher magnification shows a diffuse region in between parental chromatin domains. **C**) The mono-pronuclear zygote depicted in 3a after staining. Two not overlapping chromatin domains in close proximity are observed. Higher magnification shows a clear separation of the parental domains. **D**) A mono-pronuclear zygote obtained after ICSI in which the oocyte is activated by the sperm (indicated by arrow) but no further nuclear decondensation of the sperm has occurred. **E**) A mono-pronuclear zygote obtained after ICSI which contains paternally derived chromatin only. The positive domain in proximity of the PN is a polar body (see page 72).

Chapter 4.1

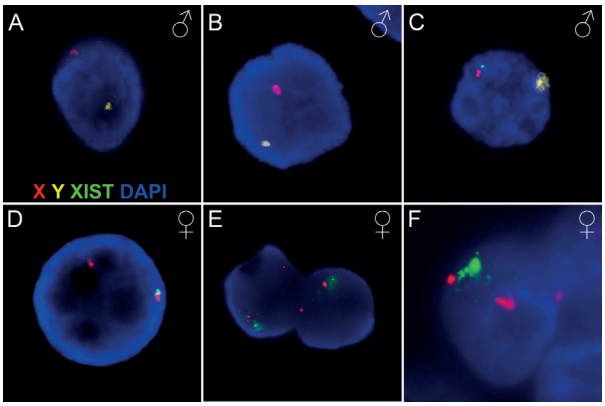


Figure 1. *XIST* expression in male and female human pre-implantation embryos. RNA and DNA FISH staining with probes to detect *XIST* RNA (green) ,the X (red) and Y chromosome (yellow) and DAPI counterstain. Human male embryos **(A-C)** do not generally show *XIST* signals at the 8-cell stage (A) or at the morula stage (B). A minority of male cells at the morula stage show a pinpoint of *XIST* staining (C). Female embryos **(D-F)** show an *XIST* pinpoint in the majority of embryos at the 8-cell stage (D). (E) Two cells at the morula stage each show a beginning cloud of XIST on one of their two X chromosomes. (F) At the blastocyst stage this has further accumulated to a full cloud on one of the two X chromosomes. A third diffuse red signal is an X chromosome from an adjacent cell that is in a different focal plane (see page 87).

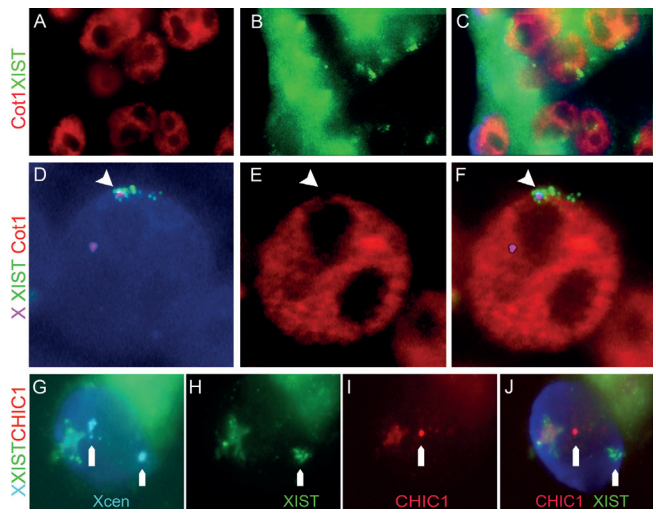


Figure 2. Transcriptional changes on the inactive X chromosome. **(A-F)** *Cot1* exclusion around *XIST*. **(A-C)** Cells of a female blastocyst embryo with staining for *Cot1* RNA (red in A) and *XIST* RNA (green in B) showing depleted regions of *Cot-1* RNA around the *XIST* signals indicating the position of the inactivated X (merged in C). **(D-F)** Representative cell of a female blastocyst with staining for the X centromeres and *XIST* RNA (D, Xcen in magenta, *XIST* in green) together with *Cot1* (red in E). Transcription of *Cot1* RNA was absent in a region that overlaps with *XIST* RNA staining (F), while the active X without *XIST* staining overlaps with a *Cot1*-positive region. **(G-J)** Female blastocyst cell with two X centromeres (cyan in G) has a single *XIST* cloud on one X chromosome (green in H) and monoallelic expression of *CHIC1* on the other X chromosome (red in I, merged in J). A dust spot is visible in all colors and is therefore nonspecific staining (see page 89).

