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Unravelling chromosomal instability in mammalian preimplantation embryos using single-cell genomics

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Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

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Supervisor: Professor Andres Salumets, PhD
Department of Obstetrics and Gynaecology /
Institute of Biomedicine and Translational Medicine,
University of Tartu, Estonia

Professor Joris Robert Vermeesch, PhD
Laboratory for Cytogenetics and Genome Research /
Center for Human Genetics, KU Leuven, Belgium

Professor Ants Kurg, PhD
Department of Biotechnology / Institute of Molecular and Cell Biology, University of Tartu, Estonia

Reviewers: Professor Alireza Fazeli, PhD
Department of Pathophysiology / Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

Dr. Martti Laan, PhD
Department of Molecular Pathology / Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

Opponent: Professor Joyce Harper, PhD
Department of Reproductive Health / Institute for Women's Health / University College London, United Kingdom

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Fundamentally, the embryo of a higher animal form
Never resembles the adult of another animal form
But only it's embryo
Karl Ernst von Baer
"Über Entwickelungsgeschichte der Thiere"

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles:

I. Destouni, A.; Zamani Esteki, M.; Catteeuw, M.; **Tšuiko, O.**; Dimitriadou, E.; Smits, K.; Kurg, A.; Salumets, A.; Van Soom, A.; Voet, T.; Vermeesch, J.R. (2016). Zygotes segregate entire parental genomes in distinct blastomere lineages causing cleavage stage chimaerism and mixoploidy. *Genome Research*, 26(5): 567−578.

II. **Tšuiko, O.**; Catteeuw, M.; Zamani Esteki, M.; Destouni, A.; Bogado Pascottini, O.; Besenfelder, U.; Havlicek, V.; Smits, K.; Kurg, A.; Salumets, A.; D'Hooghe, T.; Voet, T.; Van Soom, A.; Vermeesch, J.R. (2017). Genome stability of bovine in vivo-conceived cleavage-stage embryos is higher compared to in vitro-produced embryos. *Human Reproduction,* 1;32(11):2348–2357.

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Study I – Participation in wet-lab experiments and data analysis; revision of the manuscript

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Study III – Participation in the study design, wet-lab experiments and data analysis; preparation of the original manuscript

ABBREVIATIONS

aCGH – array comparative genomic hybridization
ADO – allele drop-out
AI – artificial insemination
aMTOCs – acentriolar microtubule organizing centers
APC/C – anaphase-promoting complex/cyclosome
ART – assisted reproductive technologies
ATP – adenosine triphosphate
BAF – B-allele frequency
BF – blastocoel fluid
BFB – breakage-fusion-bridge cycle
BSA – bovine serum albumin
CCS – comprehensive chromosome screening
CHM – complete hydatidiform mole
CIDR – controlled internal drug release
CIN – chromosomal instability
CNV – copy number variation
COS – controlled ovarian stimulation
CSF – cytostatic factor
DOP-PCR – degenerate oligonucleotide primed PCR
DSB – double-strand break
GTD – gestational trophoblastic diseases
EGA – embryonic genome activation
EGF – epidermal growth factor
FACS – fluorescence-activated cell sorting
FCS – fetal calf serum
FET – frozen embryo transfer
FISH – fluorescence *in situ* hybridization
FSH – follicle-stimulating hormone
GnRH – gonadotropin-releasing hormone
GV – germinal vesicle
HR – homologous recombination
i.m. – intramuscularly
ICM – inner cell mass
ICSI – intracytoplasmic sperm injection
IVF – *in vitro* fertilization
IVM – *in vitro* maturation
IVP – *in vitro* production
k-MT attachment – kinetochore-microtubule attachment
Kb – kilobase
LA-PCR – ligation-mediated PCR
LCM – laser-capture microdissection
LH – luteinizing hormone

MI – meiosis I
MII – meiosis II
MALBAC – multiple annealing and looping-based amplification
Mb – megabase
MDA – multiple displacement amplification
miRNA – microRNA
mRNA – messenger RNA
MT – microtubule
MZT – maternal-to-zygotic transition
NGS – next-generation sequencing
NHEJ – non-homologous end-joining
OHSS – ovarian hyperstimulation syndrome
OPU – ovum pick up
PBS – phosphate buffered saline
PEP-PCR – primer extension pre-amplification PCR
PCOS – polycystic ovarian syndrome
PCR – polymerase chain reaction
PGT-M – preimplantation genetic diagnosis
PGT-A – preimplantation genetic screening
p.i. – post insemination
piRNA – PIWI-interacting RNA
PVP – polyvinylpyrrolidone
QC – quality control
ROS – reactive oxygen species
SAC – spindle assembly checkpoint
SCNT – somatic cell nuclear transfer
siCHILD – single-cell haplotyping and imputation of linked disease variants
SNP – single nucleotide polymorphism
SOF – synthetic oviductal fluid
TE – trophectoderm
TLI – time-lapse imaging
UPD – uniparental disomy
WGA – whole-genome amplification
ZP – zona pellucida

PROLOGUE

"The decline of fertility is one of the most fundamental social changes that happened in human history"
Max Roser, creator of "Our World In Data"

It seems ironic, even a little paradox, that with rapid world population growth voices started to emerge, raising concerns about the diminished fertility rates. This is especially evident in the developed countries that face the impending population decline. After the post-World War II baby boom, the birth rates started to decline in 1970s and this trend is persisting until today. According to the United Nations (U.N.) data for 2015, the global fertility rate has reached its unprecedented low levels, but differences in childbearing exist among various countries and regions. Low-fertility countries include all of Europe and Northern America, and many countries in Asia and Latin America and the Caribbean. In contrast, women in most of the countries in the sub-Saharan Africa, where child mortality rate is still high, have five or more children over their lifetime. Of course, social, cultural, economic and political climates are the key factors influencing birth figures. The fertility rate drop can also be attributed to the developing countries own efforts to control their rapidly expanding populations. One of the most significant population planning programs was initiated in 1979 in China with the introduction of the One Child Policy, in which it was discouraged to have more than one child. Notorious India compulsory sterilization program was also implemented for population control in 1976, a practice that is still being used in some regions of Bangladesh. Although the national fertility rate is still high in those countries, both India and Bangladesh have encountered a steady decline over the years of 2000s. In the Western world, technological progress, economic reforms, changing religious norms and family values, women's empowerment and higher costs of childcare and education have all reflected in the declining fertility rate. In some countries, including Estonia, the average age at first pregnancy has reached 30 years of age. At the same time, the life expectancy continues to increase, creating a substantial imbalance in the ratio of elderly dependents to working-age people. The U.N. predicts that by 2100 almost 30% of the population will consist of people >60 years, which may lead to fears of economic stagnation, if there will be not enough workers to support the elderly. As a result, a new two-child policy became effective from January 1, 2016 in China to address the aging issue, while the Western world countries offer substantial financial incentives and subsidies to encourage women to have children.

The role of women in society has changed, giving them better education and increased employment opportunities. With more options for self-improvement and with increased cost of living in developed countries, women take the advantage of these opportunities and delay motherhood until later in life. But then there is another side to the story…

INTRODUCTION

Women and men continue to delay parenthood worldwide due to various personal and socioeconomic reasons. As a consequence, involuntary childlessness and age-related infertility became a common medical problem, affecting 10–20% of couples of reproductive years. The increasing prevalence of infertility has led to an explosion in the use of assisted reproductive technologies (ART). The most commonly used ART technique is *in vitro* fertilization (IVF), where *in vitro* fertilized oocytes are cultured under artificial conditions and the developed embryos are transferred back to the uterus. Today, fertility treatment has become standard care in many countries and the field of assisted reproduction has progressed rapidly over the last decade. According to European statistics, almost half a million IVF cycles are performed annually, resulting in the birth of 100,000 newborns, which account for up to 5% of all babies born in Europe. Despite scientific efforts, ART is still powerless to help many couples, as IVF success rate remains moderate with only 30% of patients getting pregnant per single treatment. The low efficiency of IVF poses significant social and financial burden to all patients that can result in emotional distress and feelings of stigmatization. Although IVF outcome depends on many clinical, demographic and lifestyle factors, genomic integrity of IVF embryos and the functional endometrium play the most crucial role. Hence, the wish to improve IVF treatment has triggered great interest towards deeper understanding of human reproduction.

Recent years have seen a plethora of technological breakthroughs, especially in the field of single-cell research. Owing to innovative single-cell approaches, it has become prominent that one of the inherent features of human IVF embryos is chromosomal instability (CIN), which is manifested by an increased rate of full chromosome aneuploidies, segmental deletions and duplications, uniparental disomies and translocations. These events in embryonic cells also contribute to chromosomal mosaicism that can be fatal to the embryo, causing developmental arrest, implantation failure, miscarriage or, in the worst case scenario, the birth of a child with chromosomal disorder. To tackle this issue, preimplantation genetic testing for aneuploidy (PGT-A) has been implemented into the clinics to detect embryonic aneuploidy and ensure uterine transfer of the most viable embryos. On the other hand, CIN can be seen as a driver of genomic evolution that may also occur in natural conceptions. The existence of embryonic CIN in natural reproduction is indirectly supported by the birth of mosaic and mixoploid/chimeric individuals.

By using cutting-edge single-cell technologies, the current thesis addresses the topic of embryonic CIN with the general aim to expand our knowledge on the origin of genomic instability in preimplantation embryos. Given the ethical restrictions, associated with human embryo research, this work utilizes bovine as an animal model to investigate the nature of chromosomal instability during early development *in vivo* and *in vitro*. Additionally, we investigate the feasibility of newly advocated blastocoel fluid biopsy for PGT-A – a topic that has gained much controversial attention.

I. LITERATURE OVERVIEW

1. Biology of reproduction: from oogenesis to embryogenesis

1.1. Basics of oocyte development

Oocyte is the largest cell in the human body that develops in the ovarian follicle, and it is large enough to be visible to the naked human eye without the aid of a microscope. Apart from providing half of the genetic material, the oocyte supplies the zygote with nearly all cytoplasmic and transcriptional factors necessary for successful early embryo development. One of the crucial aspects of oogenesis is the meiotic maturation of the oocyte, which is achieved through two sequential asymmetric cell divisions – meiosis I and meiosis II – that result in the formation of a fully functional haploid egg and two non-viable polar bodies. Yet, to achieve reproductive success, the oocyte must first gain developmental competence to resume meiosis, to become fertilized, to guide first post-zygotic cleavages and to maintain early embryonic survival (Sirard et al., 2006). Hence, the road to oocyte maturation goes through complex and carefully regulated stages of oogenesis in parallel with folliculogenesis that take their roots already in human prenatal development. It is generally accepted that the final number of oocytes and follicles is established during fetal period of life and no new germ cells are produced after birth, but this view has been challenged recently, suggesting that potential postnatal *de novo* oogenesis may exist (Johnson et al., 2004; Virant-Klun, 2015).

The journey of oocyte development starts already in the fetal ovaries and is accompanied by a transition from mitosis to meiosis (Fig. 1). First, primordial germ cells differentiate into small immature diploid germ cells, called oogonia. Oogonia then undergo rapid mitotic proliferation from the second to seventh month of gestation period, forming roughly 7 million cells. Surprisingly, oocyte formation is an incredibly wasteful event, because it is accompanied by an extensive embryonic germ cell loss due to mitotic arrest and apoptosis, so only around 20% of the original cohort will remain by the time of birth (Hartshorne et al., 2009). Those oogonia that successfully enter meiosis will undergo meiotic recombination at prophase stage, forming primary oocytes. Primary oocytes do not complete the first meiotic division, but rather maintain a meiotic arrest in the protracted prophase stage, termed dictyate, until puberty. At this stage of development the nucleus of an oocyte is called germinal vesicle (GV), and the cell is embedded within the primordial follicle, surrounded by a single layer of flat pregranulosa cells (Fig. 1). Although GV oocytes remain in a prolonged resting phase, they are still transcriptionally active to assure a sufficient level of housekeeping functions (Susor et al., 2015). Primary oocytes that are not surrounded by somatic cells will undergo apoptosis, so once again a substantial amount of oocytes are lost by the time of puberty as only 300,000–400,000 follicles remain.

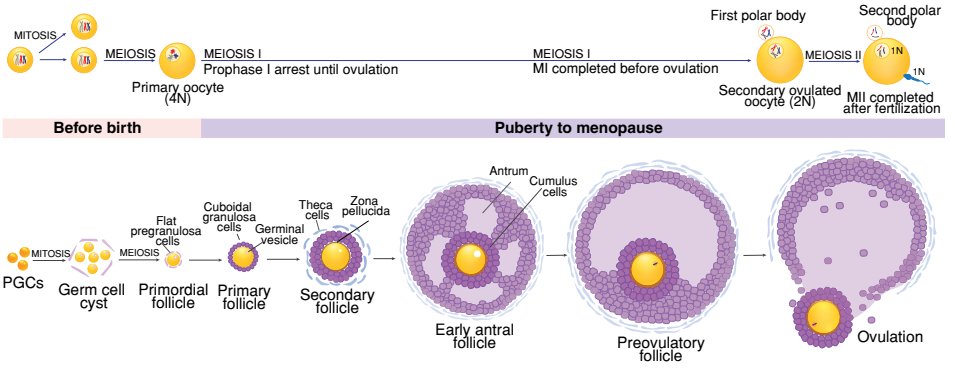

Figure 1. Main stages of folliculogenesis and oocyte development. Oocyte maturation (top) is coupled with long process of ovarian follicle maturation (bottom). First, primordial germ cells (PGCs) rapidly proliferate and differentiate into oogonial stem cells, encapsulated in the germ cell cyst. As cysts break down, oogonia enter meiosis, forming primordial follicle. Upon initiation of folliculogenesis, primordial follicles become primary follicles with embedded immature oocyte that is in a prolonged resting phase of meiosis I, also known as germinal vesicle stage. Next, proliferating granulosa cells create several layers around the oocyte, forming secondary, early antral and finally preovulatory follicles with two distinct follicular somatic cell populations: cumulus cells (dark purple) and mural granulosa cells (light purple). Upon luteinizing hormone (LH) surge, only one dominant follicle ovulates, releasing the mature secondary oocyte, while other follicles will undergo follicular atresia.

Upon puberty and onwards, primordial follicles are continuously activated by follicle-stimulating hormone (FSH) to initiate folliculogenesis and oocyte growth. Granulosa cells, surrounding the oocyte, start to proliferate to form first primary and then secondary follicles. The rise of estrogen levels, produced by the growing follicles, eventually triggers luteinizing hormone (LH) pre-ovulatory surge that signals the oocyte to overcome dictyate arrest and proceed with meiosis: the germinal vesicle breaks down, the first meiotic division is accomplished and the meiotic cell cycle progresses until the oocyte reaches nuclear maturation and arrests again at metaphase of meiosis II, ready to be fertilized (Park et al., 2004). Apart from chromosome reduction and haploidization, the newly formed secondary oocyte acquires cytoplasmic maturation that involves translational and post-translational protein modifications, as well as organellar structural and functional changes essential to support early stages of embryo development [reviewed in (Coticchio et al., 2015)]. Hence, the zygote will inherit maternal factors to meet the demands of a cleaving embryo until maternal-to-zygotic transition (MZT). It is important to note that oocyte nuclear and cytoplasmic maturation is constantly supported by follicular somatic cells that provide nutrients and metabolites, like amino acids, lactate and pyruvate, which are used to generate ATP in mitochondria and maintain ion- and redox homeostasis in the growing oocyte (Collado-Fernandez et al., 2012). Granulosa/cumulus cells also exchange many regulatory signals that control oocyte

metabolism, cytoskeletal remodeling, maintenance of meiotic arrest and subsequent meiotic resumption – all of which are key events necessary for successful oocyte fertilization and support of early embryogenesis. In turn, oocyte-secreted factors contribute to granulosa cell differentiation and cumulus cell expansion (Hussein et al., 2005; Hussein et al., 2006; Diaz et al., 2007), so a carefully orchestrated cross-talk between the oocyte and the surrounding follicular cells is essential for achievement of both oocyte competence and follicle maturation.

Although a pool of primary oocytes/follicles is recruited each month during menstrual cycle, only a small fraction will reach maturity and ovulate, while the rest will undergo follicular death, or atresia. This continued gradual loss of germ cells drastically influences female reproductive capacity, as out of the initial thousands and thousands of cells only 300–400 develop into mature eggs during women's reproductive lifespan. Finally, the depletion of ovarian reserves results in menopause and cessation of oogenesis (Faddy et al., 1992). Currently, it is not yet fully understood how the fate of germ cells is determined and whether the whole process follows the strict "survival of the fittest" rule or the selection of the oocytes to develop is purely stochastic.

1.1.1. Molecular control of meiotic division in oocytes

Meiosis is one of the defining milestones of human gametogenesis by which haploid gametes are generated from diploid precursors, following two specialized cell divisions, known as meiosis I (MI) and meiosis II (MII). Meiotic division is also called a reduction division that involves the disjunction of homologous chromosomes in MI, followed by segregation of sister chromatids during MII. Hence, genome integrity of germ cells strongly relies on intact cellular and molecular mechanisms involved in accurate chromosome segregation during meiotic progression. However, a number of sex-specific differences make female meiosis more complex. The most obvious difference occurs already at the late prophase of MI: male gametes proceed quickly through the first meiotic division, while oocytes encounter meiotic arrest that can last for decades. Second, unlike developing spermatocytes, oocytes lose their centrioles during meiotic prophase and subsequently segregate their chromosomes on an acentrosomal spindle (Manandhar et al., 2005). Instead, meiotic spindle is assembled by numerous acentriolar microtubule-organizing centers (aMTOCs), at least in mouse oocytes (Schuh and Ellenberg, 2007). These aMTOCs have similar microtubule nucleation properties as centrosomes, suggesting that they can substitute their function, leading to self-organization of the meiotic spindle. Whether human oocyte spindle assembly operates via similar mechanism is yet to be determined. Finally, male germ divisions are symmetric and produce four equally sized gametes, while eccentric positioning of oocyte meiotic spindle results in an asymmetric cell division that yields a large fertilizable egg and small non-functional structures known as the first and

second polar bodies (Brunet and Verlhac, 2011). Such asymmetric division also allows the oocyte to retain maximum of its cellular cytoplasm.

Female meiosis I is preceded by a round of pre-meiotic DNA replication that forms pairs of homologous chromosomes (one paternal and one maternal), each containing two sister chromatids (Fig. 2). Before the onset of the first meiotic division, homologous chromosome pairs are initially zipped together by the synaptonemal complex protein structure and undergo reciprocal exchange of genetic material between homologous chromosomes via homologous recombination (HR). HR is initiated at DNA double-strand breaks (DSBs), generated across the genome by endonuclease SPO11 (Baudat et al., 2013). These DSBs recruit repair proteins that search for homologous chromosomes, promoting pairing and synapsis of homologs. DSB repair results in two outcomes: generation of crossovers that link homologs and generation of non-crossovers that result in gene conversion. The maturation of meiotic DSBs to crossovers is mediated by the DNA mismatch repair protein complex MLH1-MLH3 (Lenzi et al., 2005). The formation of crossovers has two essential purposes: (i) the exchange of genetic material at crossover sites promotes the genetic diversity in offspring, and (ii) the establishment of a physical linkage between homologs (chiasma) at the crossover sites ensures accurate homolog segregation upon MI division. As a consequence of crossover and chiasmata formation during meiotic recombination, parental homologs acquire a unique bivalent chromosome configuration. Maintenance of the bivalent structure is regulated by the cohesin protein complex that binds homologs together at the crossovers and sister chromatids at the centromere [reviewed in (Webster and Schuh, 2016)]. At this stage, the oocyte remains quiet at the state of dictyate arrest, maintained by high levels of cAMP (Conti et al., 2002; DiLuigi et al., 2008), and will not resume meiosis until puberty. Following LH surge in adulthood, oocyte cAMP levels decline and MI progresses: meiotic spindle gets assembled and bivalents align on the metaphase I plate. Monopolar attachment of homologs to spindle microtubules (MTs) is mediated by kinetochores (k-MT attachment) that are assembled on the centromeres of homologous chromosomes to generate tension by counteracting the pulling force of spindle MTs. Meiotic spindle then migrates to the oocyte cortex by actin-dependent mechanism to facilitate the first polar body formation (Schuh and Ellenberg, 2008). During MI division, only cohesin holding the two homologs is removed by protease separase, while the sister-chromatid cohesion in the centromeric region is maintained by REC8 protein that also orients kinetochores, so that sister chromatids move to the same pole (Watanabe and Nurse, 1999). REC8 itself is protected from cleavage by Shugoshin proteins (Sho), preventing premature segregation of sister chromatids at anaphase I (Kitajima et al., 2004; Lee et al., 2008; Llano et al., 2008). As a result of the first asymmetrical division, the oocyte segregates half of the homologous chromosomes into the diploid polar body I that gets extruded from the oocyte upon cytokinesis (Fig. 2). In subsequent meiosis II, the second meiotic spindle is formed and already sister chromatids align on the MII spindle. At this stage the oocyte is ovulated and remains arrested at metaphase

II, awaiting fertilization. MII arrest is mediated by cytoplasmic activity, known as cytostatic factor (CSF), that blocks cell cycle progression by inhibiting a core component of the cell cycle regulatory machinery – anaphase-promoting complex/ cyclosome (APC/C) (Schmidt et al., 2006). The CSF activity and inhibition of APC/C is regulated by early mitotic inhibitor 2 (EMI2) as a part of the Mos-MAPK pathway, which is required for maintaining CSF-mediated arrest (Tung et al., 2005; Inoue et al., 2007). Once fertilized, the oocyte overrides metaphase arrest, centromeric cohesin is eliminated and bipolar k-MT attachment segregate sister chromatids towards opposite spindle poles, resulting in MII oocyte and a haploid polar body II.

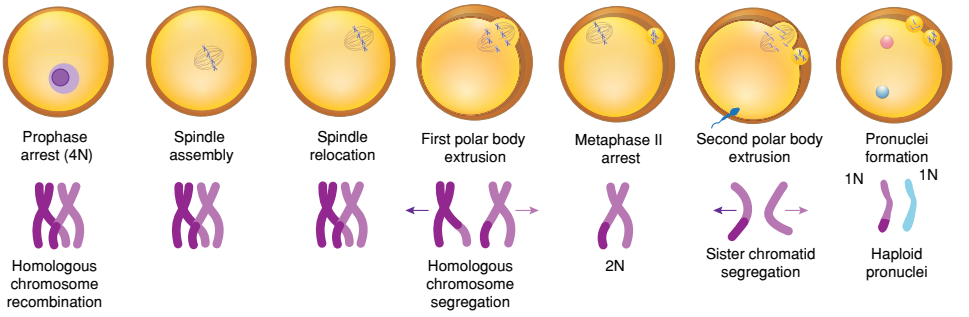

Figure 2. Schematic representation of oocyte nuclear maturation during meiosis. Following DNA replication, homologous chromosomes undergo pairing, synapsis and recombination, after which primary oocytes remain in meiotic arrest. Hormonal surge in adulthood triggers ovulation and exit from prophase arrest: meiotic spindle assembles and migrates to the oocyte cortex. Upon completion of meiosis I, half of homologous chromosomes segregate into the first polar body and the oocyte now contains a diploid set of chromosomes. A second meiotic spindle assembles around the remaining diploid chromosomes of the oocyte and the ovulated egg arrests again at second meiotic metaphase until fertilized. Finally, fertilization of the ovulated egg triggers the onset of sister chromatid segregation into the second polar body and completion of the second meiotic division. The resulting zygote now contains two haploid pronuclei, one from the mother and from the father. Figure based on (Clift and Schuh, 2013).

1.2. Fertilization

The essence of fertilization in all higher animals is the fusion of male and female gametes that will give rise to a new organism. The fertilizable life span of the ovulated human oocyte is relatively short and is estimated to be 12–24 hours after ovulation. At this point, the mature oocyte is surrounded by two substantial layers: (i) a glycoprotein matrix, or zona pellucida (ZP), that was synthesized and secreted by the oocyte during follicular development and (ii) a layer of expanded cumulus cells. During normal fertilization, only one sperm must penetrate these layers to fuse with oocyte plasma membrane. Prior to fertilization, the sperm undergoes several biochemical changes (known as sperm

capacitation) that increase sperm motility and prepare it for acrosome reaction, necessary to penetrate the egg's tough membrane, release it from meiotic sleep and form a zygote (Evans and Florman, 2002). Hence, fertilization encompasses a sequence of coordinated molecular events that involve sperm-egg binding and fusion, oocyte activation and subsequent cellular reprogramming to transform highly specialized haploid germ cells into a totipotent diploid zygote [reviewed in (Clift and Schuh, 2013)].

1.2.1. Sperm-egg binding and oocyte activation

Oocyte fertilization initially begins with sperm cell binding to zona pellucida of the egg, which is made of a few glycoproteins, designated as zona pellucida binding protein 1 (ZP1) to ZP3 in mice and ZP1 to ZP4 in humans (Gupta et al., 2012). Zona pellucida proteins are modified with specific oligosaccharides that enhance gamete binding affinity (Pang et al., 2011). Although the precise mammalian sperm-egg interaction mechanisms remain elusive, earlier studies suggest that ZP3 is the key sperm-binding receptor in mouse oocytes, but in humans also ZP1 and ZP4 participate in binding of capacitated spermatozoa (Gupta et al., 2012). Subsequent sperm-egg interaction is preceded by acrosomal exocytosis that releases a mixture of glycohydrolytic and proteolytic enzymes at the binding site to help the sperm to digest through zona pellucida and fuse with egg's plasma membrane (Abou-Haila and Tulsiani, 2000). Spermatozoa that fail to undergo acrosome reaction will not be able to digest ZP of intact oocytes, making acrosome a crucial player in the fertilization process.

Following fertilization and fusion of egg and sperm membranes, the oocyte establishes zona pellucida and plasma membrane block to preclude the attachment of additional sperms and prevent polyspermic fertilization (Gardner et al., 2007). In mammals, the polyspermy block is achieved through cortical reaction, triggered by Ca^{2+} oscillations in the egg upon fertilization (Fig. 3). The wave of Ca^{2+} is likely to be initiated by the sperm phospholipase Cζ (PLCζ), which promotes the release of free Ca^{2+} from endoplasmic reticulum by activating the Ca^{2+} channel, targeting inositol phosphate cascade (Miyazaki et al., 1992; Saunders et al., 2002; Hachem et al., 2017). As a result of Ca^{2+} induced egg activation, the oocyte releases Golgi-derived cortical granules from its cortex, which contain several enzymes that modify zona pellucida proteins to ensure monospermic fertilization and proper preimplantation embryo development (Liu, 2011). One of the key components of cortical granules is an oocyte-specific protease, ovastacin, that breaks down the sperm-docking domain of ZP2, altering the topology of zona pellucida and making it impenetrable to sperm (also referred to as zona hardening) (Burkart et al., 2012).

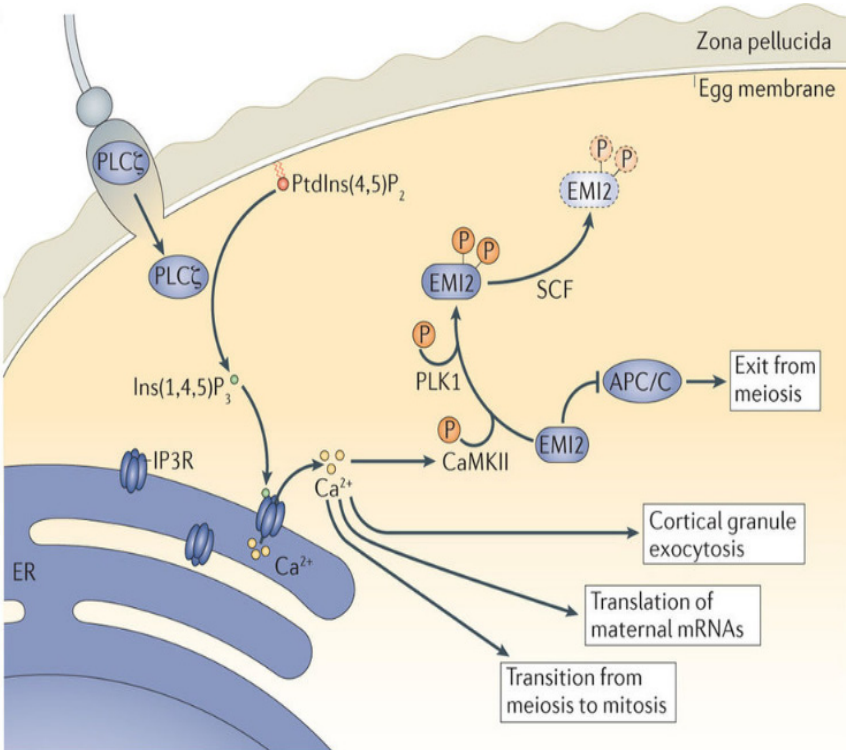

Figure 3. Fertilization-induced events in the oocyte. Following sperm-egg binding, sperm-derived phospholipase Cζ (PLCζ) is released into the oocyte cytoplasm, where it promotes the generation of ligand IP3 (inositol 1,4,5-trisphosphate) that targets Ca^{2+}-channels, causing the release of Ca^{2+}-ions from the endoplasmatic reticulum. As a result, Ca^{2+} oscillations (i) trigger cortical granule exocytosis to block polyspermy, (ii) activate CAMKII pathway, releasing APC/C from EMI2-mediated arrest to promote exit from meiotic metaphase II arrest, and (iii) induces the onset of maternal mRNA translation to promote the transition from meiosis to mitosis. Adapted by permission from (Clift and Schuh, 2013).

In addition to polyspermy block, changes in cytosolic Ca^{2+} levels trigger exit from meiosis and translation of maternal mRNAs (Fig. 3), which is the last phase of oocyte activation (Ducibella et al., 2002). The rise of intracellular Ca^{2+} concentration within the oocyte activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which releases APC/C from EMI2-mediated CSF arrest (Rauh et al., 2005). This event involves sequential phosphorylation of EMI2 by CaMKII, and recruited polo-like kinase 1 (PLK1) that, together with SCF (SKP2–cullin 1–F-box protein) ubiquitin ligase complex, mark EMI2 for proteosomal degradation (Schmidt et al., 2005). With EMI2 out of the way, activated APC/C eliminates key cell-cycle regulators, such as cyclin B and securin, and promotes meiotic resumption, followed by the formation of a second polar body and a female pronucleus. At the same time, the sperm undergoes a series of morphological and biochemical transformations, including

chromatin remodeling, which lead to the development of a functional male pronucleus (McLay and Clarke, 2003). Finally, the formation of maternal and paternal pronuclei delineates the completion of fertilization and the oocyte is now denoted a zygote. The zygote then prepares to enter its first post-zygotic cell division.

1.2.2. Transition from meiotic to mitotic cell cycle

The transition from oocyte to embryo is one of the most complex and fascinating phases of early mammalian development. The newly formed zygote must abandon the inherited germ cell-specific traits and erase its epigenetic memory to achieve totipotency. In addition, it needs to reprogram its cell-cycle machinery from the reductional meiotic division to equational mitotic division, so the extensive re-organization of the zygote includes epigenetic reprogramming, switch from acentrosomal meiotic spindle to centrosomal mitotic spindle and from asymmetric to symmetric cell divisions [reviewed in (Clift and Schuh, 2013)].

Epigenetic reprogramming

Epigenetic reprogramming in zygotes is a highly dynamic process that is pivotal for correct embryonic development and accurate chromosome segregation during first post-zygotic cleavages. Initially, sperm DNA is highly coated with protamines that allow tight packaging of the paternal genome into the sperm head. Such difference in chromatin assembly creates an epigenetic asymmetry between paternal and maternal genomes in the zygote that needs to be resolved for successful mitotic progression, as epigenetic asymmetry makes paternal chromosomes more susceptible to kinetochore attachment and missegregation errors (Burton and Torres-Padilla, 2014; van de Werken et al., 2015). Most likely, the genome-wide epigenetic reprogramming of parental genomes is predominantly controlled by oocyte-specific factors. This view is supported by somatic cell nuclear transfer (SCNT) techniques, in which the anucleated oocyte, containing oocyte-specific factors in its remaining cytoplasm, is able to reprogram a fully differentiated somatic cell into a newly developing embryo. During maturation, growing oocytes acquire histone-assembly activity by accumulating and translating mRNAs that encode histone chaperons, required for nucleosome assembly (McLay and Clarke, 1997; McLay et al., 2002). Shortly after fertilization, the sperm undergoes decondensation, by which protamines are removed from paternal pronucleus and replaced by maternally-derived histones (e.g. by H3 variant H3.3), establishing *de novo* nucleosomes (Torres-Padilla et al., 2006; Lin et al., 2014; Wen et al., 2014). As the embryo develops, chromatin becomes more accessible and new sites appear at promoters, as well as at exonic, intronic and intergenic regions (Lu et al., 2016).

When sperm chromatin protamines are replaced by oocyte cytoplasmic histones, the paternal genome undergoes active DNA demethylation to create a hypomethylated epigenome in the embryo (Fulka et al., 2004; Guo et al., 2014; Smith et al., 2014). The key modulator of this process is TET3 enzyme that oxidates 5-methylcytosine into 5-hydroxylmethylcytosine, which serves as an intermediate state between methylated and unmethylated DNA (Gu et al., 2011). The maternal genome remains stable at this point, as it is protected from TET3-mediated oxidation by the DNA demethylation controlling protein DPPA3, which prevents binding of TET3 to chromatin (Nakamura et al., 2007; Bakhtari and Ross, 2014). Apart from TET3-mediated active DNA demethylation, several other parallel pathways may also facilitate this process, like DNA-repair based mechanisms (Hajkova et al., 2010; Ladstatter and Tachibana-Konwalski, 2016). Subsequently, both parental genomes undergo passive replication-dependent DNA demethylation during embryo cleavage-stage development (Guo et al., 2014). However, during epigenetic reprogramming, both maternal and paternal pronuclei are not entirely demethylated and retain imprinted loci, established during male and female gametogenesis (Li and Sasaki, 2011). The maintenance of imprinted genes ensures the parent-of-origin specific gene expression, necessary for normal pre- and postnatal development.

Transition from meiotic to mitotic spindle

One of the first steps in erasing the meiotic footprint is re-organization of the chromosome cohesin complex. The meiosis-specific cohesin complex that contains REC8, which is essential for reductional division, is rapidly replaced by the mitotic cohesin complex, containing SCC1 (Tachibana-Konwalski et al., 2010). Although SCC1 has no role in meiosis, its accumulation in the oocyte is likely a prerequisite for the rapid switch from meiotic to mitotic cohesin complex immediately after fertilization. The equitable mitotic division also depends on the formation of a centrosomal bi-polar spindle. During mitosis the cell's two centrosomes, which consist of a pair of centrioles surrounded by an amorphous pericentriolar matrix, move towards the opposite poles and facilitate capture and alignment of chromosomes before equally segregating them between the two daughter cells. However, oocytes do not contain canonical centriole-containing centrosomes, as centrioles are eliminated during oogenesis by unknown mechanisms, and so conventional centrosomes are unable to form.

In most mammals, including human, the incoming sperm contributes a centriole to the egg at fertilization. Paternally-derived centrioles can already be visualized in the human zygote, suggesting that the switch from acentrosomal meiotic spindle to centrosomal mitotic spindle in humans embryos occurs immediately after fertilization (Sathananthan et al., 1996; Sathananthan, 1998). In contrast, mouse and other rodents are the only mammals, where sperm centriole gets degenerated during spermatogenesis and first post-zygotic divisions also occur in the absence of centrioles, following the meiosis-like pattern

of spindle assembly. The *de novo* centriole production in mice establishes gradually and the spindle adopts typical mitotic features upon embryo developmental progression (Courtois et al., 2012). However, a question remains, whether the sperm centriole-mediated spindle assembly is the only exclusive mechanism in human zygotes, taking into account the ability of human oocytes to spontaneously cleave without being fertilized, a phenomenon known as parthenogenesis (Winston et al., 1991; Paffoni et al., 2007; de Fried et al., 2008).

Transition from asymmetric meiotic to symmetric mitotic division

Mammalian oocyte maturation is accompanied by asymmetric cell division that produces a large egg and a small polar body. In contrast, the first post-zygotic division needs to be symmetric to produce two cells of the same size that equally inherit both genetic material and maternal cytoplasmic material. In order to achieve that, two parental pronuclei need to be centrally positioned. Prior to the formation of the first mitotic spindle in zygotes, microtubule-dependent movement of maternal and paternal pronuclei towards each other takes place, referred to as karyogamy. In mice and rodents, pronuclei centering depends on dynamic actin networks that maintain central spindle positioning and ensure symmetrical first mitotic division (Chew et al., 2012; Chaigne et al., 2016). In other mammalian species, the paternally inherited centrosome nucleates microtubules to form the sperm aster, which most likely allows the female pronucleus to associate with microtubules upon their expansion and move towards the male pronucleus in a motor dynein-dependent manner (Reinsch and Gonczy, 1998; Payne et al., 2003). Subsequently, the sperm centrosome generates the mitotic spindle that segregates chromosomes to two equal embryonic cells, called blastomeres.

1.3. Stages of preimplantation embryo development

Preimplantation embryo developmental processes are highly conserved among mammals, as they all progress through the same morphological stages, i.e. from zygote to blastocyst (Fig. 4). In humans, the first cytokinesis is estimated to occur after 14–15 hours post fertilization (Chavez et al., 2012; Vera-Rodriguez et al., 2015) and the first division itself occurs ~26–28 hours post fertilization (Gardner and Balaban, 2016). Subsequently, the developing embryo undergoes a series of rapid cellular proliferations that increase the number of blastomeres without changing the overall size of the embryo. Progression through 2-cell, 4-cell and 8-cell is termed cleavage-stage of development, which is characterized by rather synchronous cell divisions, leading to morphologically identical blastomeres. At the end of cleavage-stage, blastomeres develop gap junctions and initiate embryo compaction to form a morula on day 4 of develop-

ment, which contains 16–32 cells and marks first morphogenetic and cellular differentiation. During the process of compaction, blastomeres increase intercellular adhesion and undergo cell polarization, creating outer polarized cells and inner apolar cells (Zernicka-Goetz et al., 2009). Consequently, polarity and cell division orientation lead to lineage specification into inner cell mass (ICM) and trophectoderm (TE) cells. Shortly after the development of morula, ion pumps become assembled on the membranes of TE cells that actively transport sodium, creating the osmotic gradient and promoting the formation of a fluid-filled cavity, known as blastocoele (Houghton et al., 2003). Generation of the blastocoele cavity also defines the beginning of the blastocyst formation. The progressive accumulation of blastocoele fluid (BF) coupled with continuous cell proliferation leads to the expansion of the blastocyst and thinning of zona pellucida that has been surrounding the embryo during first days of development. At this point, different cell lineages can be clearly distinguished from each other: the outer layer of TE cells will contribute to uterine wall invasion and formation of the placenta, while ICM will give rise to the future fetus. Eventually, zona pellucida develops a crack and the embryo escapes from its enclosure, leaving behind an empty zona pellucida shell. The zona-free hatched blastocyst is now ready to implant and continue its post-implantation development.

1.3.1. Embryonic genome activation

Early embryo development begins in transcriptional silence and the genetic control of first cell divisions initially rely on maternally-derived RNAs and proteins, the majority of which will be degraded during the maternal-to-zygotic transition. The duration of embryonic quiescence differs between mammalian species, but all embryos gradually develop the ability to transcribe genes from their newly formed genomes. The requirement for embryonic genome activation (EGA) is absolutely essential for embryogenesis, as it takes the embryo from a state of almost no transcription to a state of self-sustained cellular control, where up to thousands of genes are transcribed to ensure normal embryo development and survival. Mouse embryos exhibit the earliest transition from maternal to embryonic transcription already at 2-cell stage (Hamatani et al., 2004). The shift to EGA in humans occurs on day 3 after fertilization approximately at 4–8-cell stage (Fig. 4) (Braude et al., 1988; Dobson et al., 2004), which is similar to non-human primates (Wang et al., 2017). Changes in nuclear and chromatin structure and cytoplasmic content appear to regulate periods of transcriptional activation [reviewed in (Lee et al., 2014)].

The transition to EGA has been extensively studied in model organisms, especially in the mouse, but detailed information for human embryos has also recently emerged. In mammalian embryos, including humans, initiation of EGA consists of two waves, which are characterized by a dramatic transcriptional turnover (Xue et al., 2013; Graf et al., 2014). In the first wave, which in humans

occurs during the progression to the 4-cell stage, the maternally inherited transcripts are either significantly downregulated or degraded (Fig. 4).

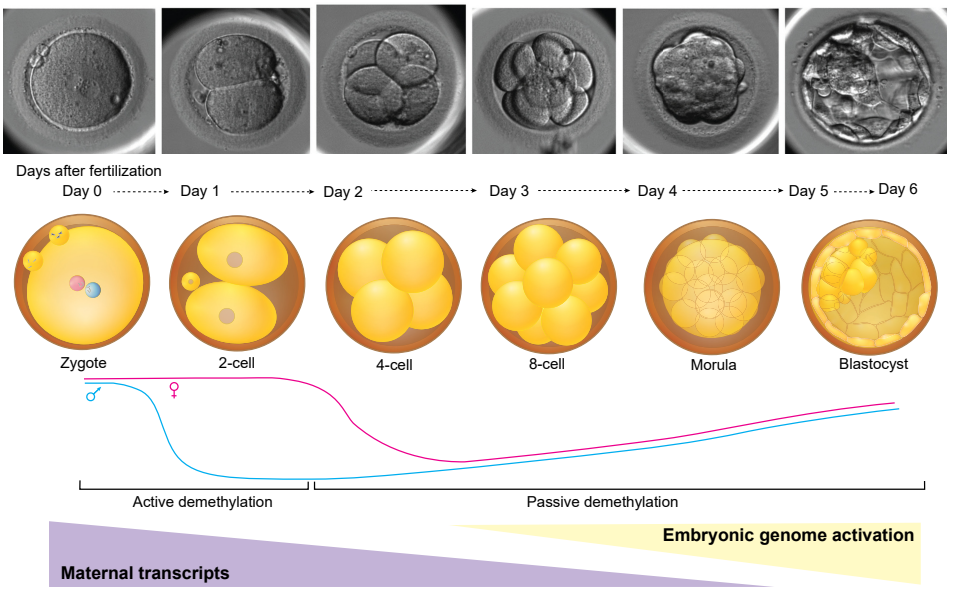

Figure 4. Preimplantation development of human embryos. Key morphological features are represented for each embryonic stage, accompanied by the corresponding time-lapse image. During cleavage-stage development the transition to embryonic genome activation (EGA) occurs: maternally inherited transcripts are gradually degraded in the first wave of EGA that lasts up 4-cell stage and in the second wave, which occurs after 8-cell stage, embryo genome becomes accessible to transcription factors that activate embryonic gene expression. Time-lapse images adapted by permission from (Gardner and Balaban, 2016).

In the second wave, which in humans occurs after 8-cell stage, there is a significant upregulation of genes, involved in RNA metabolism and processing, indicating that embryonic transcriptional machinery is being established. By the blastocyst stage of development, already genes that guide cell linage commitment become activated (Zhang et al., 2009; Vassena et al., 2011; Yan et al., 2013). Importantly, timely discard of maternal mRNAs is a necessity for successful embryonic reprogramming, as failure to do so can result in developmental defects and early embryonic arrest (DeRenzo and Seydoux, 2004; Dobson et al., 2004; Wong et al., 2010). The main purpose of eliminating maternal material is to discard proteins, necessary for oocyte maturation and homeostasis, and erase the germline program to pave the way for somatic development. Although the precise molecular mechanisms of maternal decay in mammals are still being elucidated, two forms of maternal clearance have been described in *Drosophila* and *Xenopus* models: the maternal mode, which relies on maternal factors, and the zygotic mode that takes advantage of the *de novo* zygotic transcription

[reviewed in (Yartseva and Giraldez, 2015)]. The maternal mode of mRNA decay is thought to be initiated upon oocytes activation and involves RNA-binding proteins that destabilize maternal transcripts through mRNA deadenylation. This process is most likely regulated by the small non-coding PIWI-interacting RNA (piRNA) pathway that triggers maternal mRNA degradation (Rouget et al., 2010). In the zygotic mode of clearance, *de novo* expression of another small non-coding RNAs, microRNAs (miRNAs), is a common strategy to induce maternal mRNA translation repression, deadenylation and decay. Together these two activities shape the embryonic gene expression landscape to give rise to a reprogrammed embryo.