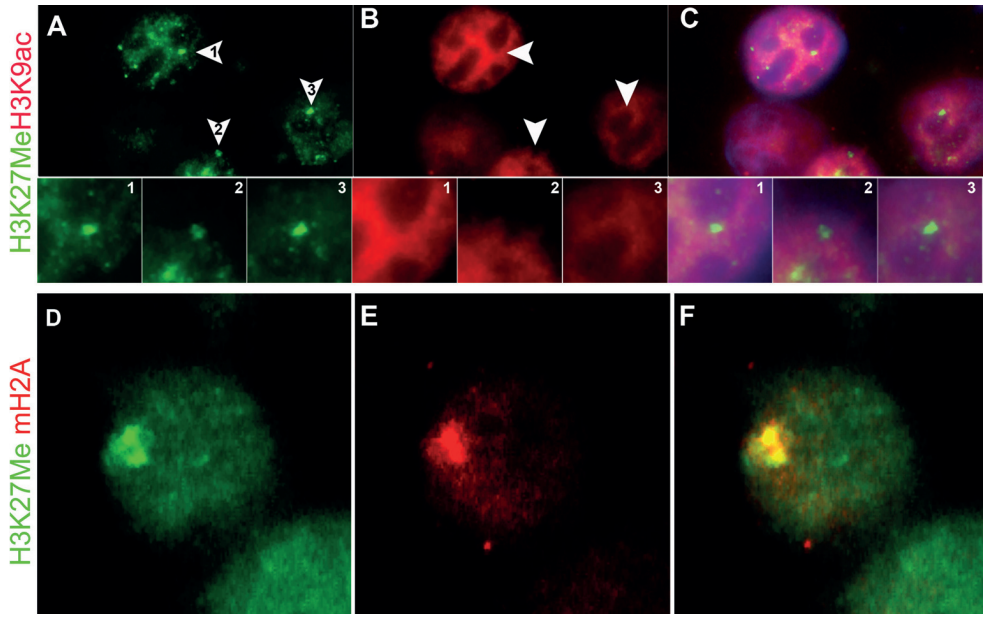


Figure 3. Epigenetic changes on the inactive X chromosome. **(A-C)** Three adjacent blastocyst cells show H3K27Me3 hypermethylation (arrowheads in J and enlarged panels 1-3) and staining for H3K9 acetylation (B, 1-3) shows a H3K9ac-depleted region overlaying the H3K27Me3 accumulation (C, 1-3) indicating the position of the Xi chromosome. **(D-F)** Representative blastocyst cell shows H3K27 hypermethylation (green, D) and enrichment for macroH2A (red, E) with a clear overlap (yellow, F) analogous to the signal around an Xi chromosome (see page 90).