2. Technology of reproduction: from assisted reproduction to single-cell analysis

2.1. Assisted reproductive technology

Reproductive medicine is one of the fastest developing fields that has made tremendous progress in fertility treatment in a relatively short time: from the development of *in vitro* fertilization (IVF) in the late 1970s and intracytoplasmic sperm injection (ICSI) in 1990s to the successful implementation of the ovarian and uterine transplant that both resulted in birth of healthy babies in 2014 (Brannstrom et al., 2015; Demeestere et al., 2015). One of the best examples in reproductive medicine, where fundamental and translational science performed in research laboratories goes hand-in-hand with clinical practice, is assisted reproductive technology (ART) that utilizes different techniques of oocyte, sperm and embryo manipulations to improve fertility treatments. Over the past decades, there has been a profound impact of ART on biomedical research and the use of assisted reproduction continues to increase globally (Dyer et al., 2016). Since the birth of the first IVF baby (Steptoe and Edwards, 1978), over 6 million children have been conceived via ART and fertility treatments have become standard care in many countries (Maheshwari et al., 2016). This undoubtedly leads to the increasing need to transfer knowledge from bench to bedside, but despite the progress made in clinical and laboratory ART protocols, including embryo selection for transfer, the pregnancy rate per embryo transfer after single treatment cycle still lingers at around 30% worldwide (Dyer et al., 2016). This highlights the importance of understanding *in vivo* regulation of oocyte maturation and subsequent preimplantation development in order to refine assisted reproduction, so that *in vitro* techniques can mimic natural conditions as closely as possible.

2.1.1. In vitro fertilization

In vitro fertilization and embryo transfer is the most commonly performed procedure in ART. Initially, IVF was indicated for tubal factor infertility, endometriosis, male factor infertility and idiopathic or unexplained infertility. Nowadays, given the social and financial pressure encountered in many countries, especially in the Western world, women continue to postpone the age of first pregnancy for various socioeconomic and educational reasons. As a consequence, more and more couples turn to IVF to overcome age-related sub-fertility. Whether for medical or non-medical reasons, standard IVF procedure involves controlled ovarian stimulation (COS), oocyte retrieval, *in vitro* oocyte fertilization and *in vitro* embryo culture, followed by embryo transfer back to the uterus. The improved molecular understanding of folliculogenesis and oocyte maturation has enabled to implement COS as a part of routine IVF process that allows to retrieve numerous oocytes for fertilization per cycle and to enhance the cumulative pregnancy rate. The procedure comprises three basic elements: (i) stimulation of multi-follicular development by exogenous gonadotropins, (ii) suppression of premature ovulation by co-treatment with gonadotropin-releasing hormone agonist or antagonist and (iii) triggering final oocyte maturation by human chorionic gonadotropin administration 36–38 hours prior to oocyte retrieval (Pacchiarotti et al., 2016). In humans, COS is considered to be a safe procedure, since embryo quality, implantation rates and pregnancy outcomes are comparable between stimulated and natural IVF cycles (Ziebe et al., 2004; Sunkara et al., 2016). Following COS, oocytes are retrieved via transvaginal puncture and subsequent IVF procedure may involve conventional *in vitro* fertilization, in which prepared sperm is added to the dish with cultured oocytes, or in case of severe male infertility by performing ICSI, in which fertilization is achieved by injecting single sperm into the oocyte (Palermo et al., 1992). When performing ICSI, the cumulus cell layer is removed from the oocyte and sperm is injected only into the mature oocytes, while in case of conventional IVF, cumulus cells remain intact. For this reason ICSI can also be the preferred method for fertilization, if embryo testing is indicated, as it would minimize the risk of contamination. Fertilization is then assessed 16–20 hours post insemination (p.i.) by inspecting the pronuclei status and polar body formation. About 70% of oocytes will be fertilized and further embryo development will be evaluated under the stereomicroscope or monitored using time-lapse imaging (TLI) system that takes digital images of an embryo at set time intervals (Armstrong et al., 2014). Finally, only good quality embryos are transferred back to the uterus on either day 3 or day 5, depending on a doctor-patient agreement. With the development of embryo cryopreservation techniques, frozen embryo transfer (FET) became available, which can potentially improve IVF outcome compared to fresh embryo transfer due to a better embryo-endometrium synchrony (Roque et al., 2013). Major advances in vitrification protocols also led to an incredible story of a woman, who gave birth to a baby that developed from an embryo cryopreserved 24 years ago, holding the all-time

record for the longest-frozen embryo to come to birth (http://www.bbc.com/news/world-us-canada-42420864). In parallel, there have also been substantial improvements in human embryo culture conditions, including the development of a two-step protocol, based on embryos metabolic needs at different stages: the first medium, high in lactate and pyruvate, is used for the first three days of culture, while rich in glucose second medium is used from day 3 to day 5 (Gardner et al., 1998). However, in recent years the growing awareness about the impact of different culture conditions on embryonic development sparked a strong demand for full transparency, concerning commercially available culture medium composition (Morbeck et al., 2014; Chronopoulou and Harper, 2015; Salvaing et al., 2016; Sunde et al., 2016; Morbeck et al., 2017).

2.1.2. Oocyte in vitro maturation

Oocyte *in vitro* maturation (IVM) refers to the maturation of immature oocytes in culture after their recovery from antral follicles. *In vitro* embryo production coupled with IVM has been the main strategy for producing offsprings in agricultural breeding programs. The ability to obtain oocytes, destined for atresia, has major benefits for domestic farm animal production, and the vast application of IVM technology likely results in over 100,000 births per year in cattle (The International Embryo Transfer Society Report for 2016). This is in complete contrast to the estimated >5,000 human births worldwide (Sauerbrun-Cutler et al., 2015). The main advantage of using IVM in humans is the ability to eliminate the risk of developing ovarian hyperstimulation syndrome (OHSS) and to reduce the overall costs associated with hormonal stimulation. Although achieving pregnancy and live birth via IVM in humans is less successful than by using conventional IVF/ICSI (Buckett et al., 2008; Gremeau et al., 2012), it might be a safer and more beneficial option for women with polycystic ovarian syndrome (PCOS) or cancer patients, for whom hormonal stimulation is contraindicated. In PCOS patients, standard COS can lead to potentially lethal OHSS, so the alternative option for these patients is to collect immature follicles during natural or mildly stimulated cycle (Shalom-Paz et al., 2012; Siristatidis et al., 2015). In the field of oncofertility, IVM and oocyte cryopreservation can be a treatment option for patients with estrogen receptor positive cancer history, as COS elevates serum estradiol levels, and for young women in general, who are undergoing gonadotoxic cancer therapy that can lead to loss of ovarian function and infertility (De Vos et al., 2014). Despite the attractive features of fertility preservation, providing culture system that is similar to follicular environment will be the most important challenge for the development of IVM protocols.

2.2. Single-cell technology

Our understanding about the structure and regulation of the genome is largely based on millions of cells analysed in bulk, but such approach would neglect any heterogeneity present in a subset of cells within the cellular population. For this reason, the past decade can be regarded as one of the most exciting in science due to the advent of powerful single-cell technologies that enable studying life down to the very core unit – one cell. The field of single-cell research represents a turning point in cell biology that, with its rapid advance, can provide novel insight into complex biological networks, ranging from the diversity of microbiome to pathogenesis of human disease (Saadatpour et al., 2015; Proserpio and Lonnberg, 2016; Tolonen and Xavier, 2017). Pioneering single-cell assays are now available for genome, epigenome, transcriptome, metabolome and proteome, and integrating all these disciplines into a single multi-omics assay will undoubtedly revolutionize biomedical research (Bock et al., 2016; Macaulay et al., 2017). Here, only a minor fraction of single-cell technology possibilities will be discussed, focusing on single-cell genomics that scrutinizes genomic architecture of individual cells and characterizes the incidence and consequence of genomic instability in physiology and disease (Wang et al., 2012; McConnell et al., 2013; Demeulemeester et al., 2016). In embryo research, single-cell genomics aims to unravel the onset and mechanistic origins of embryonic aneuploidy and evaluate the role of chromosomal mosaicism (the presence of cells with different genomic constitution) on embryo viability.

2.2.1. Single-cell isolation

The first step in any single-cell workflow is isolation of individual cells. A variety of well-established techniques can be used for efficient isolation of cells from a population, ranging from either manual or automated single-cell isolation, such as fluoresce-activated cell sorting (FACS) and microfluidic, to more complex procedures of laser capture microdissection (LCM) and single nucleus isolation (Table 1). Manual micromanipulation is a low-throughput method performed under the stereomicroscope, when only a small number of cells need to be analyzed. By using a simple mouth-pipette system or micropipettes that utilize special capillaries for cell disassociation, it is possible to visually confirm successful cell isolation and collection. Micropipetting is used, when working with embryos, although it carries a risk of operator-bias. For other cellular samples, a more robust cell isolation can be achieved by high-throughput and user-friendly FACS-based sorting, which uses either cell-type specific biomarkers or DNA dyes. Before separation, a cell suspension is made and the cells are labelled with fluorescent probes. FACS-based sorting has been successfully implemented in cancer research (Navin et al., 2011; Baslan et al., 2012), however the major disadvantage of this method is that it requires a large starting number of cells in suspension, so FACS fails to isolate single cells from

a low quantity cell population. Secondly, the rapid flow in the machine can damage the cells or isolate more than one cell per well, leading to biased biological data. In recent years, various microfluidic devises have been introduced that allow both isolation and subsequent amplification of single cells, an approach that has been widely adopted for different single-cell studies (Rowat et al., 2009; Wang et al., 2012; Xin et al., 2016). Microfluidic systems are a clear example of lab-on-a-chip devices that allow the compartmentalization and controlled management of nanoliter reactions using fabricated microfluidic chips. For instance, the automated Fluidigm C1™ system (Fluidigm Corporation, USA) captures cells from a cell suspension and provides visual confirmation that a single cell entered the reaction chamber for DNA or RNA amplification. Similarly to FACS, the main risk associated with the method is that multiple cells can enter the reaction chamber or some samples can be lost during sorting. Recently developed DEPArray™ technology (Silicon Biosystems, Italy) combines both microfluidics and microelectronics together to collect single cells. Following immunofluorescent staining, cells are entrapped in so-called "dielectrophoretic" cages on the microfluidic cartridge that can be repositioned, allowing movement and isolation of individual target cells. Hence, the DEPArray™ automated platform enables to identify and recover viable specific or rare individual cells from a heterogeneous sample (Carpenter et al., 2014; Polzer et al., 2014).

Table 1. Methods for single-cell isolation

I. Single-cell suspension and manipulation		
Manual micropipetting	***Single-cell fluorescence activated cell sorting (FACS)***	***Microfluidics***
+ visual confirmation + only a few cells need to be collected – operator bias – low throughput	+ high throughput + sorting based on cell phenotype and cellular biomarkers – large cell suspension required – "leaking" of more than one cell – putative damage to cell	+ highly standardized and automated nanoliter reactions + high throughput isolation with visual confirmation – putative loss of cells
II. Lasure capture microdissection	**III. Single nucleus suspension and sorting**	
+ retain topological information of the cell + isolation of cells from fixed solid or frozen tissues – potential damage to cell – contamination by neighbouring cells	+ isolation of single nuclei from fresh or frozen tissue + eliminates the need to isolate intact cells + can be used for both DNA and RNA studies – potential loss of micronuclei and/or genetic material	

Many of the above mentioned approaches require cells or nuclei in suspension; so alternatively, LCM method can be used to cut cells from fixed, frozen or solid tissues that require harsh mechanical and enzymatic digestion. LCM retains the spatial information of isolated cells in the context of tissue topography (Nichterwitz et al., 2016), but there is a high risk of contamination from neighboring cells. Additionally, the laser can damage target cells, so this methodology is not widely used for single-cell analysis due to various technical difficulties.

2.2.2. Single-cell amplification

One diploid human cell contains only about 6–7 pg of DNA, while genomics platforms require up to several hundred nanograms of input material. Hence, once the cell is isolated, it needs to undergo rounds of whole-genome amplification (WGA) prior to application of high-throughput technologies. There are several commercially available WGA kits, which are either polymerase chain reaction (PCR)-based or use isothermal multiple displacement amplification (MDA) (Fig. 5), and the choice of WGA strongly depends on the study question, as some methods are more suitable than others to detect specific genetic variations (Macaulay and Voet, 2014). PCR-based methods include primer extension pre-amplification PCR (PEP-PCR) (Zhang et al., 1992), ligation-mediated PCR (LA-PCR) (Klein et al., 1999) and degenerate oligonucleotide-primed PCR (DOP-PCR) (Cheung and Nelson, 1996) (Fig. 5B). Because PCR-based WGA suffers from low genome coverage and lack of proofreading of used thermostable polymerases that introduce artifacts during amplification steps, these methods are mostly preferred for copy-number profiling of single cells (Treff et al., 2011; de Bourcy et al., 2014; Ning et al., 2015). In contrast, MDA is based on an isothermal reaction, which utilizes high-fidelity phi29 polymerase with high processivity and strand displacement activity (Fig. 5A). However, like PCR-based amplification, MDA is an exponential amplification that causes non-reproducible overamplification in certain genomic regions and underamplification in other regions. For this reason, copy-number variation (CNV) calling is distorted by noisy profiles and ineffective normalization that skew data interpretation. Instead, MDA is the most suitable method for single nucleotide polymorphism (SNP)-genotyping, as it produces high molecular weight products and offers greater genome coverage with lower error rates than the initial PCR-based methods (Treff et al., 2011).

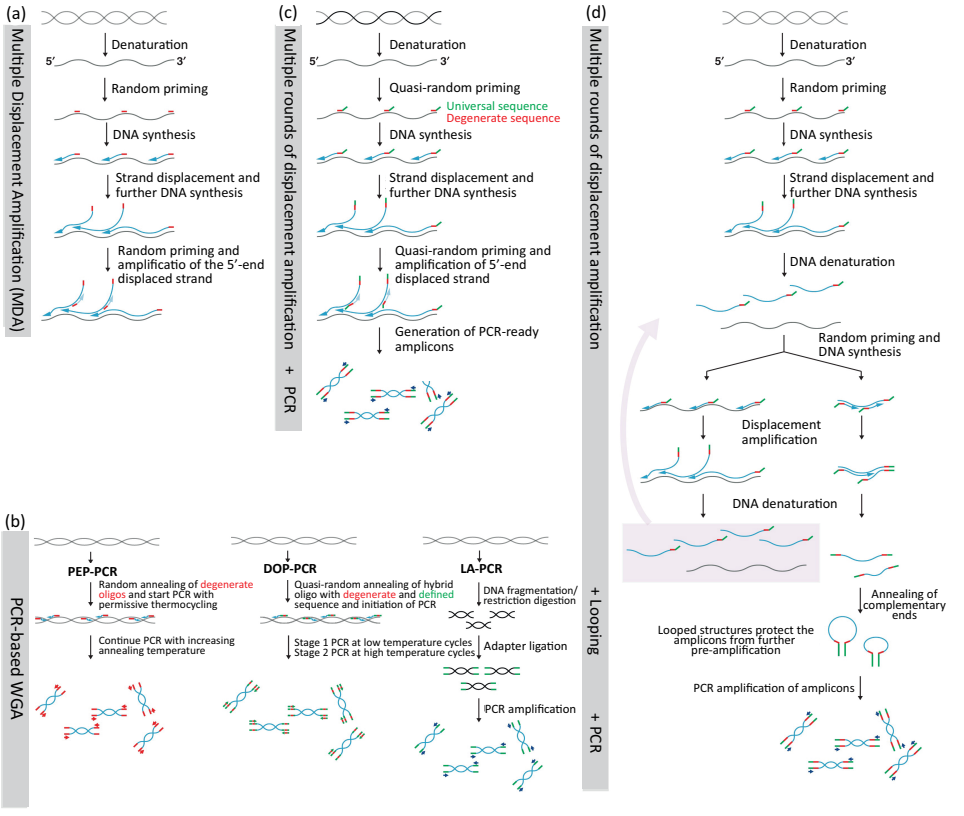

Figure 5. Whole-genome amplification methods. (a) In multiple displacement amplification (MDA), an isothermal reactions starts by annealing random hexamer primers to the template and DNA synthesis is carried out by high-fidelity phi29 DNA polymerase with strand displacement activity. **(b)** PCR-based WGA technologies: (i) In primer-extension pre-amplification (PEP)-PCR DNA is amplified with oligonucleotides of complete degenerate sequence using permissive thermocycling with increasing annealing temperatures. (ii) Degenerate-oligonucleotide primer (DOP)-PCR applies hybrid oligonucleotides containing degenerate and defined sequence and is carried out in two steps: first thermocycling at low annealing temperatures for semi-random priming, followed by PCR at high annealing temperatures for non-random priming. (iii) In linker adaptor (LA)-PCR, DNA is sheared into small fragments and adaptors with universal sequence are ligated to DNA-ends, creating universal sequences for PCR amplification. **(c)** Combined displacement amplification with PCR (DA-PCR) undergoes multiple rounds of displacement pre-amplification to generate unique DNA sequences for PCR amplification. **(d)** MALBAC uses specific oligonucleotide design that allow full amplicons to form looped pre-amplification products that are protected from further pre-amplification until PCR amplification, resulting in a more uniform representation of the genome. Adapted by permission from (Van Loo and Voet, 2014)

In an attempt to overcome the low coverage of PCR-based approaches and lack of uniformity of isothermal MDA, recent WGA methods have combined features of both MDA- and PCR-based amplifications, in which case multiple rounds of displacement pre-amplification of the single-cell genome is followed by subsequent PCR amplification of generated DNA fragments. A modified version of this approach involves multiple annealing and looping-based amplification (MALBAC), which forms circular DNA fragments that will no longer be available as a template until subsequent PCR amplification of generated amplicons (Fig. 5D) (Zong et al., 2012). Due to generation of loops during the first round of amplification, MALBAC results in a more uniform representation of the genome. Similarly to PCR-based WGA, both methods use low-fidelity polymerases compared to MDA, making them more suitable for copy-number profiling. However, the demand for unbiased single-cell genome representation still inspires researches to develop novel WGA protocols (Picher et al., 2016; Chen et al., 2017) or even omit this costly artifacts-introducing step from the single-cell workflow (Xi et al., 2017; Zahn et al., 2017).

2.2.3. Single-cell DNA microarrays

Single-cell array comparative genomic hybridization

The array comparative genomic hybridization (aCGH) technology uses specially designed slides, usually made out of glass, carrying unique DNA segments in certain positions, known as probes. The size and the genomic distance between these probes (probe spacing) determine the resolution of aCGH. As the name implies, for aCGH analysis, the extracted test DNA sample and reference DNA with known karyotype are first labelled with different fluorescent dyes. Next, both denatured genomic DNAs are co-hybridized onto the DNA-microarray containing single-stranded probes. By measuring the fluorescence intensity ratio of the test and reference samples, DNA copy-number alterations can be detected across the genome to a resolution as low as 1–2 Kb on high-density arrays (Conrad et al., 2010). However, aCGH experiments require substantial amount of input DNA material for reliable signal detection and copy-number analysis, so it has mainly been performed using bulk DNA samples. The first successful attempts to detect full and segmental chromosome losses and gains from whole-genome amplified single cells used arrays that contained large genomic clones, known as bacterial artificial clones or BACs (Le Caignec et al., 2006; Fiegler et al., 2007). Compared to fluorescence *in situ* hybridization (FISH) that can evaluate a few loci at time, the ability to screen all 24 chromosomes was a defining moment for single-cell genomics, but it became clear that WGA-induced background noise reduces the sensitivity and specificity of conventional copy-number calling algorithms. Allele drop-out (ADO) and preferential amplification, as well as chimeric DNA molecules generated during WGA, can bias the fluorescent signal, leading to false positive findings. Importantly, cell-

cycle stage can also interfere with accurate copy-number profiling, as cells in S-phase undergo DNA replication that can be misinterpreted as genomic imbalance (Dimitriadou et al., 2014). To overcome these issues, several custom data analysis pipelines have been developed over the few years, including the commercialized 24Sure Microarrays platform (Illumina Inc., currently discontinued), that sought to reduce WGA-bias and enable reliable copy-number detection (Cheng et al., 2011; Konings et al., 2012; Czyz et al., 2014).

Single-cell SNP-genotyping

Despite the improvements, the main disadvantage of aCGH technology is that it can only detect numerical chromosomal abnormalities, but provides no information on the presence of chromosomal mosaicism or copy-number neutral losses of heterozygosity, such as uniparental disomy (UPD), nor determines the parental origin of *de novo* genomic rearrangements. To compensate these drawbacks, several groups made one step further and successfully hybridized single-cell WGA products on high-density SNP-arrays that were initially developed to conduct genotyping procedures (Iwamoto et al., 2007; Vanneste et al., 2009; Johnson et al., 2010; Treff et al., 2010). SNP-array technology allows to genotype hundreds of thousands up to millions of SNPs, which are polymorphic in the human population, by hybridizing the DNA sample to array probes, using the same principle of complementary base pairing, utilized in aCGH. In a normal scenario, a variant is either homozygous (AA or BB) or heterozygous (AB), where A and B represent the two alleles with distinct nucleotide residues. As mentioned, the number of SNPs called, as well as genotyping and copy-number calling accuracy can be significantly affected by different WGA methods (Treff et al., 2011), but the ability to exploit single-cell genotype calls together with their copy-number status enables to differentiate a genuine genomic aberration from WGA artifacts. By using SNP-arrays combined with sophisticated bioinformatical approaches, unbalanced translocation, UPDs, complex structural rearrangements and parental origin of chromosomal aberrations were accurately identified in single embryo blastomeres (Vanneste et al., 2009; Treff et al., 2011; Voet et al., 2011; van Uum et al., 2012).