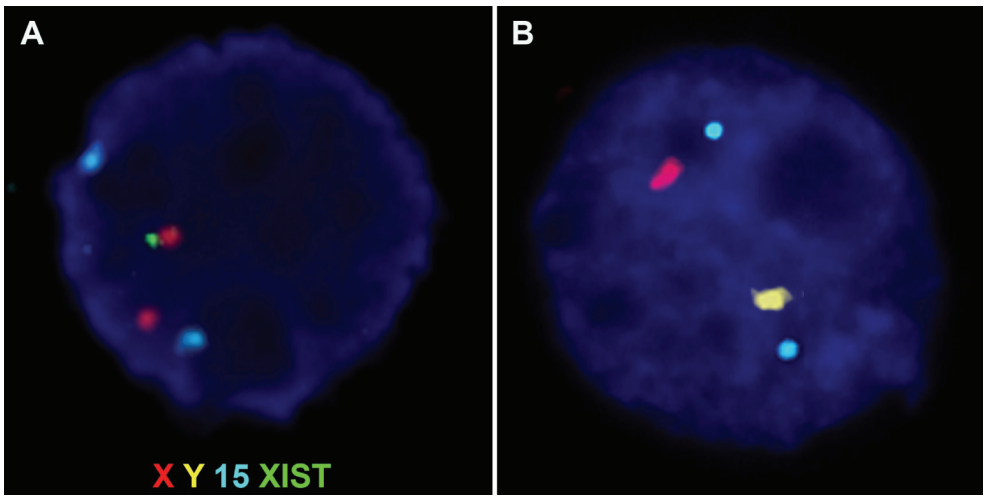


Figure S1. Single cell RNA/DNA FISH analysis of human embryos. **(A)** Diploid blastomere from a female 8-cell embryo displaying two X centromeres (red), two chromosome 15 centromeres (aqua), and an *XIST* pinpoint (green). **(B)** Diploid male blastomere from a 12-cell male embryo with an X chromosome (red), a Y chromosome (yellow) and two chromosomes 15 (aqua). *XIST* RNA was absent (see page 99).

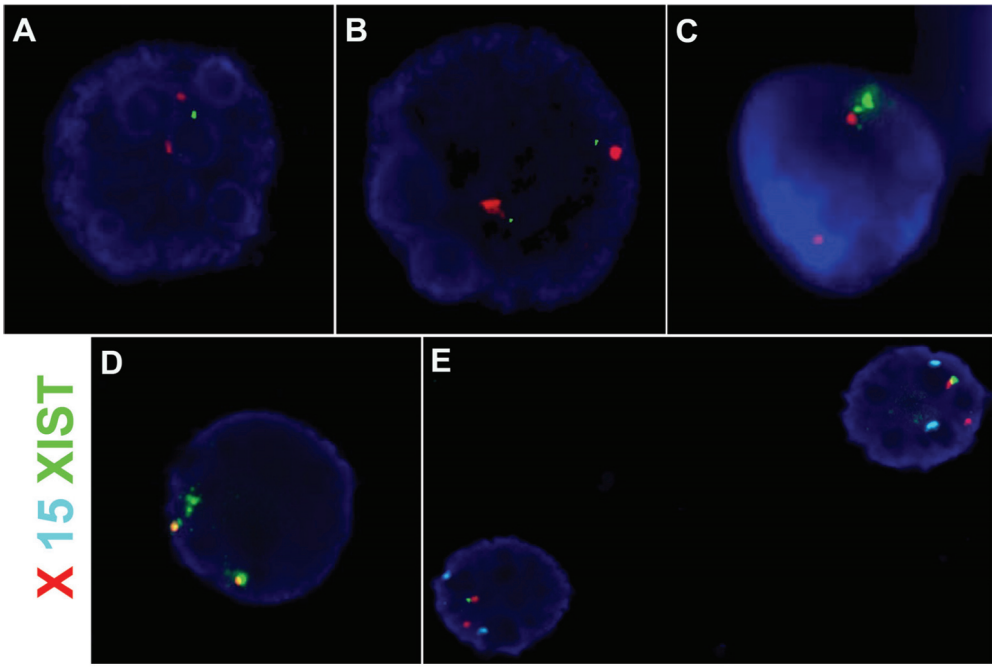


Figure S2. Examples of different patterns of *XIST* RNA signals in female embryos. **(A)** Single pinpoint of *XIST* (green) near the X centromere (red) at the 8-cell stage. **(B)** Two pinpoint signals of *XIST* at morula stage. **(C)** Single cloud signal in blastocyst. **(D)** double cloud signals of *XIST* in late morula embryo. **(E)** Two cells from a morula, one with a pinpoint *XIST* signal and the other with an intermediate cloud of *XIST* RNA (see page 99)**.**

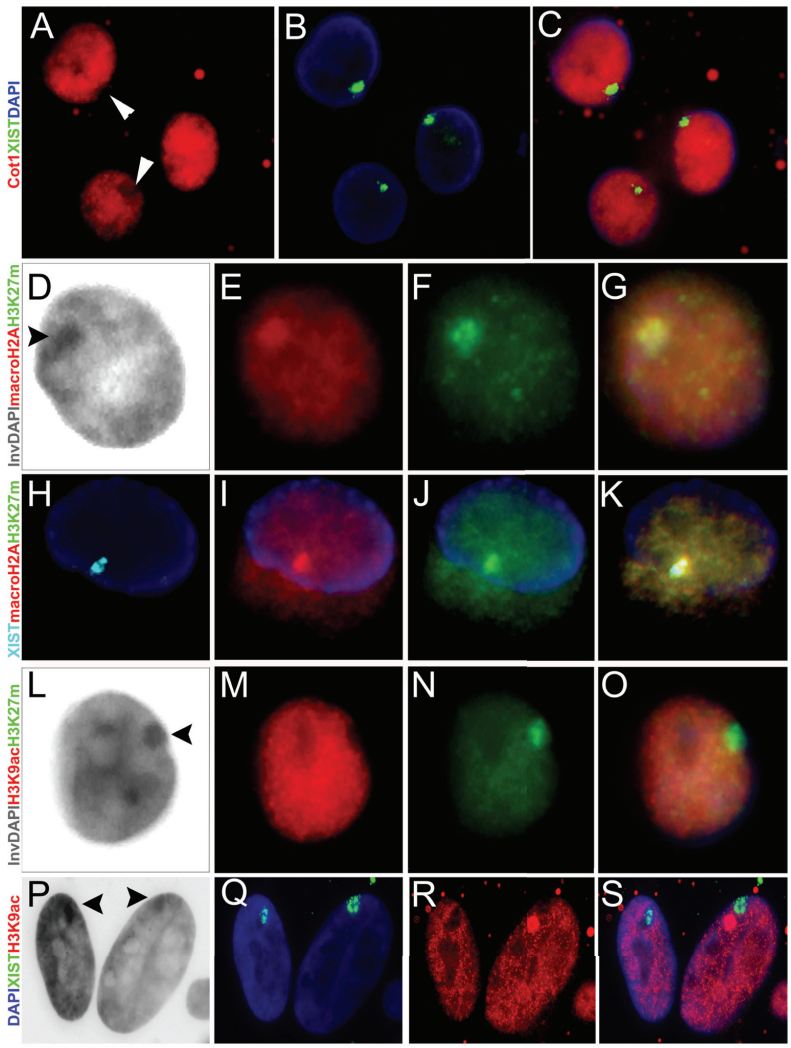


Figure S3. Epigenetic changes on the inactive X chromosome in human cumulus cells **(A-G, L-O)** and female amniocytes **(H-K, P-S).** RNA FISH and immunocytochemistry of human female cumulus cells with probes and antibodies commonly used to characterize the inactive X chromosome. *Cot1* RNA FISH staining of cumulus cells (red in A) shows an excluded area (indicated with arrowheads), that overlaps with *XIST* RNA staining (green in B) as shown in the overlay in (C). The third nucleus is not in focus and the *Cot1* depleted region can thus not be seen. Inverted DAPI staining reveals the position of the inactive X/Barr body (arrowhead in D) in cumulus cells that overlaps with macroH2A staining (red in E) and H3K27Me3 (green in F), merged in G. **(H-K)** In female amniocytes, *XIST* RNA (H) shows a complete overlap with the accumulated nuclear domains of macroH2A (red in I) and H3K27me3 (green in J) as shown in the overlay (merged in K). (L) Cumulus cell with Barr body that is indicated with an arrowhead in the inverted DAPI image . H3K9 acetylation staining shows exclusion of the Barr body area (red in M) and H3K27Me3 gives a strong localized signal staining (green in N) that overlaps exactly with the exclusion of H3K9ac and the position of the Barr body (merged in O). In female amniocytes the Barr body (arrowheads in P) overlaps the *XIST* cloud (green signal in Q) and at this area H3K9ac is depleted (red in R) as shown in the overlay (merged in S) (see page 100).