2.2.4. Single-cell haplotyping

The obtained SNP-genotypes can be used to reconstruct haplotypes, which represent a combination of genetic variants along a single chromosome, and determine the inheritance patterns of particular sets of DNA variants (Browning and Browning, 2011). Once again, the accurate detection of genetic variants and long-range haplotypes from SNP-array data remains challenging due to erroneous WGA. To date two genome-wide single-cell haplotyping methods have been developed that use SNP-array data: Karyomapping (Handyside et al.,

2010) and single-cell haplotyping and imputation of linked disease variants (siCHILD) (Zamani Esteki et al., 2015). Due to a number of attractive features that address the issues of FISH and PCR-assays, both Karyomapping (provided by Illumina) and siCHILD (licensed to Agilent Technologies and marketed under OnePGT Solution) have been clinically implemented for embryo diagnostics (Natesan et al., 2014; Dimitriadou et al., 2017). First, both methods are generic and use available SNP genotypes from the parents and a close relative to reconstruct haplotypes of an embryo, including the locus of interest. Because the analysis is performed on a genome-wide scale, simultaneous screening for multiple Mendelian disorders and traits is possible. Both methods also minimize the issues associated with ADO, because there is no direct mutation analysis. Instead, the inheritance of DNA mutations is deducted from reconstructed haplotype blocks and even if some polymorphic markers will result in ADO, the significant part of variants, flanking the region of interest, will still remain. Finally, both methods can pinpoint to meiotic homologous recombination sites, which can break down the linkage of a pathogenic variant with its nearby SNPs, and also determine genome-wide copy-number profile of a single cell. However, Karyomapping only uses discrete SNP calls, therefore it struggles with reliable detection of post-zygotic chromosome gains compared to siCHILD, which, in addition to SNP genotypes, also relies on continuous BAF-values and can also distinguish the meiotic segregation errors from mitotic ones, based on the recombination patterns. The shortcoming of both genome-wide haplotyping approaches that can limit the use of these techniques is the need for additional DNA from family members and inability to detect *de novo* mutations, for which no carrier family member exists [reviewed by (Vermeesch et al., 2016)]. The modified version of siCHILD methodology (siCHILD-bovine) was at the core of the current thesis research (explained in detail in Chapter 3.2.3).

2.2.5. Single-cell sequencing

Massive parallel sequencing, also known as next-generation sequencing (NGS), is a high-throughput technology that allows billions of sequencing reactions in parallel in a time- and cost-effective manner. NGS has a number of advantages over SNP microarrays that can improve the resolution and accuracy of variant calling. Importantly, unlike SNP-arrays that use a limited amount of known SNP probes, NGS can examine virtually every nucleotide from the amplified cell, allowing the discovery of a full spectrum of *de novo* DNA mutations (Voet et al., 2013). The continuous reduction of sequencing costs has rendered NGS as a promising methodology for single-cell genomics studies, as the principles applied for SNP-array data analysis can be translated to sequences of single cell. For single-cell sequencing, WGA product is first shattered into smaller DNA fragments, from which a library of DNA templates for sequencing is generated. The resulting short sequence reads are mapped to the human reference genome and further investigated for genetic variation using computational workflows.

The first single-cell sequencing was performed to study copy-number aberrations in isolated tumor cells using DOP-PCR, which achieved ~10% physical coverage of single-genome (Navin et al., 2011; Baslan et al., 2012). Since then, substantial progress have been made over the recent years to improve genomic coverage, although the interpretation of single-cell sequencing data still remains complicated due to WGA errors that may be falsely interpreted as copy-number or nucleotide changes in the single-cell genome (Gawad et al., 2016). Single-cell sequencing has been pivotal in cancer research, but it also gradually became a reality in the field of genetic profiling of embryos. By using long-fragment read sequencing technology, genetic variants can also be assembled into a single haplotype, thus enabling to reconstruct individual genome-wide haplotypes using direct phasing (Peters et al., 2012; Peters et al., 2015). Long-range haplotyping will greatly benefit childless *de novo* mutation carriers, in whom linkage analysis cannot be performed. However, the current high cost of these sequencing techniques can prohibit their use in routine clinical practice.

2.3. Application of single-cell genomics in assisted reproduction

Over a short period of time single-cell technologies became a standard practice for diagnostic laboratories, particularly in the field of assisted reproduction for testing preimplantation embryos for genetic abnormalities. Preimplantation genetic testing (PGT) of embryos requires the removal of either one or two cells from cleavage-stage embryo or a few trophectoderm cells from the blastocyst stage, followed by biopsy processing using single-cell technology workflow. Alternatively, polar body biopsy of the oocyte can be performed (Fig. 6). In case of a blastocyst biopsy, embryos are cryopreserved until the next FET cycle, following hormone-supplemented endometrial preparation for embryo transfer. Today, PGT can be classified into (i) preimplantation genetic testing for monogenic disorders, or PGT-M (previously preimplantation genetic diagnosis), (ii) preimplantation genetic testing for aneuploidy, or PGT-A (previously preimplantation genetic screening), and (iii) preimplantation genetic testing for structural rearrangements, or PGT-SR, designed to help carriers of balanced translocations to increase chance of a successful pregnancy, as these couples are at increased risk of having a child with unbalanced karyotype. Once the necessary analyses are performed, only genetically healthy embryos will be chosen for transfer based on the results. In some of countries, especially in regions of Asia, PGT can be performed for so-called social sexing, where an embryo is selected for transfer based on the gender. In Europe, non-medical gender selection is prohibited and sexing can be performed only in case of X-linked recessive disorders, when the causal mutation is unknown.

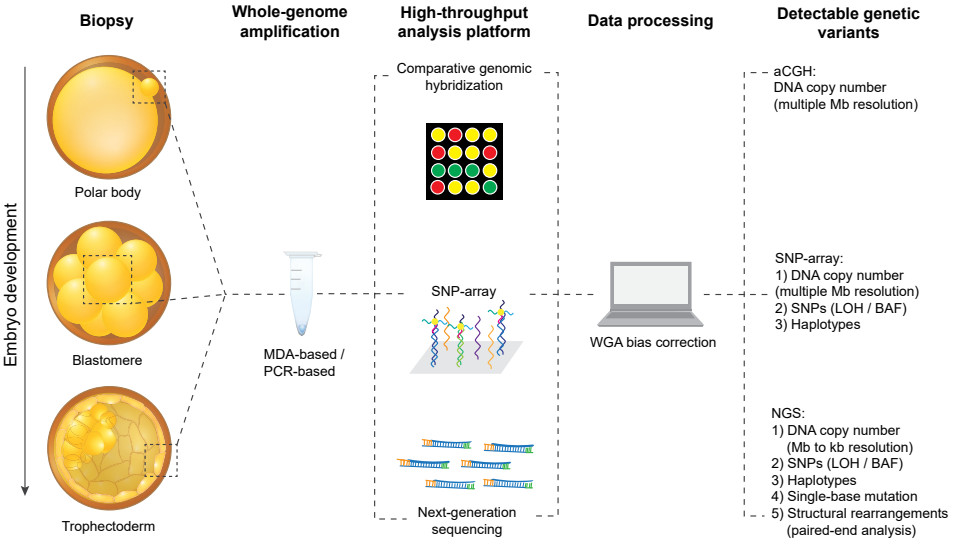

Figure 6. Single-cell genomics for preimplantation embryo testing. Preimplantation genetic testing is based on direct genetic analysis of embryonic material. During the biopsy procedure, either one or two cells are removed at cleavage-stage on day 3 or 5–10 cells from the trophectoderm at blastocyst stage on day 5–6. Alternatively, polar bodies can be biopsied to screen for maternal genetic defects only. Subsequently, biopsied material is whole-genome amplified and screened either for inherited mutations or for chromosomal imbalances by using appropriate single-cell platform. Figure based on (Van der Aa et al., 2013).

2.3.1. Preimplantation genetic testing for monogenic disorders

As an alternative to prenatal diagnosis, patients can be referred to IVF/PGT-M to avoid transmitting an inherited genetic disorder that runs in the family to their child. The first PGT-M application was done already in 1990 by performing sexing of the embryos using PCR to avoid transfer of male embryos, affected with an X-linked disorder (Handyside et al., 1990). In the following years, next successful PGT-M was reported for cystic fibrosis, based on fragment analysis (Handyside et al., 1992). Almost three decades later, PGT-M is performed worldwide and includes the analysis against Mendelian hereditary conditions with a known cause, including autosomal-recessive, autosomal-dominant and X-linked disorders (De Rycke et al., 2017). Traditionally, biopsied cells can be analyzed by either single-cell multiplex PCR coupled with short tandem repeats (STR) haplotyping (Fiorentino et al., 2006; Harton et al., 2011). However this method comes with a bouquet of limitations: they are labor-intensive and require time consuming locus- and family-specific probe design. Consequently, this could lead extensive service costs and long waiting list, which can cause unnecessary emotional stress to couples. In addition, PCR-assays need to minimize ADO effect, which is the primary reason for misdiagnosis. The

development of single-cell genomics and novel genome-wide approaches, addressed earlier, have begun to overcome these limitation (Natesan et al., 2014; Vermeesch et al., 2016; Dimitriadou et al., 2017).

From a healthcare perspective, it has been proposed that national IVF/PGT-M programs can be implemented into the modern healthcare system, offering free service for carrier couples in order to avoid lifetime costs, associated with medical treatment of patients affected by the genetic disease. The scope of PGT-M has also been expanded and the test can also be offered to couples, where one or both partners are carriers of genetic mutations, predisposing to cancer (e.g. *BRCA1* or *APC* mutations for breast and colon cancer, respectively) (Rechitsky et al., 2002; Spits et al., 2007), or adult-onset disease, such as Alzeihmer's (Verlinsky et al., 2002). Despite some of the ethical and legal issues, associated with late-onset disease screening (ASRM, 2013), the number of PGT-M cycles for these type of conditions is increasing together with controversial HLA-matching, in which PGT is employed to conceive a child, who later will become a cord blood or hematopoetic stem cell donor for saving an already affected sibling (Kakourou et al., 2018).

2.3.2. Preimplantation genetic testing for aneuploidy

In contrast to PGT-M cycles, where young fertile couples are recruited for specific mutation screening, PGT-A has been offered to couples, who are burdened with fertility issues due to advanced maternal age, recurrent miscarriage,recurrent implantation failure or severe male factor infertility. Because embryonic chromosomal aneuploidy significantly contributes to embryo implantation failure and spontaneous miscarriage, and is likely to explain the low success rate of IVF treatments, PGT-A has been established in many IVF centres worldwide to select against embryos with abnormal karyotype. Thus, PGT-A has the capacity to prevent adverse IVF and pregnancy outcome and/or provide alternatives for IVF treatment, such as use of egg donors, especially in women with advanced age (Munne et al., 2005; Lathi et al., 2008; Rubio et al., 2013; Munne et al., 2016). However, after the development of advanced single-cell technologies that can screen all 24 chromosomes at once, the topic of PGT-A has become quite controversial. The first alarming paper came out in 2009 demonstrating that 70–80% of human IVF cleavage-stage embryos is made up of cells bearing signatures of chromosomal instability (CIN), which is a hallmark of tumorigenesis, characterized by an increased rate of whole or segmental chromosome aberrations (Vanneste et al., 2009). Further independent studies also confirmed this phenomenon (Johnson et al., 2010; Chavez et al., 2012; Mertzanidou et al., 2013; Chow et al., 2014), which put the whole rationale of PGT-A under question, as high-degree of mosaicism at cleavage-stage, even in young fertile patients, may not adequately represent the genomic status of the whole embryo. These findings also had a direct clinical significance that partially explained why cleavage-stage biopsy lacks improvement in IVF baby-

take-home rate, as revealed by randomized controlled trials in patients undergoing PGT-A (Mastenbroek et al., 2007; Hardarson et al., 2008; Debrock et al., 2010). Moreover, cleavage-stage embryos with abnormal cells may also develop into normal blastocysts (Northrop et al., 2010), highlighting the complex and dynamic nature of human early embryogenesis. Ineffectiveness and potential embryo damage associated with cell removal at cleavage-stages of development (Scott et al., 2013) raised an important discussion at that time: what's next for PGT-A (Vanneste et al., 2009; Harper et al., 2010)? This question also prompted an investigation into novel biopsy strategies. In recent years, genomic analysis has shifted towards TE biopsy that is now widely adopted for PGT-A, because of the ability to analyze multiple cells of the trophoblast lineage without disturbing the inner cell mass that will give rise to the future foetus. TE biopsy is also thought to be less harmful to the overall developmental capacity of embryos, and currently chromosome analysis from the blastocyst stage may provide the most reliable representation of the embryonic genome due to the lower impact of mosaicism (Yang et al., 2012; Forman et al., 2013; Scott et al., 2013; Scott et al., 2013). There is also an important technical aspect, as WGA performs better on TE biopsies with 5–10 cells, compared to only one single cell derived from cleavage-stage biopsy. In addition, NGS techniques were also implemented in PGT-A, proving to be a more sensitive method for aneuploidy screening in embryos, because of the ability to reliably detect chromosomal mosaicism (Munne et al., 2017; Munne and Wells, 2017). Recently, results of a multicenter randomized controlled trial were revealed, demonstrating the potential benefit of PGT-A at the blastocyst stage on pregnancy outcomes, especially in women with advanced age, using NGS-based aneuploidy screening (Munne et al., 2017). However, the implementation of highly sensitive single-cell sequencing for PGT-A also brought up new challenges, associated with chromosomal mosaicism in blastocysts and its effect on subsequent prenatal development, as transfer of mosaic embryos can lead to live birth (Greco et al., 2015). As such, embryonic mosaicism is currently under intense investigation to elucidate the impact of mosaic aneuploidies on pregnancy outcome and to provide proper patient counselling in everyday clinical practice (Fragouli et al., 2017; Munne et al., 2017).

Although current techniques for PGT-A enable whole-genome chromosome screening of single (or few) cells with high sensitivity and specificity, they require a potentially harmful and, in case of TE biopsy, technically challenging invasive removal of embryonic cells. Thus, non- or less invasive approaches have been explored as an alternative to conventional day 3 or day 5 embryo biopsy, including blastocoel fluid sampling and analysis of embryonic cell-free DNA (cfDNA) in culture medium. However, up-to-date only a few studies have explored these options and most of the data were only recently published with either poor or contradicting results for both blastocoel fluid (Perloe, 2013; Gianaroli et al., 2014; Tobler et al., 2015; Magli et al., 2016) and cfDNA analysis (Shamonki et al., 2016; Xu et al., 2016; Feichtinger et al., 2017; Vera-Rodriguez et al., 2018). In addition, there are still concerns regarding the true

origin of genetic material in blastocoel cavity and in spent media, and whether interpretation of the genetic results can be biased due to maternal contamination (Hammond et al., 2016). Thus, additional research is warranted for further investigation and evaluation of non-invasive approaches for embryo aneuploidy screening.

3. Chromosomal instability in preimplantation embryos

One of the first crucial milestones of mammalian embryogenesis is the formation of a blastocyst capable of implantation. Yet as mentioned, the development of human IVF embryos is compromised by a high rate of chromosomal mosaicism, observed at cleavage-stages. This prompted researches to investigate the nature and prevalence of embryonic aneuploidy in greater detail. In general, aneuploidy and CIN are envisioned as a "vicious cycle", where single aneuploidy can increase the likelihood of CIN, which in turn drives aneuploidy (Potapova et al., 2013). The advent of powerful single-cell technologies and time-lapse imaging (TLI) has provided novel insights into mechanistic origins of CIN in human preimplantation development (Fig. 7). TLI has revealed that chromosome imbalances in IVF embryos are not always underlined only by mitotic errors, but are often accompanied by aberrant cleavage patterns with or without cellular fragmentation. Atypical cell morphokinetics, such as entire blastomere fusion or failure of cytokinesis, significantly contribute to genomic instability and can be observed in >50% of aneuploid embryos (Hardy et al., 1993; Chavez et al., 2012). Recently, fusion of a second polar body was detected by whole-genome haplotyping of trophectoderm biopsy derived from human IVF blastocyst (Ottolini et al., 2015). The persistence of polar body DNA in the embryo until the blastocyst stage was rather unexpected, giving the fact that polar bodies typically undergo fragmentation after extrusion from the oocyte upon fertilization.

Different studies on animal models have demonstrated that aneuploidy and mosaicism in early embryos are not restricted only to humans, but have also been observed in farm animals (Viuff et al., 2000; Zudova et al., 2003; Rambags et al., 2005) and non-human primates (Dupont et al., 2009; Dupont et al., 2010). In contrast, spontaneous aneuploidy rate in mouse embryos is very low, so chromosome segregation errors in mice are usually induced by using different inhibitors (Lightfoot et al., 2006; Bolton et al., 2016). Using this approach, a recent study in mice investigated the developmental fate of induced mosaic aneuploidies and demonstrated that aneuploid cells become progressively depleted from the embryo at later stages of development (Bolton et al., 2016). This finding together with the fact that mosaic embryos can successfully result in live birth of healthy babies in human (Greco et al., 2015) challenges the hypothesis that aneuploid embryos are not as developmentally competent as their euploid counterparts. At the same time, these observations highlight our limited knowledge on the molecular causes and the developmental

fate of human embryonic CIN and raise important questions regarding both the burden and nature of aneuploidy that can be tolerated throughout the development.

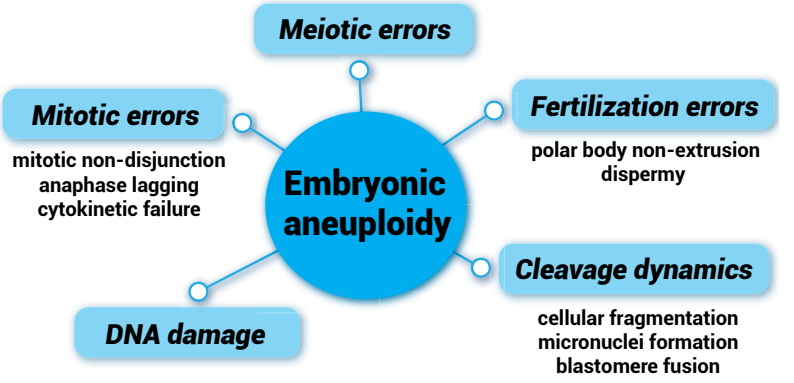

Figure 7. Causes of aneuploidy in preimplantation embryos. Different events may contribute to the generation of embryonic aneuploidy, such as mitotic and meiotic defects, DNA damage, fertilization errors and aberrant cleavage dynamics. Both meiotic and mitotic aneuploidy can increase the likelihood of chromosomal instability within an embryo. Similarly, aberrant fertilization and impaired cleavage morphokinetics can lead to blastomeres with unstable genomes or chaotic chromosome patterns. Adapted by permission from (Tsuiko et al., 2018).

3.1. Maternal contribution to embryonic aneuploidy

Genome integrity of the cells strongly relies on intact molecular mechanisms involved in accurate chromosome segregation during meiotic and mitotic divisions. Errors in either first or second meiotic division lead to zygotic inheritance of meiotic-based aneuploidies that can consequently cause spontaneous miscarriages, stillbirth or congenital birth defects. Given the complexity of meiosis during oogenesis, compared to spermatogenesis, it is not surprising that the majority of meiotic errors in the embryo are of maternal origin (Hassold and Hunt, 2001). Moreover, the incidence of maternal aneuploidy rises abruptly in the mid-thirties and reaches up to 80% by the age of 45 (Franasiak et al., 2014). As woman ages, her meiotic recombination machinery becomes less efficient in time, because it is constantly influenced by the environmental and age-related factors that accumulate in the ovary. In turn, this can potentially lead to altered genomic recombination patterns, which were suggested to be a risk a factor for trisomy 21 (Lamb et al., 2005; Lamb et al., 2005). In general, meiotic recombination in oocytes is surprisingly error-prone, as oocytes are inefficient in translating DSBs into crossovers, while lower genome-wide recombination rates can increase the risk of developing vulnerable crossover configurations that can interfere with proper segregation of sister chromatids (Ottolini et al., 2015; Wang et al.).

The considerable time between the initiation and the completion of meiosis may also likely contribute to the alteration or degradation of factors important for proper chromosome segregation. Numerous morphological and cellular defects that occur in the oocyte, especially aged ones, will consequently impact fertilization rates and reduce embryo development potential (Miao et al., 2009; Keefe et al., 2015). Notably, age-related oocyte aneuploidy is multifactorial, originating from diminished cell-cycle regulation, aberrant spindle formation, loss of proper checkpoint signalling and compromised centromeric cohesion between sisters chromatids [reviewed in (Webster and Schuh, 2016)]. Defective spindle assembly can also be attributed to the age-related chaotic microtubule dynamics that result in multipolar spindles and predispose the oocyte to chromosome segregation errors (Nakagawa and FitzHarris, 2017). This, in combination with general oocyte-specific spindle assembly checkpoint (SAC)-mediated control of meiosis, which is not able to block meiotic progression upon misalignment of a single chromosome, results in an increased aneuploid egg formation in older women and propagation of aneuploidy to developing embryo (Nagaoka et al., 2011). In contrast, male cell-cycle checkpoint mechanisms seem to be more robust and can successfully eliminate cells with meiotic errors regardless of paternal age (Vrooman et al., 2014).

One of the reasons for the age-related decrease of cellular functions in the oocyte might be the influence of age on oocyte transcriptional activity, necessary to guide cytoplasmic maturation, meiotic divisions and post-translational process (Steuerwald et al., 2007; Grondahl et al., 2010). Second, the age-related downregulation of protein ubiquitination in the mammalian oocytes can lead to breakdown of ubiquitin-proteasome pathway, resulting in failure to support meiosis, cell cycle progression and polar body I extrusion (Sun et al., 2004; Ben-Eliezer et al., 2015; Yu et al., 2015). The inhibition of proteosomal activity can also interfere with maternal clearance in the zygote and impair early embryonic development (Shin et al., 2010; Yang et al., 2017). Third, dysfunction of genes and proteins involved in meiotic cell cycle and metaphase spindle formation may also partially explain the elevated prevalence of three pro-nuclear (3PN) zygotes in older women undergoing IVF or ICSI treatment that results from the compromised ability to extrude polar body II (Grondahl et al., 2017). Finally, both zona pellucida and plasma membrane of aged oocytes undergo structural changes that may likely predispose the aged oocyte to fertilization abnormalities, resulting in either reduced fertilization rates or increased incidence of polyspermy due to compromised membrane block to prevent polyspermic egg penetration (Wortzman and Evans, 2005).

The decreased cellular function in the oocyte has also been extensively associated with altered mitochondrial biogenesis, morphology and function (May-Panloup et al., 2016). Cell division is energy demanding and alterations in mitochondrial dynamics and metabolic pathways can impair the process (Salazar-Roa and Malumbres, 2017). In support of this, deficit in mitochondria-produced ATP was shown to cause spindle instability and impaired chromosome segregation in MII mouse oocytes (Zhang et al., 2006). Because ATP is

required for the movement of microtubules during anaphase and cytoskeletal actin filament contraction during cytokinesis, the decreased mitochondrial DNA content in the oocytes may fail to synthesise sufficient amount of ATP, contributing to aneuploidy formation in oocytes and future embryos (Wilding et al., 2001; Eichenlaub-Ritter et al., 2004; Fragouli et al., 2015). Interestingly, embryos seem to possess a compensatory mechanism for inherited mitochondrial deficiency by initiating premature mitochondrial biogenesis, however this untimely response seems to lead to reduced developmental potential (Fragouli et al., 2015). Thus, defective maternal resources, together with the relaxed or absent cell-cycle checkpoint discussed above, may contribute to embryonic aneuploidy, compromising preimplantation development.