Chapter 4.2

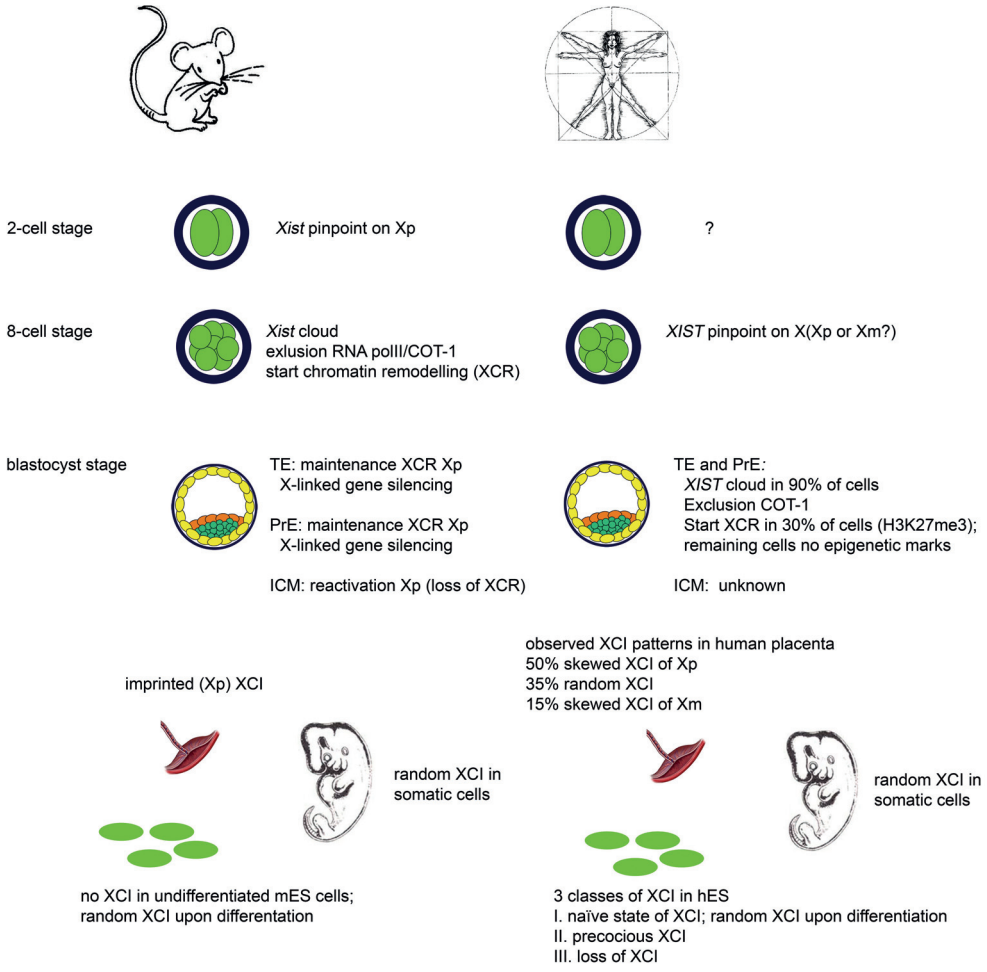


Figure 1. Schematic overview of the three different steps of XCI in mouse and man.
Mouse: In the mouse 2-cell stage embryo, imprinted XCI begins with pinpoint Xist expression from the paternal X. At the 8-cell stage the Xp chromosome is remodeled (XCR, see text for details) with *COT-1* exclusion and epigenetic marks; this remodeled X chromosome generally becomes inactivated (XCI) at the blastocyst stage. Mouse blastocyst ICM cells reactivate the paternal X while the TE and PrE retain the imprinted form of XCI (see text for more detailed description). The imprinted form of XCI is maintained in the placenta while the epiblast converts to a random XCI mechanism. Human: No data are available for single human 2-cell stage embryos regarding the level and location of *XIST* expression. At the 8-cell stage most cells have a single pinpoint of *XIST* expression but whether this is an imprinted XCI is not yet known. Human blastocysts have a full cloud of *XIST, COT1* exclusion, epigenetic marks and mono-allelic expression of a gene adjacent to XIST in a portion of the cells indicative of XCR and the initiation of XCI [47]. Data on XCI in human placenta point towards a preferential silencing of the paternal allele, although random XCI patterns are often observed. The model hES cell lines to study random XCI are not as good in humans as they are in mice: undifferentiated mouse ES cells have two active X chromosomes and upon differentiation one X is randomly silenced. However, undifferentiated human ES cells are extremely variable in *XIST* expression and so far three classes have been described (see text). ICM = inner cell mass, TE = trophectoderm, PrE = primitive endoderm (see page 109).