3.2. Paternal contribution to embryonic aneuploidy

Up to 80% of chromosome imbalances in IVF human embryos are mitotic in origin and lead to chromosomal mosaicism. In case of mitotic mosaicism, male factors are often suspected, as increased DNA damage of human spermatozoa can negatively impact fertilization rates and/or early embryo development (Duran et al., 2002; Benchaib et al., 2003; Silber et al., 2003). Because male germ cells divide continuously, DNA lesions start accumulating over time in the testis, mostly due to oxidative stress. Failure to repair oxidative DNA damage can result in genomic integrity decline in the sperm, especially in males with advanced paternal age (Paul et al., 2011). Reactive oxygen species (ROS), along with apoptosis and abnormal chromatin packaging, can also lead to sperm fragmentation, which is one of the causes of male subfertility and infertility. Spermatozoa with fragmented and/or unrepaired DNA are able to fertilize oocytes, but they can significantly contribute to the generation of chromosomal aberrations in the embryo, compromising its development (Garcia-Ferreyra et al., 2015).

Aside from providing the paternal genome, sperm cell also contributes its centrosome to generate the mitotic spindle that segregates chromosomes to two sister blastomeres during the first post-zygotic division (the only exception is the mouse, where centrosome is maternally inherited). However, in some fertilized oocytes the first post-zygotic cleavage does not occur as expected. Under *in vitro* conditions almost 12% of human zygotes divide into three cells (Chamayou et al., 2013). Time-lapse analysis of human IVF embryos has revealed that tripolar division is frequent in dispermic zygotes with three pronuclei (3PN), carrying an extra set of haploid paternal or maternal chromosomes, but bi-pronuclear (2PN) embryos also exhibit abnormal first post-zygotic cleavage (Joergensen et al., 2014). Hence, sperm centrosome anomalies and formation of abnormal tripolar spindle result in chaotic chromosome profiles of the embryo due to disorganized first mitotic division (Moomjy et al., 1999; Silber et al., 2003).

Lastly, in addition to inheritance of centrosome components, several studies have implied that sperm retains coding and non-coding RNAs that are necessary

for successful fertilization and may be potentially relevant in regulating gene transcription and cellular processes in early embryos (Boerke et al., 2007; Sendler et al., 2013; Teperek et al., 2016; Selvaraju et al., 2017). However, unlike maternal transcripts, the role of RNAs detected in paternal gametes has remained uncertain and the impact of aberrant paternal factors on embryo genome stability requires further elucidation.

3.3. Post-zygotic origins of embryonic aneuploidy

As embryonic cleavage proceeds, errors in post-zygotic division, such as centrosome overduplication, impaired spindle assembly, chromosome cohesion/segregation dynamics and cytokinesis, can contribute to chromosome missegregation and aneuploidy (Silkworth and Cimini, 2012). Indeed, knockout studies in mouse pre- and peri-implantation embryos have revealed that disruptions in key genes involved in the centrosome/kinetochore structure or cell-cycle checkpoints lead to aneuploidy, mitotic arrest, abnormal mitotic division and early embryonic lethality (Artus et al., 2006). Although there are numerous possible mechanisms leading to post-zygotic errors in cleavage-stage embryos (Fig. 8), anaphase lagging and mitotic non-disjunction are the most frequent causes of embryonic aneuploidy and mosaicism. During mitotic non-disjunction, chromatids fail to separate at the centromere during cell division, resulting in loss of a chromosome in one daughter cell and a reciprocal gain in the other. In contrast to chromosome non-disjunction, anaphase lagging occurs when one of the sister chromatids fails to connect to the spindle apparatus or is prematurely dissociated from it and is lost upon cytokinesis, resulting in one diploid daughter cell and one cell with a monosomy.

In addition, erroneous merotelic kinetochore-spindle microtubule attachments can also contribute to the onset of aneuploidy and CIN in early embryos. Just like in meiosis, spindle assembly and chromosome alignment on the metaphase plate during mitosis is regulated by SAC machinery that prevents premature separation of sister chromatids until proper chromosome alignment has been achieved and all kinetochores have established a stable connection to bipolar spindles. Once tension is detected, SAC is inactivated, MTs shorten and two sister chromatids become separated until finally cytokinesis divides the cell into two daughter cells with complementary sets of chromosomes. In correct amphitelic attachment, k-MTs attach each sister kinetochore to opposite spindle poles, establishing bi-oriented chromosome orientation that is necessary for proper chromosome segregation. In merotelic k-MT attachments, single kinetochores attach to microtubules extending from both spindle poles. Because kinetochores are attached by MTs from both sides and support chromosome alignment on a metaphase plate, merotely avoids detection by SAC, which leads to failure of chromatid segregation and lag of one chromosome near the central spindle, while the other chromosome moves towards the spindle pole (Thompson and Compton, 2011). Because merotely is not detected by SAC, it

may cause anaphase lag at the spindle equator or centromeric breakage of the chromosome, leading to aneuploidy or sub-chromosomal imbalances, respectively. It has been proposed that merotely can also induce centromere-localized DNA breaks, leading to sub-chromosomal imbalances that can be accompanied by breakage-fusion-bridge (BFB) cycle and/or result in complex genomic rearrangements observed in human IVF embryos (Vanneste et al., 2009; Voet et al., 2011; Voet et al., 2011). Consequently, the lagging chromosomes can form micronuclei in the cytoplasm, providing the basis for cellular fragmentation in human embryos (Chavez et al., 2012). Once trapped within micronuclei, chromosomes can undergo defective DNA replication, resulting in DNA damage and frequent double-strand breaks (DSB) (Crasta et al., 2012). In turn, DSB can trigger an error-prone non-homologous end-joining (NHEJ) repair mechanism in micronuclei (Ly et al., 2017) that can result in chromosomal aberrations. These events may also lead to chromosome pulverization within the micronuclei. Moreover, chromosomal fragments can be incorporated back into the genome of a developing embryo upon fusion of the micronuclei with blastomeres, resulting in chaotic chromosome aberration patterns (Chavez et al., 2012).

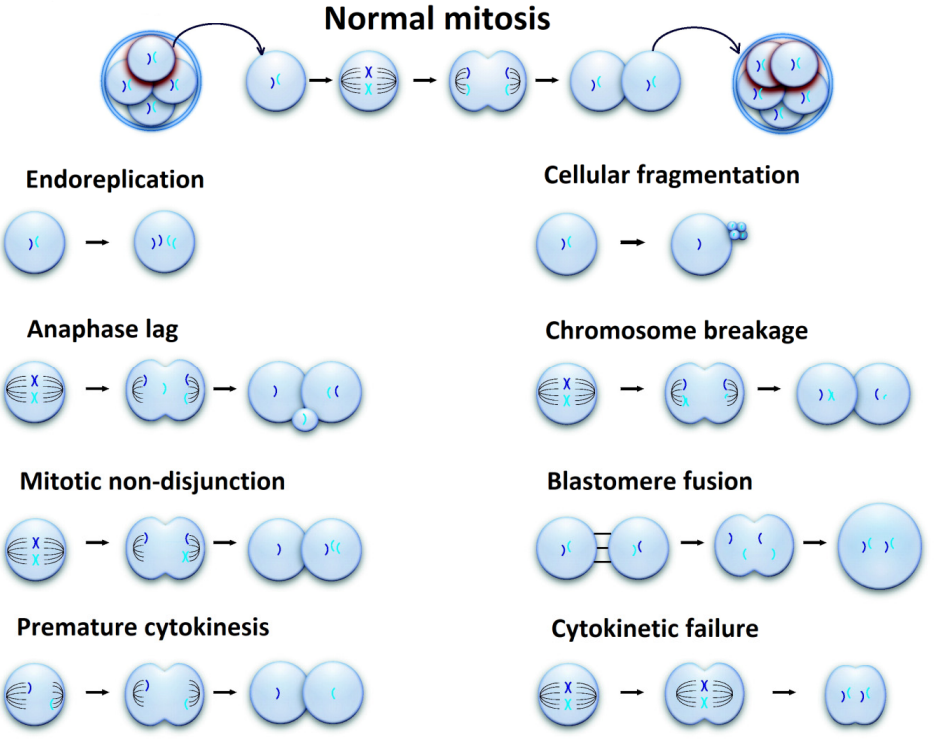

Figure 8. Schematic overview of post-zygotic events, leading to aneuploidy in preimplantation embryos. Adapted by permission from (Mantikou et al., 2012).

II. AIMS OF THE STUDY

The introduction of IVF into clinical practice made it possible to study human embryos at their earliest stages of development. The application of single-cell genomics revealed that cleavage-stage embryos are burdened with aneuploidy, which significantly contributes to implantation failure, first trimester miscarriage and low IVF success rate. This knowledge also prompted the investigation into novel biopsy and embryo comprehensive chromosome screening (CCS) strategies for preimplantation genetic testing for aneuploidy (PGT-A). However, some of the post-zygotic events observed *in vitro* may also occur *in vivo*, as only 30% of human conceptions result in live birth, while in at least half of all spontaneous miscarriages chromosomal abnormalities can be detected. Owing to considerable legal and ethical limitations, associated with human oocyte and embryo research, the possibilities to study genetic and molecular features of human embryo development *in vivo* are lacking. As such, the use of appropriate animal models is inevitable to understand the origins of embryonic chromosomal instability (CIN) that drives error-prone stages of preimplantation development. Ultimately, the overall objectives of the current thesis were:

1. To investigate the genomic architecture of *in vitro* bovine cleavage-stage embryos by concurrent haplotyping and copy-number profiling of single blastomeres.

2. To investigate the prevalence and nature of CIN in naturally conceived *in vivo* embryos, using the established bovine model for studying genomic instability in preimplantation embryos.

3. From the clinical perspective, to investigate the use of blastocoel fluid aspiration as an alternative less invasive biopsy approach for obtaining embryonic genetic material for PGT-A.

III. MATERIALS AND METHODS

3.1. Donor animals and embryo production protocols (Study I & II)

The general scheme of different embryo production protocols used in Study I and Study II is depicted in Figure 9. Oocytes from eight slaughter house-derived Belgian blue cows (*Bos taurus*) and semen from two Holstein-Friesian bulls (*Bos taurus*) were used for bovine *in vitro* embryo production (IVP) in Study I. Because haplotyping requires parental genotypes for phasing, ovarian tissue from donor cows (mothers) and semen from two bulls (fathers) were used to extract bulk DNA (DNeasy Blood and Tissue kit, Qiagen, Germany). In addition, bulk DNA was obtained from the parents of the bull (parental grandparents).

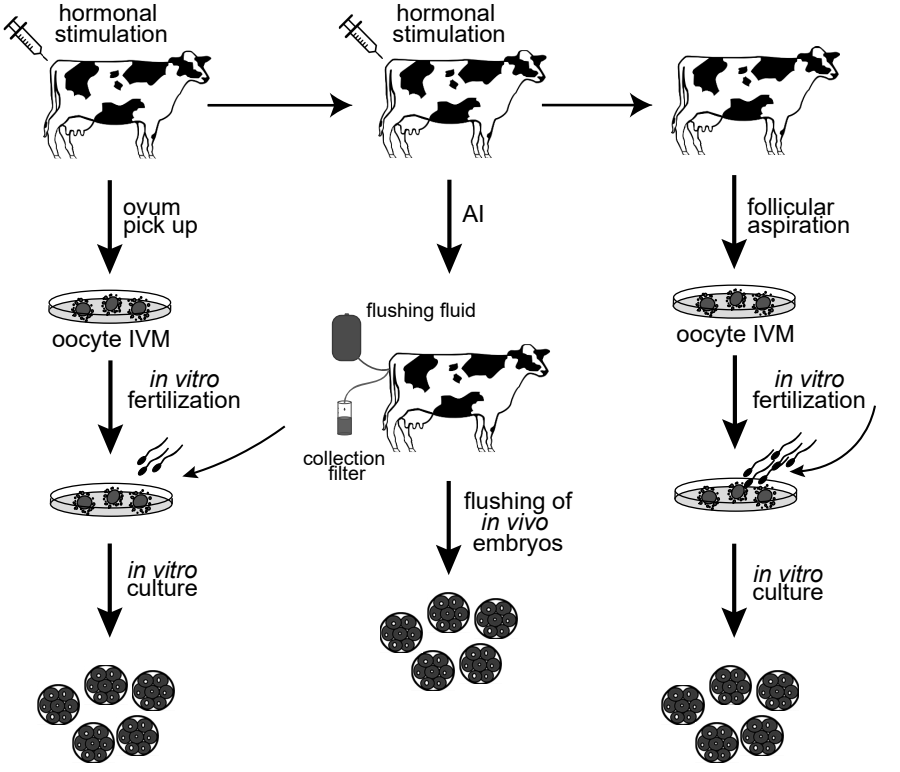

Figure 9. Schematic overview of different embryo production protocols used for Study I and II. For Study II, five donor cows were used to obtain *in vitro* embryos produced by ovum pick up with ovarian stimulation (OPU-IVF) (left). Next, naturally conceived in vivo embryos were obtained from the same donor cows via oviduct flush (middle). Finally, donor cows were slaughtered and *in vitro* embryos were produced from *in vitro* matured oocytes retrieved without ovarian stimulation (IVM-IVF) (right). For Study I, only IVM-IVF embryos from eight donor cows were used for the analysis [Fig. 1 in (Tsuiko et al., 2017)].

For Study II, five young, healthy, cycling Holstein Friesian heifers (*Bos taurus*) between 18 and 36 months of age were used as oocyte and embryo donors. Similarly, blood samples from the donor cows (mothers) and semen from the bull (father) were used to extract bulk DNA (DNeasy Blood and Tissue kit, Qiagen, Germany). Bulk DNA was also obtained from the parents of the bull (paternal grandparents) and the available parents of the cows (maternal grandparents). All animal experiments were approved by the Ethical Committee of the Faculty of Veterinary Sciences of Ghent University, Belgium (EC2013/197, EC2015/71).

3.1.1. Stimulation protocol and ovum pick up

Basic Eagle's Medium amino acids, Minimal Essential Medium (MEM) non-essential amino acids (100×), TCM-199-medium, kanamycin and gentamycin were purchased from Life Technologies Europe (Ghent, Belgium). All other necessary components were obtained from Sigma (Schnelldorf, Germany), unless otherwise stated. Before usage, all the media were filter-sterilized through a 0.22 μm filter (Pall Corporation, Ann Arbor, MI, USA).

Stimulation protocol for ovum pick up (OPU) was used to generate OPU-IVF embryos and was performed 3 to 6 times in all animals with at least one week interval between OPU sessions. On day 0, heifers were given an epidural anesthesia using 3 mL of Procaine Hydrochloride 2% (VMD, Belgium) to decrease peristalsis and discomfort. An ultrasound probe was inserted in the vagina, and dominant follicles larger than 5 mm were removed by puncturing the ovaries to promote follicular growth. Animals received dinoprost ($PGF_{2\alpha}$) intramuscularly (i.m.) (Dinolytic®, Zoetis, Belgium) and a CIDR (controlled internal drug release, Progesterone, Zoetis, Belgium) was administered in the vagina. In following days, pFSH injections (Stimufol®, Reprobiol, Belgium) were given i.m. twice a day. The CIDR was removed 40 to 44 hours after the last pFSH injection and OPU was performed. Prior to OPU procedure, animals were given an epidural anaesthesia using 3 mL of Procaine Hydrochloride 2%. All follicles were aspirated using an ultrasound probe, a 7.5 MHz transducer and a stainless steel guide. Puncturing was performed using disposable 19G needles connected to a 50 mL tube via silicon tubing. Needles were changed between ovaries of the same animal and between different animals. Follicular fluid containing the oocytes was collected in 5 mL HEPES-buffered TCM-199 supplemented with 18 IU/mL heparin, 50 μg/mL gentamicin and 0.1% fetal calf serum (FCS). The collected follicular fluid was immediately filtered through a 75 μm mesh filter with HEPES-buffered TCM-199. Oocytes were grouped per donor and embryos were produced according to the standard *in vitro* embryo production protocol.

3.1.2. Bovine *in vitro* embryo production

Bovine *in vitro* embryos were produced per donor by previously described methods (Catteeuw et al., 2017). Briefly, oocytes retrieved via OPU procedure and oocytes retrieved from ovaries of slaughtered animals were placed per donor in 500 µL maturation medium for 22 h at 38.5 °C in 5% CO_2 in humidified air. The maturation medium consisted of modified bicarbonate-buffered TCM-199 supplemented with 50 µg/mL gentamycin and 20 ng/mL epidermal growth factor (EGF). After maturation, frozen-thawed semen of a previously tested Holstein Friesian bull was used for fertilization. Spermatozoa were separated over a discontinuous Percoll gradient (45 and 90%; GE Healthcare Biosciences, Uppsala, Sweden) and sperm concentration was adjusted to 1×10^6 spermatozoa/mL using IVF-TALP medium, which is supplemented with 6 mg/ml bovine serum albumin (BSA, Sigma A8806) and 25 µg/ml heparin. Matured oocytes were co-incubated with spermatozoa in 500 µL IVF-TALP medium for 21h at 38.5 °C in 5% CO_2 in humidified air. Presumptive zygotes were transferred to synthetic oviductal fluid (SOF) supplemented with essential and non-essential amino acids, 0.4% BSA (Sigma A9647) and ITS medium (5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium) and were placed in a droplet of culture medium per donor. The droplet size differed between donors, depending on the number of zygotes, maintaining a 1:2 embryo:medium ratio. The minimal droplet size was 20 µL. Each droplet was covered by mineral oil and incubated at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2.

3.1.3. Collection of *in vivo*-derived bovine embryos by oviductal flush

The *in vivo* collection of embryos was performed by oviductal flush, as described earlier (Besenfelder et al., 2008). First, oestrous cycles of donor animals were pre-synchronized by i.m. administration of 2 ml PGF2α (500 µg Cloprostenol, Estrumate, Belgium) twice within 11 days. Forty eight hours after both $PGF_{2\alpha}$ treatments, the animals received i.m. 21 µg of Gonadotropin Releasing Hormone (GnRH) (Receptal®, MSD AH, Belgium). Dominant follicles were ablated nine days after heat detection. Thirty six hours after follicle ablation, pFSH was administered in decreasing dosages twice a day for four consecutive days (1.5 mL, 1.4 mL, 1.2 mL, 1.1 mL, 0.8 mL, 0.6 mL, 0.5 mL and 0.5 mL, respectively), and in total 380 µg of follitropine was given. The donor animals received two $PGF_{2\alpha}$ treatments 60h and 72h after the initial pFSH treatment. Finally, 24h after the last pFSH treatment, 21 µg GnRH was administered to induce ovulation; simultaneously animals were artificially inseminated (AI) with frozen-thawed semen. AI was repeated 12h and 24h later and embryos were flushed bilaterally 36h after the last AI. Briefly, donor animals were given epidural anaesthesia using 5 mL of Procaine Hydrochloride 2% and an embryo flushing catheter was directed through the cervix and fixed in the uterine horn. An integrated device, consisting of a universal tube, an

endoscope and flushing system was inserted through the vaginal wall into the peritoneal cavity, which was passively filled with air. The flushing system consisted of a 20 mL syringe, a perfusor tube and a metal tube with numerous lateral holes covered by a silicon tube. The curved end of the metal tube was inserted via the infundibulum into the ampulla. Oviducts were flushed with 40 to 60 mL flushing medium, consisting of phosphate buffered saline (PBS), supplemented with 1% FCS, to get the embryos pass the uterotubal junction. To avoid medium reflux, slight pressure near the flushing tube was applied. Once in the uterine horn, flushing medium, containing the embryos, was collected via the uterus flushing catheter into an embryo filter. The uterine horn was then flushed with another 300 to 500 mL medium through the uterine flushing catheter. The whole flushing procedure was repeated for the other oviduct and uterine horn. Finally, the collected medium per donor was transferred to Petri dishes and examined for embryos under a stereomicroscope.

3.2. Single-cell workflow for analysis of bovine cleavage-stage embryos (Study I & II)

3.2.1. Single blastomere isolation and whole-genome amplification

All obtained bovine embryos were treated with 0.1% pronase (S. *griseus,* Sigma P88110) in Hepes-buffered TCM-199 (Life Technologies Europe, Belgium) to dissolve zona pellucida, except for *in vivo*-derived embryos, where 1% pronase solution was used. The zona-free embryos were then washed in HEPES-buffered TCM-199 with 10% FCS followed by Ca^{+2}/Mg^{+2} free PBS with 0.05% BSA to stimulate blastomere dissociation. Next, each blastomere was washed in three droplets of wash medium (Ca^{+2}/ Mg^{+2} free PBS with 0.1% polyvinyl-pyrrolidone, PVP) and subsequently transferred into a 0.2-mL PCR tube containing 2 µL of PBS. Blastomere pick-up and tubing was manually performed with the use of either mouth-pipette system or the STRIPPER™ micropipetter (Origio, Denmark) using 75 µm capillary. The isolated blastomeres were immediately snap-frozen on dry ice and stored in –20 °C prior to WGA. The DNA from single blastomeres was whole-genome amplified using commercially available MDA kits (REPLI-g Single Cell Kit, Qiagen; and GenomiPhiV2, GE Healthcare Biosciences™, UK) according to the manufacturer's protocols. In case of REPLI-g Single Cell kit, a fast 3 h protocol was performed according to instructions. WGA products were then purified with SPRI-beads (Beckman Coulter Inc., USA) at 0.8× total reaction volume.

3.2.2. Single-cell SNP-genotyping

After purification, WGA samples were normalized to 600 ng/µl and bulk DNA from the parents and grandparents was normalized to 50 ng/µl prior to the downstream use of Illumina Infinium HD assay super protocol, according to the manufacturer's instructions (Illumina Inc.). The WGA and bulk DNA samples were genotyped using BovineHD Genotyping BeadChip. Subsequently, the discrete SNP genotypes, log-R ratio (logR) and B-allele frequency (BAF) values were obtained for each sample by applying the GenCall algorithm, embedded in the GenomeStudio software Genotyping Module v.3.1 (Illumina Inc.). SNP genotypes were called by setting the GenCall threshold score at 0.75, based on the optimization steps described previously (Zamani Esteki et al., 2015). The obtained raw logR- and BAF-values, as well as SNP genotypes were applied in a modified version of siCHILD computational workflow (siCHILD-bovine) to acquire genome-wide haplarithm plots for each sample (as described below).

3.2.3. Genome-wide analysis of single bovine blastomeres

In this research, a newly developed methodology – termed haplarithmisis (Greek for haplotypes numbering) – was used to investigate the genome-wide haplotype architectures of all single blastomeres, comprising bovine cleavage-stage embryos. The step-by-step process of haplarithmisis is depicted in Figure 10 and is described in greater detail by (Zamani Esteki et al., 2015). Briefly, haplarithmisis uses single-cell SNP BAF-values and phased parental genotypes to determine genome-wide haplotypes, copy-number state of reconstructed haplotypes, as well as the parental and segregational origin of putative haplotype anomalies in the cell. First, parental genotypes are phased via SNP genotype calls derived from a close relative, e.g. sibling or the grandparents (Fig. 10). Next, informative SNP loci are identified, in which case one parent is heterozygous and the other parent is homozygous for a particular SNP. These informative SNPs are then categorized as paternal, if the father's genotype is heterozygous and mother's genotype is homozygous, or maternal, when mother's SNP is heterozygous and father's SNP is homozygous. Next, specific combinations of phased parental genotypes are retrieved (P1, P2, M1 and M2; Fig. 10) that consequently define single-cell SNP BAF-values. Those BAF-values, for which called SNPs have a BA genotype in either of the parent, are mirrored around the 0.5x axis and all the retrieved BAF-values for consecutive SNPs in the genome are segmented and visualized as paternal and maternal haplarithm plots (Fig. 11). Importantly, the concept of haplarithmisis also enables to detect homologous recombination sites between the parental homologs.

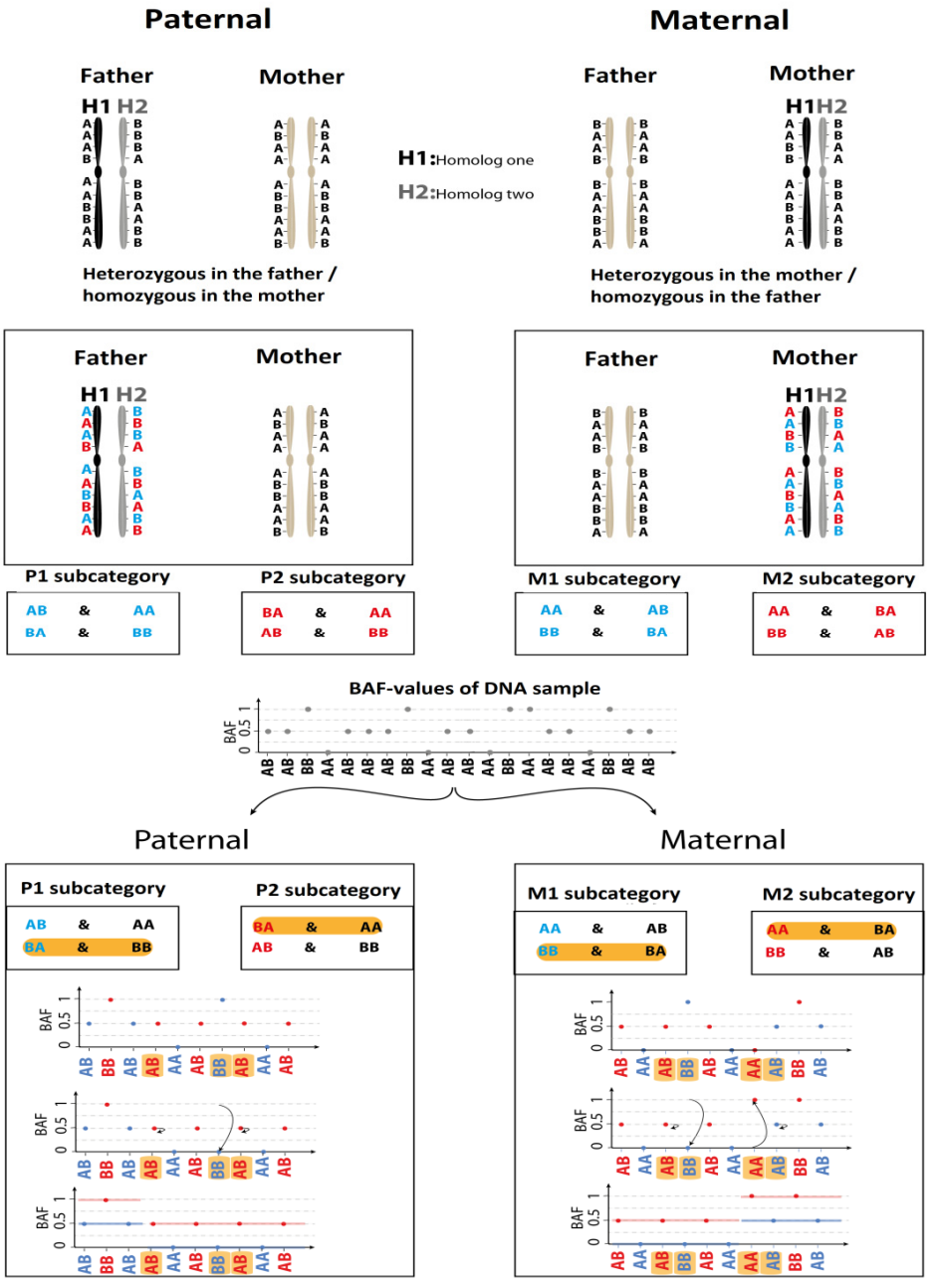

Figure 10. The principle of haplarithmisis. The parental genotypes are first phased via SNP genotype calls derived from a close relative, after which paternal and maternal homologue 1 (H1) and homologue 2 (H2) are determined. Based on the defined phased parental genotype combinations, the SNP–BAF values of a blastomere are binned into paternal-informative SNP subcategories P1 (blue box) and P2 (red box); a similar approach is used for maternal-informative SNPs to obtain M1 (blue box) and M2 (red box) subcategories. When the cell inherits H1 from the father, and either H1 or H2 from the mother, the P1 SNP–BAFs have values of either 0 or 1 (corresponding to homozygous AA and BB genotypes in the cell, respectively) and the P2 SNP–BAFs have a value of 0.5 (corresponding to heterozygous AB genotypes in the cell). In contrast, when the cell inherits H2 from the father, the P1 SNP–BAFs have a value of 0.5 and the P2 SNP–BAFs have a value of either 0 or 1. A defined subset of the single-cell BAF values (indicated in orange) are then mirrored around the 0.5 axis, allowing to segment single-cell P1 and P2 BAF values for consecutive SNPs in the genome. The resulting P1 and P2 BAF segments (depicted in blue and red, respectively) now define the haplotype blocks inherited from paternal H1 and H2. The same approach is used to impute maternal M1 and M2 BAF-segments. The reconstructed haplotype blocks of the cell, and concomitantly the copy number information of these haplotype(s) are then visualised in the parental haplarithm plots. Courtesy of Dr. Masoud Zamani Esteki; adapted by permission from https://hiva.esat.kuleuven.be/.

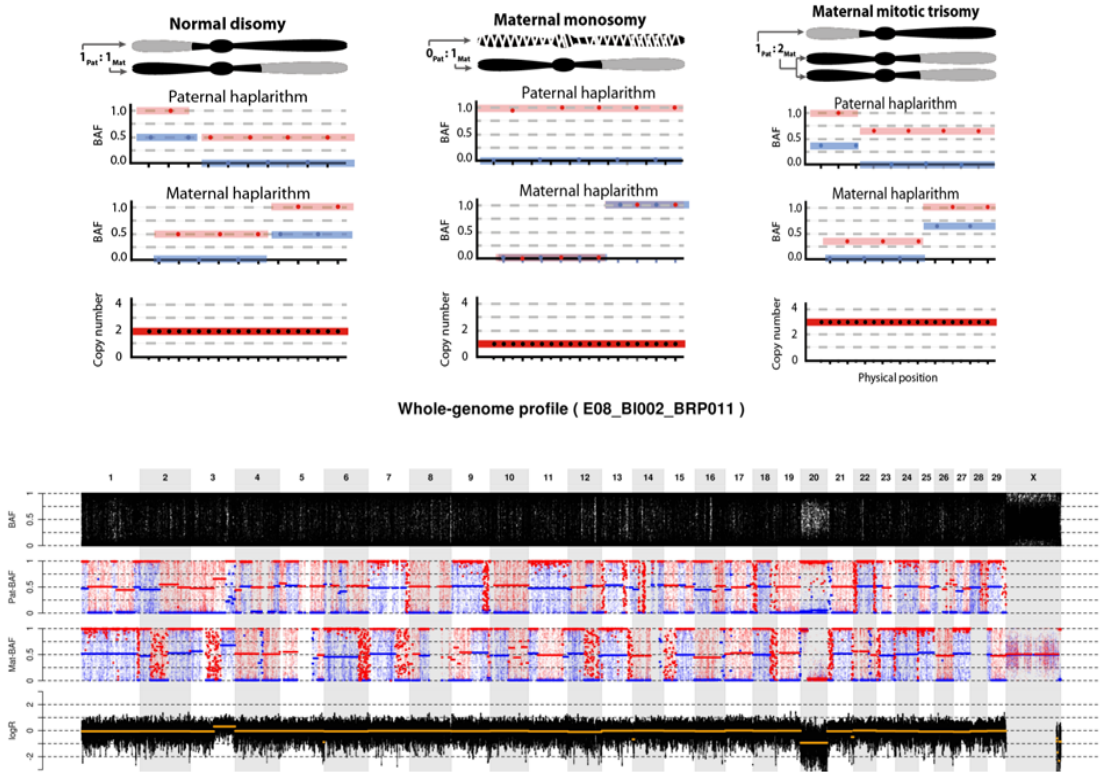

Figure 11. Genome-wide haplarithm profile of an individual bovine blastomere. The top row signifies different haplarithm profiles for normal disomy, as well as monosomy and trisomy of maternal origin. Defined single-cell BAF-values of the segmented P1, P2, M1 and M2, as well as the distance between the P1-P2 or M1-M2 denote the origin and nature of copy number (CN) alterations. The normalized logR-values are integrated with haplarithm profiles for copy-number profiling. The pairwise breakpoints in the segmented P1-P2 and M1-M2 in the haplarithm profiles signify homologous recombination sites, accompanied by the switch in the haplotype blocks. Based on the recombination patterns, the haplarithm signatures also allow tracing chromosomal aberrations back to meiotic I (MI), meiotic II (MII) or mitotic segregation errors (with the exception of monosomies). The genome-wide plot is also depicted for a single bovine blastomere (bottom), carrying chr3 segmental duplication, chr20 monosomy and chrX nullisomy, accompanied by respective changes in the haplarithm patterns and logR ratio.

3.2.4 Statistical analysis

For Study II, statistical calculations were performed using GraphPad Prism 6 software (GraphPad Software Inc., USA). The prevalence and nature of CIN were compared between the three embryo groups and corresponding single blastomeres by two-tailed Fisher's exact test with Bonferroni correction for multiple testing. The differences in the frequencies of CIN between the three embryo cohorts were considered to be statistically significant when the multiple testing corrected *P*-value was <0.01. When comparing two groups of monospermic embryos, a *P*-value <0.05 was considered to be statistically significant.

3.3. Human embryo biopsy and sample processing (Study III)

Embryo biopsy and sample collection was performed at the Tomsk regional perinatal center (Tomsk, Russia) and the Krasnoyarsk Center for Reproductive Medicine (Krasnoyarsk, Russia). The study was approved by the Bioethics Committee of the Biological Institute of the National Research Tomsk State University and all patients have signed an informed consent. All micromanipulations were performed under a hood in a high-quality standard IVF laboratory. The schematic representation of study design is depicted in Figure 12.

Embryo biopsy was performed on cryopreserved blastocysts, donated for research by patients, who have undergone IVF treatment. Cryopreservation and thawing of blastocysts were done according to the manufacturer's VT601-TOP/ VT602-KIT protocol (Kitazato Corporation, Japan). According to the study design, BF was first aspirated, and ICM and TE cells were further isolated and collected for separate chromosome analysis. Blastocyst micropuncture and aspiration of BF was performed after blastocyst thawing by previously described methods (D'Alessandro et al., 2012; Gianaroli et al., 2014). Briefly, blastocysts were immobilized by a holding pipette, mounted on a micromanipulator, and BF from the cavity of expanded blastocysts was aspirated by an ICSI micropipette (Origio, Denmark), which was inserted between the TE cells to minimize the possible cell damage. The use of ICSI micropipette also minimizes the risk of cross-contamination by intact TE or ICM cells. A single aspiration was performed, avoiding aspiration of any cellular material. A volume of ~0.01µl of blastocoel fluid was retrieved from each blastocyst, which was then expelled into the 2.5 µl of 1x PBS. After BF aspiration and blastocyst re-expansion, OCTAX Laser Shot™ microsurgical laser system (MTG, Germany) or ZILOS-tk® (Hamilton Thorne, USA) were used to separate TE and ICM cells. All biopsied materials were immediately whole-genome amplified and stored in –20 °C until further processing.

3.3.1. Whole-genome amplification and next-generation sequencing.

Whole-genome amplification of all samples was performed by ligation-mediated PCR-based commercial PicoPLEX kit according to manufacturer's protocol (Rubicon Genomics, USA). The quality of DNA amplification was controlled by 1% agarose gel electrophoresis and the amount of DNA was quantified by Qubit® dsDNA HS Assay kit (Thermo Fisher Scientific, USA). Next, the most widely used NGS-based platform for preimplantation genetic screening (VeriSeq™ PGS, Illumina Inc.) was utilized for embryo chromosome analysis. Successfully amplified sample processing and library preparations were done according to the manufacturer's VeriSeq™ PGS kit protocol. The obtained libraries were sequenced with Illumina MiSeq® system. Data analysis and genome-wide profile visualization was performed by applying standard settings on Illumina BlueFuse Multi v4.3 Software with embedded aneuploidy calling algorithm. The detection sensitivity and the degree of mosaicism were determined by BlueFuse Multi v4.3 numerical values.

3.3.2. Statistical analysis

For Study III, statistical calculations were performed using GraphPad Prism 6 software. The prevalence of chromosomal aberrations, including mosaic aneuploidies, in BF, TE and ICM was assessed with Chi-square test and the difference in the number of affected chromosomes between diffeent embryo biopsies was considered to be statistically significant, when multiple testing corrected *P*-value was <0.002. When comparing the karyotype concordance of ICM between either BF or TE, two-tailed Fisher's exact test was applied to determine the potential value of BF-DNA use for aneuploidy screening and *P*-value was <0.05 was considered to be statistically significant.

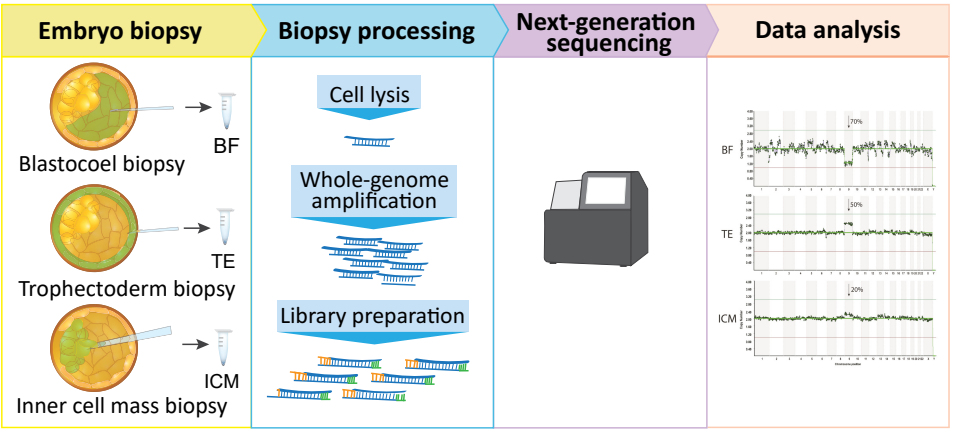

Figure 12. The schematic representation of Study III worklow. After initial sample collection, the embryonic material was whole-genome amplified and sequencing libraries were prepared according to standard VeriSeq™ PGS kit protocol and sequenced with Illumina MiSeq® system.

IV. RESULTS AND DISCUSSION

4.1. Characteristics of CIN in bovine *in vitro* cleavage-stage embryos (Study I)

Understanding the mechanism guiding early human preimplantation development is the key to implementing potential improvements in ART, as low efficiency of IVF treatments poses significant social and financial burden to the patients. However, human embryo research is very restricted due to ethical considerations; hence many studies on cellular and molecular mechanisms of early embryogenesis inevitably rely on animal models. Historically, the relatively cheap mouse model has been the most useful and popular animal model for studying biochemical and physiological regulations of mammalian embryo development. However, when it comes to studying origins of CIN in early embryos, mouse model may be the least suitable due to high genomic stability. A major difference between the early human and mouse embryogenesis is the timing of EGA. Because mouse embryonic genome is activated already at the 2-cell stage of development, it can potentially ensure more efficient guarding of the genome due to earlier onset of mitotic checkpoints, compared to human (Wells et al., 2005; Wei et al., 2011). In addition, human oocytes have a poorer competence to maintain genomic stability in embryos prior to EGA, compared to mice (Wang et al., 2017). For these reasons, an investigation into a new animal model for studying CIN in early embryos was warranted. In contrast to mice, EGA in bovine and non-human primates occurs at 8- to 16-cell stage and the 6- to 8-cell stage of development, respectively (Graf et al., 2014; Wang et al., 2017), which is also accompanied by high levels of aneuploidy, observed in these species after IVF (Viuff et al., 2000; Dupont et al., 2009; Dupont et al., 2010). The upkeep cost of non-human primates is very demanding, but the analogies between bovine and human reproduction, including endocrine characteristics, folliculogenesis and live-birth rates per embryo transfer (Malhi et al., 2005) render the bovine preimplantation embryo as an ideal candidate for studying the mechanisms of embryonic CIN in humans. By applying comprehensive single-cell genome analysis methodology in Study I, we aimed at providing deeper insight into the incidence and nature of CIN in bovine cleavage-stage embryos.

To achieve this goal, we genotyped about 777,000 uniformly spanned SNPs across the genomes of 160 single blastomeres derived from 25 *in vitro* produced bovine cleavage-stage embryos, of which 72% (n = 116) were analyzed by haplarithmisis following quality control (QC). To determine the genomic constitution of embryos, only those embryos were included, in which more than half of the blastomeres passed QC (23/25 embryos met this criteria). In our cohort, six embryos (26%) were normal diploid in all analyzed cells and 17 were abnormal (74%), containing at least one blastomere with segmental or full chromosome aberration (Fig. 13A-D). Out of 17 abnormal embryos, 15 (88%) were mosaic, including four embryos with a mix of normal and abnormal cells

(normal/aberrant) and 11 aberrant/aberrant embryos that contained various genomic abnormalities in different embryonic cells. A staggering 39% (9/23 embryos) also had blastomeres burdened with full genome anomalies, including genome-wide loss of heterozygosity, UPDs, triploidy, tetraploidy and haploidy. For these embryos, single-cell haplarithm profiles and parental haplotypes uncovered the presence of only paternal (androgenetic) or only maternal (gynogenetic) genomes in a single blastomere and enabled triploid blastomeres to be classified as diandric or digynic in origin (some of these embryos will be discussed in greater detail in Chapter 4.2). Among the embryos with blastomeres affected by whole-genome anomalies, one contained only maternal genome in all the cells, indicating a parthenogenetic activation of the oocyte without any paternal contribution.

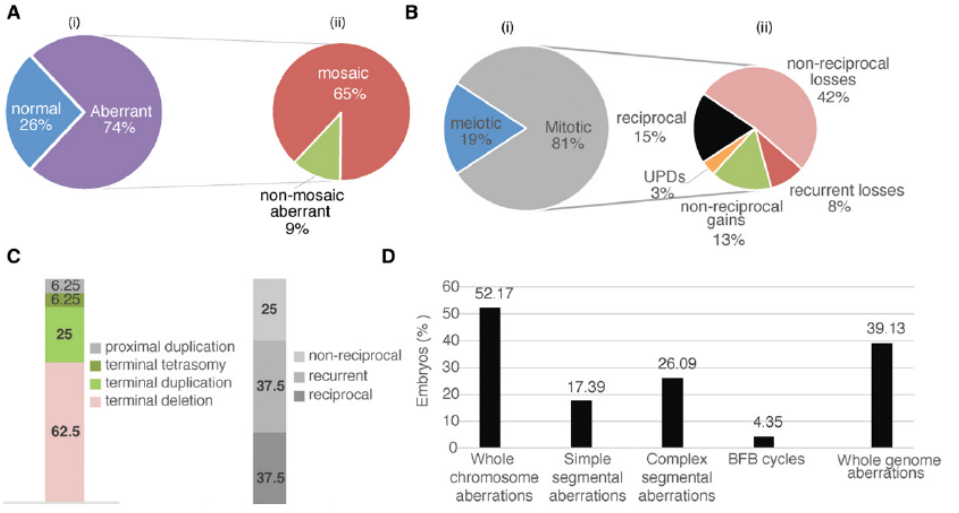

Figure 13. The frequency and nature of chromosomal instability in bovine cleavage-stage embryos. (A) General characteristics of bovine embryos based on their genomic constitution, including the (i) proportion of normal diploid and abnormal embryos and (ii) proportion of abnormal mosaic and non-mosaic embryos. (B) The mechanistic origin (i) and nature (ii) of identified whole-chromosome imbalances. (C) The frequency and nature of segmental imbalances. (D) The proportion of embryos with different genetic anomalies [Fig. 1 in (Destouni et al., 2016)].

In abnormal embryos without uniform ploidy changes, whole-chromosome abnormalities were the most frequent errors that included losses (monosomies and nullisomies), gains (trisomises, tetrasomies) and uniparental disomies (12/23 embryos, 52.17%; Fig. 13D). Whole-chromosome meiotic errors were encountered only in 19% of embryos, in which case embryos showed the same chromosomal aneuploidy in all sister blastomeres. The rest of whole-chromosome imbalances were mitotic in origin (81%, Fig. 13B). Whole-chromosome losses, which had a reciprocal duplication in a sister blastomere, represented

15% of the mitotic numerical chromosome aberrations that can be explained by mitotic non-disjunction, in which one sister cell receives an extra chromosome, while the second sister cell loses the same chromosome. The majority of mitotic chromosomes are, however, neither reciprocal nor recurrent (42%) in sister blastomeres. Such losses of chromosomes are likely due to merotelic attachments of microtubules to kinetochores that lead to mitotic anaphase lagging and exclusion of one of the chromatids from both sister blastomeres. Whole-chromosome gains entail trisomies, tetrasomies and >4 copies of the genome, which represent 26%, 3% and 1% of all mitotic numerical chromosome anomalies, respectively. Non-reciprocal gains account for 13%, however reciprocal losses may be present in sister blastomeres that were not included in the analysis due to suboptimal quality.