Chapter 5

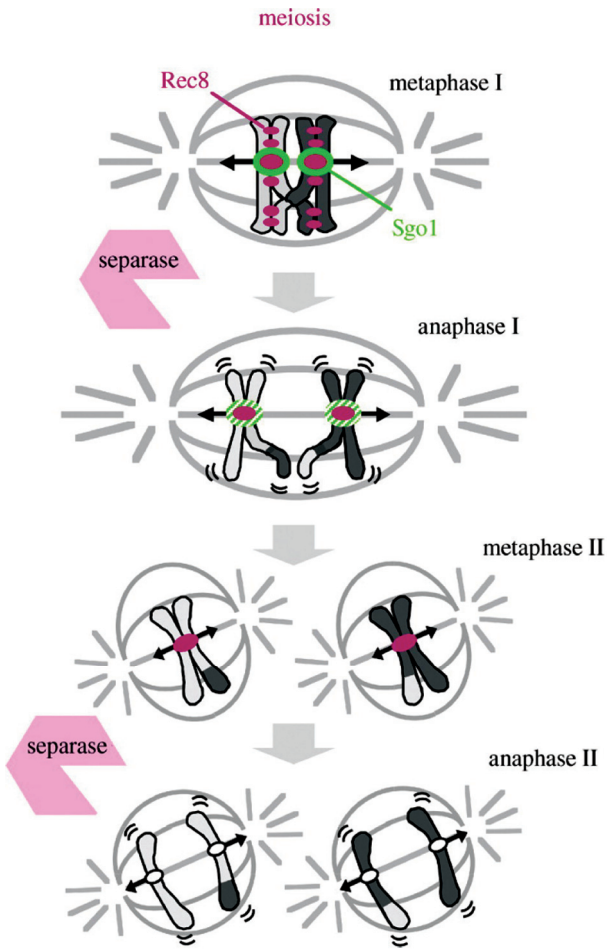


Figure 1. Stepwise cleavage of the cohesion complex in meiosis. The cohesion complex holds the two sister chromatids together. At the transition from MI to AI the REC8 cohesin between the arms of the chromatids is cleaved by Separase to allow the homologues to be separated. Centromeric cohesion is protected by the Shugoshin proteins which prevents untimely cleavage of the sister chromatids at the MI-AI transition. At the MII stage the sister chromatids are now bi-orientated to the spindle poles. This orientation creates tension on the centromeres which relocates Shugoshin from cohesin and allows REC8 to be cleaved by Separase at the MII to AII transition (adapted from [18]) (see page 129).

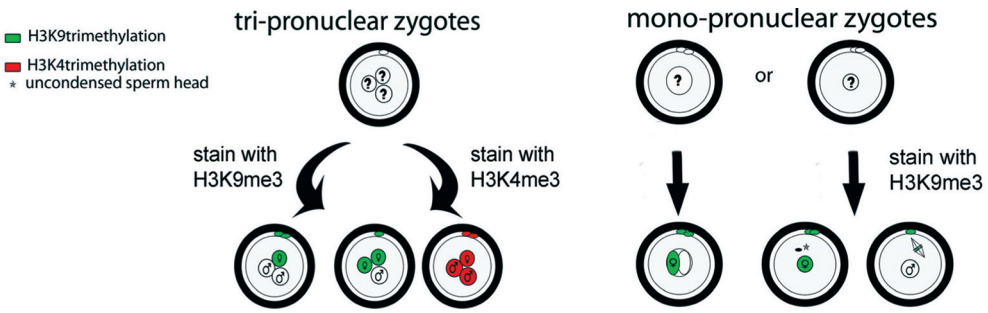


Figure 2. Schematic overview of the experimental approach of our study on human tri-pronuclear and mono-pronuclear zygotes (see page 135).