The identified segmental abnormalities were classified as simple segmental aberrations and complex segmental aberrations. Terminal deletions, terminal duplications, terminal amplifications and proximal duplications were termed simple segmental aberrations. Of the simple segmental aberrations, 37.5% are reciprocal, meaning that they have a terminal deletion in one blastomere and a terminal duplication with the same breakpoints on the same allele in the sister blastomere. Recurrent segmental imbalances (37.5%) entail only terminal losses (Fig. 15C). In contrast, terminal imbalances coexisting with other segmental aberrations for the remaining part of the chromosome were labelled complex aberrations that represented 47% of all segmental aberrations. Out of complex rearrangements, signatures characteristic of breakage-fusion-bridge (BFB) cycles were detected in three (13%) of embryos [see also Fig. 2 in (Destouni et al., 2016)].

In summary, haplarithmisis revealed a plethora of genomic abnormalities in bovine cleavage-stage embryos, some of which can be missed by conventional single-cell DNA copy-number profiling methods [see Supplementary Fig. S1A-B in (Destouni et al., 2016)]. The data presented in Study I indicates that incidence and nature of CIN in bovine embryos following IVF is similar to what has been previously observed in human IVF embryos (Vanneste et al., 2009; Mertzanidou et al., 2013; Chow et al., 2014). Hence our study proposes that bovine cleavage-stage embryo is a valuable model for providing novel insight into the mechanisms of CIN, including in *in vivo* early embryogenesis.

4.2. Spontaneous parental genome segregation in the zygote (Study I)

As uncovered by previous studies in humans and our study in bovine, a variety of post-zygotic events, such as mitotic non-disjunction, anaphase lagging, BFB cycles and cellular fusions, can contribute to the generation of mosaic embryos during early embryogenesis. Surprisingly, by using haplarithmisis that combines haplotype and copy number information, we unexpectedly disclosed for the first time that zygotes can segregate entire parental genomes into separate

cell lineages that persist and proliferate independently during embryo post-zygotic cleavages, providing basis for mixoploidy and/or chimaerism formation (Fig. 14). Aberrant parental genome segregation was not only triggered by abnormal fertilization, but was also evident in monospermic embryos. We termed this phenomenon "heterogoneic cell division" (Greek for different parental origin). The discovery of heterogoneic segregation adds to the remarkable genome plasticity of mammalian cleavage-stage development and will be discussed in the following chapters.

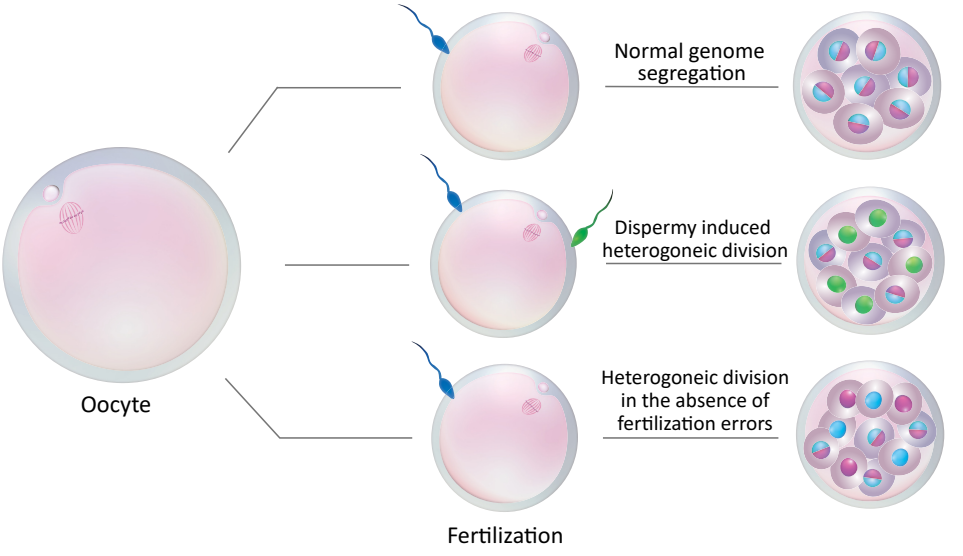

Figure 14. The discovery of heterogoneic division in bovine cleavage-stage embryos. The figure illustrates simplified genomic representation of single blastomeres in embryos after normal and abnormal segregation of parental genomes. In normal scenario, the first post-zygotic cleavage results in diploid daughter blastomeres carrying both maternal (pink) and paternal (blue) genomes that give rise to biparental cells (top). In case of dispermy-induced heterogoneic division, extra paternal genome creates a separate androgenetic cell lineage (green) that persists over embryonic cell cycles together with biparental blastomeres (middle). Spontaneous genome segregation can also occur after normal fertilization, resulting in embryonic cell lineages with only maternal or only paternal genomes that co-exist with biparental cells.

4.2.1. Heterogoneic division in abnormally fertilized embryos

As mentioned earlier, haplarithmisis revealed a number of embryos that were burdened with genome-wide anomalies. In five embryos (5/23, 22%) an aberrant inheritance pattern of paternal haplotypes was observed. In these embryos, different cells presented with two different homologous recombination sites on the paternal genome, indicating dispermic fertilization (Fig. 15). Remarkably, none of these dispermic embryos were uniformly triploid, as one might expect,

but rather contained cells with different numerical ploidy states and different parental genome contributions across the blastomeres of the same embryo. Moreover, in all dispermic embryos, one of the two paternal genomes was always segregated into a separate androgenetic cell lineage, carrying only a set of paternal chromosomes. An extraordinary example of genome dynamics can be seen in dispermic embryo E16_BRP011, consisting of three androgenetic cells with one paternal genome, one compound androgenetic cell with two different paternal genomes, two tetraploid cells with 3paternal:1maternal allelic ratio and two biparental diploid blastomeres [see Supplementary Fig. S1A-B in (Destouni et al., 2016)]. Consistent with our findings, mixoploidy and chimaerism have also been observed in human dispermic IVF embryos (Kola et al., 1987; Munne et al., 1994; Staessen and Van Steirteghem, 1997); however the allocation of different genomes into distinct blastomeres has never been demonstrated before. Although it is not surprising to see diandric triploid cells in dispermic embryos, one embryo also contained digynic triploid cells with an additional set of maternal alleles genome-wide [see Fig. 4 in (Destouni et al., 2016)]. The existence of digynic cells in dispermic embryo can be a result of either non-extrusion of the second polar body or its re-absorption back into the zygote or blastomere. Interestingly, the same embryo's androgenetic cells share chromosome abnormalities on Chr 12 (including BFB signatures), Chr 15 and Chr X, indicating that these blastomeres arose from a common progenitor.

4.2.2. Heterogoneic division in the absence of fertilization errors

Our data demonstrated that in dispermic embryos, extra set of paternal genome was always segregated into highly proliferative androgenetic blastomere lineage. Most intriguingly, we also encountered spontaneous genome segregation in two embryos that showed no signs of dispermy or digyny. For instance, embryo E19_BRP012 represents a remarkable example that further breaks down dogmas of cell division, by which daughter cells inherit both parental genomes. Namely, this particular embryo contained four androgenetic, four gynogenetic and three biparental blastomeres (Fig. 16). Mitotic errors on Chr 9, Chr 14, Chr 22 and Chr 24 were observed only in one biparental blastomere, but reciprocal losses may potentially exist in the cell that could not be analyzed. Second embryo E19_BRP005 also contained one gynogenetic, three androgenetic and two biparental blastomeres [see Supplementary Fig. S1A-B in (Destouni et al., 2016)]. Additional copy-number anomalies were detected on Chr 9, in which case the maternal allele was lost in gynogenetic blastomeres and instead was detected in androgenetic blastomeres, restoring the diploid biparental state for this chromosome. Similarly, androgenetic copy-number aberrations in androgenetic cells, affecting Chr 3, Chr 5 and Chr X, had a reciprocal change in biparental cells.

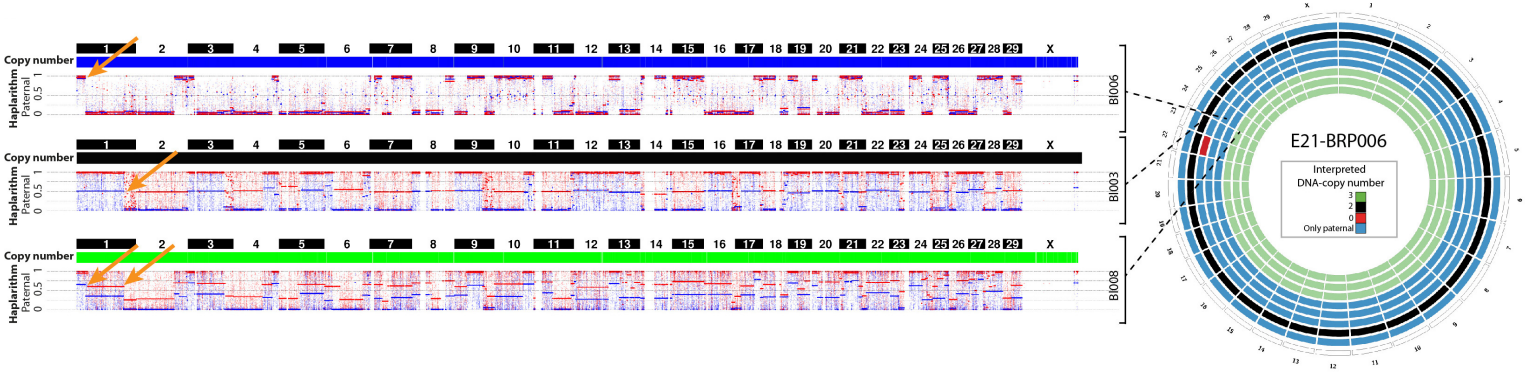

Figure 15. Dispermic fertilization, underlying mixoploidy. (A) Genome-wide paternal haplarithm of androgenetic blastomere with paternal genome only (top), normal diploid (middle) and triploid blastomeres (bottom). Orange arrows show the pairwise P1-P2 and M1-M2 breakpoints in the haplarithm profiles, indicating homologous recombination (HR) sites. Note the two different HR sites in androgenetic and diploid blastomeres, indicating the presence of two paternal genomes, and how the two HR "merge" in the triploid cells, indication contribution of two sperms to the genome of triploid cell lineage (marked with arrows). (B) Circos plot of the corresponding 8-cell dispermic embryo. [see also Fig.3 in (Destouni et al., 2016)]

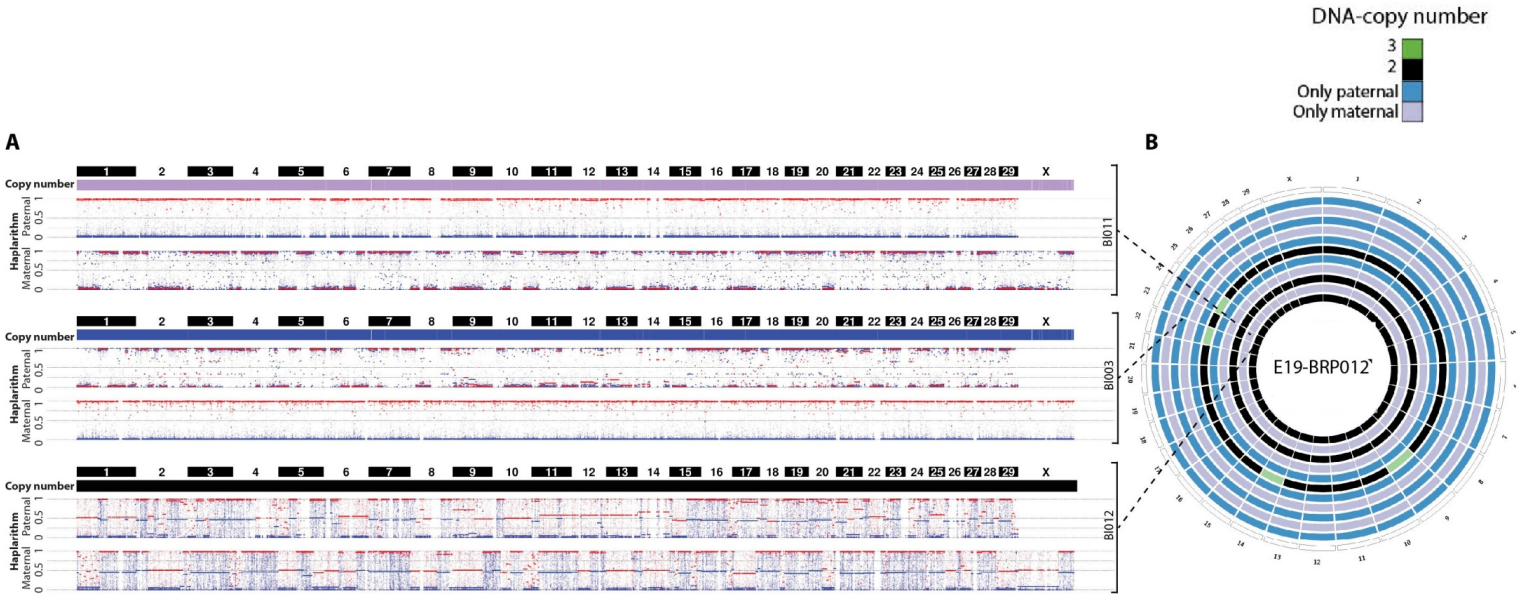

Figure 16. Parental genome segregation in a monospermic embryo. (A) Genome-wide haplarithm plots for gynogenetic (top), androgenetic (middle) and biparental blastomeres (bottom). (B) Circos plots, illustrating genome-wide copy-number profiles of all the single blastomeres [see also Fig. 4 in (Destouni et al., 2016)].

4.2.3. How can heterogoneic division occur in early embryos?

Currently, it remains unclear, how a complete parental genome can be segregated to form distinct cell lineages. Ideally, during the zygotic division, the operating mitotic spindle segregates chromosomes to two sister blastomeres. Trichotomous division directly into three cells is frequent in diandric or digynic zygotes with three pronuclei (3PN), carrying either an extra set of haploid paternal or maternal chromosomes, respectively (Staessen and Van Steirteghem, 1997; Joergensen et al., 2014). Hence, the simplest explanation for spontaneous parental genome segregation would be the direct division of the zygote into three blastomeres, resulting in different ploidy and genomic constitution of cells. In somatic cells, multiple mechanisms of aberrant division patterns have been proposed, including centrosome overduplication, which may produce supernumerary spindle poles that lead to tripolar cellular division (Maiato and Logarinho, 2014). Because the zygote is not an average somatic cell, but rather a unique entity, different zygotic stage-specific mechanism might be driving trichotomous division. Similarly to human, the first mitotic spindle in bovine zygotes is organized by paternally inherited centrosomes (Navara et al., 1994; Sathananthan et al., 1996; Sathananthan, 1998); therefore the heterogoneic divisions in case of dispermy can potentially result from an operation of two independent astral spindles, where one spindle organizes a diploid metaphase, and a second ectopic spindle organizes an additional haploid metaphase (Navara et al., 1994). Such atypical metaphase plate organization, which is most likely accompanied by karyogamy failure, would provide mechanistic basis for separation of haploid chromosomes during the zygotic cytokinesis, leading to generation of two cells lines: androgenetic and biparental (Fig. 17B). Triploid embryos can also potentially exclude one haploid genome from metaphase plate and allocate extra paternal pronucleus into a distinct lineage through non-canonical cytokinesis. This mechanism can be corroborated by the observation of pronuclear extrusion, which was originally described as the potentially haploid third cell-like structure, during the first cleavage of human triploid zygotes (Kola et al., 1987; Pieters et al., 1992).

More intriguingly, heterogoneic division was not exclusive only to abnormal fertilization events, as also monospermic embryo contained androgenetic, gynogenetic and biparental blastomeres. Similarly to dispermic embryos, we envision that the zygote has undergone trichotomous division, generating three distinct cell lineages (Fig. 17C-D). This hypothesis seems to be supported by the preliminary results of our pilot study, in which bovine IVF embryos were time-lapse monitored and individual blastomeres, derived from abnormally cleaved embryos, were analyzed immediately after the first post-zygotic division. Indeed, we have encountered evidence that aberrant first cleavage underlines spontaneous parental genome segregation (unpublished data); however the frequency and the precise cellular and molecular mechanisms of this phenomenon remain unknown.

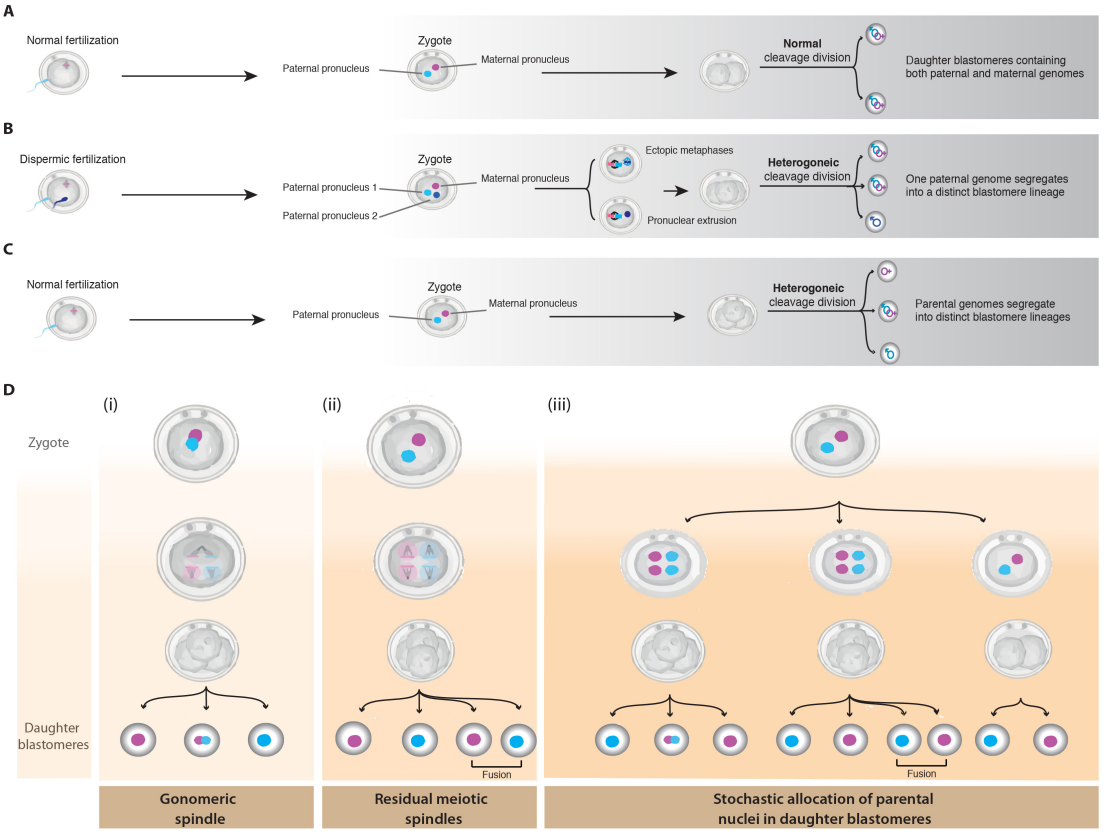

Figure 17. Hypothetic origins of heterogoneic cell division. (A) In a normal case scenario, the zygote undergoes post-zygotic mitotic division into two diploid sister blastomeres, each containing both maternal and paternal genomes. (B) After dispermic fertilization, extra paternal genome gets extruded into a separate androgenetic lineage, following pronuclear extrusion or ectopic metaphase formation. (C) Normal fertilization, followed by parental genome segregation into distinct lineages due to (D) abnormal spindle formations or stochastic allocation of parental genomes as a result of non-symmetric cytokinesis. [Fig. 6 in (Destouni et al., 2016)].

One hypothetical cause could be the development of gonomeric spindle, driven by asymmetric k-MT dynamics between parental genome, which underlies the emergence of blastomeres with distinct genomic content (Fig. 17D). Also, the co-existence of barrel-shaped meiotic spindle with an ectopic astral spindle around chromatin structures has been previously observed in so-called “silently fertilized” oocytes (Van Blerkom et al., 2004). Hence, the heterogoneic division of the zygote can also be a consequence of simultaneous operation of sperm centrosome-mediated spindle together with residual meiotic spindle that was not eliminated during oocyte-to-embryo transition. In this case, independent coordinated activity of the two spindles can result in direct cleavage into four mononucleated blastomeres, containing either maternal or paternal DNA (Fig. 17D). Finally, the stochastic allocation of parental nuclei to different blastomeres through non-canonical cytokinesis might also occur in a zygote, where both maternal and paternal pronuclei replicate, but fail to reach karyogamy.

4.3. Characteristics of CIN in naturally-conceived bovine embryos (Study II)

Humans are rather inefficient in reproduction and it might take time even for healthy fertile couples to conceive a baby. It was estimated that only 30% of natural conceptions result in live-birth and the majority of embryos are lost already during early stages of pregnancy (Macklon et al., 2002). As mentioned previously, embryonic aneuploidy is one of the leading factors of human reproductive wastage, and chromosome aberrations can often be detected in spontaneous abortion cases (Menasha et al., 2005; Levy et al., 2014). In addition, submicroscopic terminal deletions, duplications and isochromosomes are often detected in newborns (Riegel et al., 2001), and several cases of mosaic individuals, exhibiting chimaerism and mixoploidy have also been well documented (Jarvela et al., 1993; Edwards et al., 1994; van de Laar et al., 2002; Yamazawa et al., 2010). Intriguingly, traces of triploid, diploid and haploid cell lineages have been observed in these reported individuals. Finally, complete hydatidiform moles of androgenetic origin have also been described in both human (Ibrahim et al., 1989; Kwon et al., 2002; Sun et al., 2012; Obeidi et al., 2015) and in cattle (Meinecke et al., 2002). These observations, together with relatively low overall human fecundity, led us to investigate, whether CIN can also be inherent to *in vivo* embryogenesis. As naturally conceived human embryos are not available for research, CIN in human IVF and *in vivo* embryos cannot be compared directly. Alternatively, animal models have been used to investigate chromosomal abnormalities in *in vivo* preimplantation embryos (Viuff et al., 2001; Rambags et al., 2005; Coppola et al., 2007). The major limitation of these studies is the application of low-resolution traditional karyotyping methods that can neither detect submicroscopic changes nor determine the nature of genomic anomalies at a single-cell level. Hence, the

knowledge about the stability of naturally conceived embryos remained limited, largely due to the lack of robust genome analysis technologies.

In Study I, we have demonstrated that chromosome instability is conserved between human and bovine cleavage-stage embryos following IVF, making bovine a valuable animal model for studying CIN in early embryos. Equally important, gestational period of cows is nine months and only 40–55% of dairy cows produce an offspring after single insemination (Diskin et al., 2012). Although aneuploidy was reported in 22% of bovine aborted foetuses and neonates (Coates et al., 1988), this number might be an underestimate, as most of the studies in cattle were done at late gestational period, while whole-chromosome losses or gains are usually lethal already during the first trimester (Schmutz et al., 1996). Therefore, we have continued our studies using bovine as a model to investigate chromosome instability in naturally conceived embryos. We have used the same approach, as in Study I: all single blastomeres of embryos were isolated, genotyped using Illumina SNP-array technology and analyzed by haplarithmisis. In parallel, as depicted in Fig. 9 (Materials and Methods), we tested *in vitro* produced embryos, derived from *in vitro* matured and fertilized oocytes that were retrieved from the same donor animals after ovarian stimulation and ovum pick up (OPU-IVF embryos); and *in vitro* produced embryos, derived from *in vitro* matured and fertilized oocytes that were retrieved from donor animals without ovarian stimulation (IVM-IVF embryos). After sample collection and quality control, 171 out of initially 222 isolated blastomeres (77%) were considered for further analysis and data interpretation: 66 blastomeres from 13 IVM-IVF embryos, 46 blastomeres from 13 OPU-IVF embryos and 59 blastomeres from 16 *in vivo*-derived embryos. To date this is the first study that used single-cell approach to simultaneously compare three different settings of embryo production and development and to establish a comprehensive spectrum of chromosomal aberrations in single blastomeres of *in vivo*-derived embryos.

4.3.1. *In vitro* procedures exacerbate CIN in bovine cleavage-stage embryos

We first aimed to assess the prevalence of chromosomal abnormalities in IVM-IVF, OPU-IVF and *in vivo*-derived embryos. In contrast to our initial hypothesis, the number of abnormal embryos, carrying at least one blastomere with a full or segmental chromosomal aberration, increased from 18.8% in *in vivo*-derived embryos (3/16) up to 84.6% in IVM-IVF embryos (11/13) (Fig. 18A). At the same time, this result may not have been unexpected, as it was comparable to the previously published work on cattle, where 7% of naturally conceived embryos were chromosomally abnormal on day-2 post fertilization (Viuff et al., 2000). However, when we looked at the chromosome segregation patterns at a single-cell level, the difference between the three embryos groups became even more staggering, as genomic stability of *in vitro* embryos was

extremely compromised, compared to *in vivo*-derived embryos (in both cases $P<0.0001$, Fisher's exact test; Fig. 18B). For this comparative analysis blastomeres were scored as balanced, if they did not contain any whole-chromosome or segmental anomalies. However, to avoid bias in representing chromosome segregation dynamics in single cells, blastomeres with uniform genome-wide ploidy state (e.g. fully haploid or triploid cells) were also scored as balanced, unless they had additional signatures of chromosomal losses and gains.

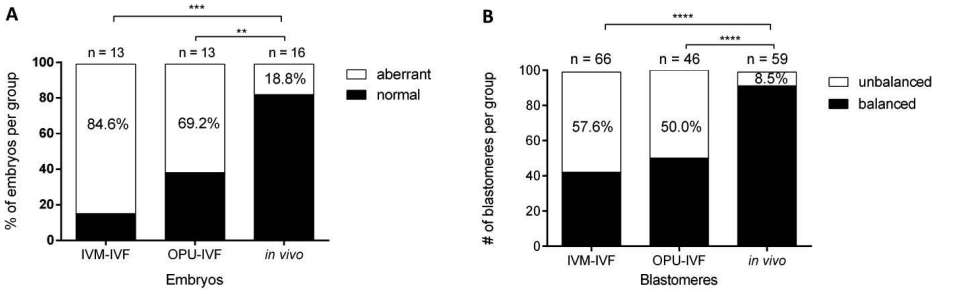

Figure 18. The rate of CIN in IVM-IVF, OPU-IVF and *in vivo* embryos. The numbers above the columns represent the total numbers of analyzed embryos (A) and blastomeres (B). Statistical difference between different groups has been calculated using two-tailed Fisher's exact test for multiple testing. (A) The proportion of normal diploid embryos and aberrant embryos in IVM-IVF (n = 13), OPU-IVF (n = 13) and *in vivo* group (n = 16)., two-tailed Fisher's exact test for multiple testing. (B) The comparison of balanced and unbalanced blastomeres represents the chromosome dynamics of single blastomeres in IVM-IVF (n = 66), OPU-IVF (n = 46) and in vivo-derived embryos (n = 59). $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. [Fig. 2 in (Tsuiko et al., 2017)].

In general, whole-chromosome aneuploidy was the most frequent type of error and it was observed in all abnormal *in vivo*, OPU-IVF and IVM-IVF embryos (at a rate of 18.8%, 69.2% and 84.6%, respectively). Segmental imbalances were also detected in all three embryo groups, hower they were the most prevalent in IVM-IVF embryos (9/13, 69.2%) when compared to OPU-IVF embryos (2/13, 15.4%, $P = 0.01$, Fisher's exact test) and *in vivo* embryos (1/16, 6.3%, $P = 0.001$, Fisher's exact test) [see also Fig. 3 in (Tsuiko et al., 2017)].

We then assessed the level of mosaicism in embryos containing chromosomally abnormal cells. The most common mosaic pattern for *in vivo* and OPU embryos was combination of diploid and aneuploid cells that mainly affected one pair of chromosomes. In contrast, almost all abnormal IVM-IVF embryos were burdened with mixoploidy that can be attributed to a high number of abnormally fertilized cases observed among this particular group (69.2%, 9/13). Also here dispermic embryos underwent heterogoneic division, where one of the extra paternal genomes segregated into a separate androgenetic cell line carrying only paternal DNA. For example, three abnormally fertilized embryos had separate androgenetic cell lineages, as well as biparental blastomeres

(E12_Cross8301, E10_Cross4770 and E06_Cross4006), while the other three embryos (E13_Cross8301, E08_Cross4770 and E09_Cross4770) also contained triploid blastomeres (Fig. 19).

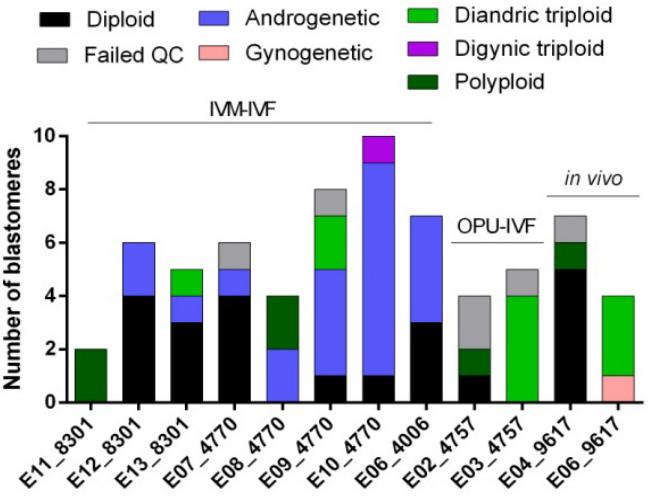

Figure 19. Examples of embryos, containing blastomeres with abnormal ploidy. The stacked bar plots depict the number of blastomeres with different genomic anomalies per embryo. Blastomeres with normal karyotype and with single aneuploidies and/or segmental losses or gains that have both maternal and paternal alleles present in their genome (diploid embryos) are depicted in black [see also Fig. 3 (Tsuiko et al., 2017)].

Intriguingly, although the genome of *in vivo* embryos is more stable than of their OPU-IVF or IVM-IVF counterparts, haplarithmisis revealed one *in vivo* case (E06_Cross9617), in which the zygote most likely also underwent a heterogoneic cell division (Fig. 19). This *in vivo*-derived embryo consisted of three diandric triploid cell lines and one gynogenetic line. In addition, based on the haplarithmisis, there was no evidence of polyspermy or meiotic errors, suggesting that genome segregation occurred after monospermic fertilization and in our case the division of the zygote into three daughter blastomeres could have given rise to gynogenetic, biparental and androgenetic cell lines. Subsequently, the androgenetic blastomere could have fused with the biparental one, creating a diandric triploid that was detected during the analysis. Unfortunately, because three out of seven blastomeres could not be analyzed, the genomic constitution of these blastomeres remained unknown and the existence of a pure androgenetic lineage could not be excluded. Still, our observation does point to a common genome segregation mechanism that operates regardless of *in vitro* or *in vivo* environment, bypassing the developmental selection barrier and possibly explaining the origin of human mixoploid and chimeric individuals. Nevertheless, together our findings suggest that even though CIN is also present in *in*

vivo embryos, *in vitro* procedures, such as *in vitro* maturation, fertilization and culture, influence the rate and nature of embryonic chromosomal abnormalities and consequently impede embryo developmental potential.

Finally, because dispermy might influence chromosome missegregation in embryos, we then analyzed only those embryos that developed from monospermic zygotes. For this purpose, we combined monospermic IVM-IVF ($n = 4$) and OPU-IVF ($n = 12$) embryos into a single group (referred to as *in vitro*) and compared them to *in vivo*-derived embryos ($n = 16$). The CIN rates confirmed a considerable difference between the *in vitro* produced and cultured embryos, and *in vivo*-derived embryos (10/16, 62.5% vs 3/16, 18.8%, $P = 0.03$, Fisher's exact test; Supplementary Fig. S2 in (Tsuiko et al., 2017)). In addition, it was clear that *in vitro* procedures can have a negative impact on CIN, when we compared the low frequency of chromosomal aberrations and aberrant ploidy states in blastomeres of *in vivo*-derived embryos (7/59, 11.9%) with the high frequency chromosomal aberrations and aberrant ploidy states of *in vitro* produced and cultured embryos (27/57, 47.4%, $P < 0.0001$, Fisher's exact test). This trend persisted even when we removed some of day-3 p.i. embryos to unify our cohorts, in which case seven day-2 p.i. *in vitro* embryos were classified as abnormal (7/11, 63.6%, $P = 0.04$), while the total number of abnormal blastomeres in the *in vitro* group reached up to 45.9% [17/37, $P < 0.001$, Fisher's exact test; Supplementary Fig. S2 in (Tsuiko et al., 2017)].

4.3.2. The relevance of bovine embryo data in the context of human IVF

Despite the progress made in clinical and laboratory ART protocols, including embryo selection for transfer, the pregnancy rate per single IVF cycle still lingers at around 30% worldwide (Dyer et al., 2016). The two main factors that determine the IVF outcome are the embryonic aneuploidy and endometrial receptivity, and here we focus on the former. Studies on early human embryogenesis are restricted due to ethical and legal concerns, so unavoidably researches must rely on appropriate animal model. Of course, often data obtained from model organisms cannot be directly translated to human and caution should be applied when doing so, but animal models do provide a valuable insight into a wide range of issues at the forefront of human reproductive health and medicine, including assisted reproduction. In our case, we demonstrated that the genomic stability of *in vivo* embryos is significantly higher compared to OPU-IVF and IVM-IVF embryos. The use of bovine model revealed that CIN is present in less than 20% of *in vivo* embryos compared to at least 70% in *in vitro* embryos, which means *in vivo*-conceived embryos are most likely more viable. The major limitation of the current work is the small number of embryos analyzed, thus more studies are warranted to corroborate our findings. Still, the observation that *in vitro* embryos are genetically compromised draws attention to the fact that potentially adverse effect of different *in vitro* procedures and culture on

early embryonic development must not be overlooked. If a negative impact of different embryo production protocols on embryonic genome integrity was observed in cattle, there is no guarantee that it will not be the same for human. During preimplantation embryo culture *in vitro,* even minor alterations in culture conditions and micromanipulation of oocytes and embryos may impact embryo quality and its subsequent development (Rizos et al., 2002; Wale and Gardner, 2016). Although it was recently demonstrated that abnormal cells get depleted during preimplantation development, there needs to be a sufficient proportion of normal cell within the embryo to ensure its survival (Bolton et al., 2016). Unfortunately, *in vitro* culture conditions can exacerbate embryonic aneuploidy and reduce this chance of survival, in turn leading to reduced IVF success rate per single cycle. This knowledge can be especially relevant, taking into account that the number of women voluntarily or involuntarily delaying motherhood is also steadily increasing, and for those women, fertility preservation and IVF procedures are becoming a mainstream approach to achieve motherhood (Lallemant et al., 2016). For these reasons, our results on genomic constitution of *in vitro* and *in vivo* bovine embryos have two major implications for human assisted reproduction: (i) directing individuals towards IVF programs should be done cautiously, as it may compromise embryo quality and consequently bring great deal of psychological distress to patients and (ii) improvements in the embryo *in vitro* environment are likely still possible to enhance ART/IVF success rate. Importantly, any modification to IVF culture conditions should be closely monitored under full transparency to avoid inconclusive and/or controversial outcomes (Chronopoulou and Harper, 2015).

This study also indirectly touches the subject of controlled ovarian stimulation. In our study, donor cows underwent hormonal stimulation to increase the number of *in vivo*-derived embryos via oviduct flush, so, unfortunately, these embryos do not fully represent the natural conception. Donor cows also received hormonal stimulation prior to OPU. Hence, both *in vivo*-derived and OPU-IVF embryos were retrieved after hormonal stimulation of donor animals, but we observed more chromosomally balanced diploid embryos and blastomeres in *in vivo*-derived embryos than in OPU-IVF embryos. This indicates that *in vitro* fertilization and culture are the major causes of embryonic aneuploidy, rather than ovarian stimulation itself. Because there is still an ongoing debate on the potential deleterious effect of ovarian stimulation on oocyte and embryo quality, more studies should be conducted on this matter; however based on our data, the effect of hormonal stimulation is expected to be minor.

Finally, we contribute to understanding why IVM of human eggs and subsequent *in vitro* fertilization and embryo culture are associated with low reproductive success. When oocytes are matured *in vivo*, they originate from ovulatory follicles that undergo strongly regulated processes of selection, growth and dominance. In contrast, oocyte IVM can perturb proper nuclear (spindle organization, chromosome segregation) and cytoplasmic maturation that is necessary for normal fertilization and oocyte-to-embryo transition (Combelles et al., 2002; Li et al., 2006; Nichols et al., 2010). As reviewed earlier, the oocyte

plays a central role in maintaining genomic integrity before the major EGA wave and first post-zygotic divisions are highly dependent on the large pool of maternal mRNAs and proteins acquired by the oocyte during maturation. In light of this, the inherited aberrant transcriptome has been associated with altered first cleavages in human embryos, highlighting the importance of maternal factors on early embryonic development (Vera-Rodriguez et al., 2015). Therefore, in the current study, the higher rate of chromosomal abnormalities in IVM-IVF embryos likely arises from the defective maternal resources in the oocyte; however more research should target the precise impact of the intrinsic quality of the oocyte on the incidence of chromosomal aberrations in cleavage-stage embryos. We also add another note of caution with respect to the use of human oocyte IVM. Namely, in human IVM procedure, subsequent oocyte fertilization is typically performed by ICSI, but traditional IVF (human IVM-IVF) has also been proposed as a better alternative to fertilize *in vitro*-matured oocytes (Soderstrom-Anttila et al., 2005; Walls et al., 2012). However, the IVM-IVF combination may not be beneficial for humans, because *in vitro*-matured oocytes have no contact with the oviductal fluid, and thus zona pellucida of IVM oocytes may become less resistant to dispermic fertilization under *in vitro* conditions (Xia, 2013). Our results seem to corroborate this view, as dispermic fertilization was almost exclusively found among IVM-IVF embryos. Of course in humans, the presence of polyspermy can be detected by checking the number of pronuclei, which is not possible in bovine zygotes because of the dense lipid content. However, it would also likely imply that a number of embryos will be immediately discarded and not considered for transfer due to abnormal PN status, reducing the total number of healthy embryos. Currently, suboptimal human IVM outcome is the main reason why oocyte IVM is rarely used in clinical practice, but nevertheless the technology is promising, especially for a subset of patients (e.g. PCOS or oncology patients), and the attempts to improve IVM protocols and conditions are still ongoing.

To sum up, bovine data presented here highlights important genomic aspects, associated with *in vitro* maturation, fertilization and culture, although excessive generalization between bovine and human should be avoided. However, in the absence of human data, ART should foremost be proposed to those couples who have a medical indication for IVF treatment, while the use of IVF for social reasons should be critically discussed, taking into account possible complications associated with assisted reproduction. If ART is to be an integral part of modern society, decision making on treatment strategies should also carefully consider all possible risks to avoid low implantation rates and/or undesired pregnancy outcomes.

4.4. Blastocoel fluid as a source of DNA for PGT-A (Study III)

Together with improvements in IVF laboratory protocols, technological advances in single-cell genomics also push the boundaries of PGT-M and PGT-A to enable faster and more efficient genetic analysis. PGT-A has been implemented into the clinics to tackle the issue of embryonic aneuploidy and to assist in the identification of euploid embryos by analyzing their chromosome profiles prior to transfer. Currently, different biopsy methods can be used to obtain embryonic material for genetic analysis, including polar body biopsy of the oocyte, single blastomere biopsy of cleavage-stage embryos and TE biopsy of blastocysts. Polar body biopsy was shown to be the least efficient way of predicting embryo status, as it allows screening for maternal meiotic errors only, without taking into account paternally-derived and/or mitotic aneuploidies (Capalbo et al., 2013; Salvaggio et al., 2014). Blastomere biopsy was long considered to be the golden standard for obtaining embryonic DNA for PGT-A, which accounted for about 80% of all embryo biopsy methods by the years 2012–2013 (De Rycke et al., 2017). However, as mentioned in Chapter 2.3.2., biopsy at earlier stages may not be a suitable approach for PGT-A, as high-degree of mosaicism at cleavage-stage raises challenges in blastomere analysis and data interpretation; hence, blastocyst biopsy became the more preferred option for obtaining embryonic material for genetic analysis.

Although TE biopsy yields good clinical results, it is a rather challenging procedure that requires skills and experience of an operator. Following TE biopsy, embryos are cryopreserved until the results of genetic testing are available. Normally, blastocyst artificial collapse can be performed prior to blastocyst vitrification to protect the embryo from ice-crystal formation induced membrane damage and improve embryo survival following cryopreservation (Chen et al., 2005; Mukaida et al., 2006). During this step, blastocoel fluid can be isolated from the embryo by a procedure, termed blastocentesis (Gianaroli et al., 2014). The discovery of amplifiable DNA in the blastocoel fluid (BF-DNA) made it an object of attention and it was proposed that BF aspiration can be used as an alternative less invasive approach for obtaining embryonic material for PGT-A. Although the volume of retrieved BF is relatively small, it was successfully whole-genome amplified for aneuploidy screening using aCGH and NGS (Palini et al., 2013; Zhang et al., 2016). Despite the promises, preliminary investigation into BF remained limited and the potential use of BF-DNA for PGT-A remained questionable, as few of the preliminary studies showed contradictive results regarding aneuploidy detection rates and karyotypic concordance between BF and different biopsied material derived from the same embryo. So far, only one group was able to achieve high concordance rate, when comparing genomic profiles of BF with TE cells, polar bodies and blastomeres (Gianaroli et al., 2014; Magli et al., 2016). Contrary to this, in other reports the discordance in karyotypes reached up to 50% between BF and TE biopsy or the rest of whole embryo (Perloe, 2013; Poli et al., 2013; Tobler et al., 2015). However, previously published studies used aCGH to compare the

consistency of diagnosis between BF-DNA and TE biopsies or the rest of the whole embryo, so they were not able to investigate the occurrence of embryonic mosaicism, which is currently a prominent topic in PGT-A. Therefore, because of the inconsistent results and lack of data on blastocyst-stage mosaicism, additional studies were warranted to investigate the potential use of BF-DNA for diagnostic purposes. Because the scientific world is slowly moving towards NGS, and clinical diagnostic laboratories are no exception, in Study III we have applied the most widely used commercial NGS-based platform for PGT-A – VeriSeq™ PGS (Illumina Inc.) – to simultaneously investigate molecular karyotypes of three different embryonic compartments (BF, TE and ICM) derived from a single blastocyst. The Veriseq™ platform is reported to reach to at least 25 million raw clusters, out of which more than 70% are mapped to the genome. With subsequent filtering, approximately 600,000–900,000 reads are then used for copy-number calling and the amount of filtered reads is sufficient enough to detect mosaic aneuploidies in a biopsied sample with a resolution of 20–80% (product description was obtained from Illumina Inc). Hence, our study design had two advantages over previously published reports: (i) the use of high-resolution NGS method with improved sensitivity enabled the detection of mosaic chromosomal rearrangements and (ii) the analysis of three different embryonic compartments allowed to investigate, to which extent do genomic profiles of BF, TE and ICM reflect each other at the blastocyst stage, and to evaluate the feasibility of blastocentesis for clinical practice.

4.4.1. Proof-of-principle experiments and embryo analysis using NGS

Before analyzing embryos, we first wanted to check, whether we would be able to achieve the same mosaicism detection sensitivity for NGS, as was reported recently (Goodrich et al., 2016; Fragouli et al., 2017). To do that, we first performed mixing experiments to mimic possible mosaic aneuploidies observed in embryos, by previously described approaches (Fragouli et al., 2017; Goodrich et al., 2017). Briefly, we obtained fibroblast cell lines with known karyotypes from the NIGMS Human Genetic Cell Repository at the Coriell Institute of Medical Research (USA). Aneuploid cell lines included trisomy 13 (47,XY,+13; GM02948), trisomy 18 (47,XY,+18; GM01359) and trisomy 21 (47,XX,+21; GM04616). The proportion of X-chromosome was evaluated by mixing XX cell lines with XY. Following culture, individual euploid and aneuploid cells were isolated and combined in different ratios, creating a mixture of six cells with different proportion of abnormal alleles of interest (0%, 17%, 33%, 50%, 66% 83% and 100%). Proof-of-principle experiments were performed in three replicates, each time creating new cell mixtures. Subsequently, cell mixtures were whole-genome amplified and analyzed via NGS. Internal validation of our mixing experiments successfully distinguished mosaic losses and gains that are present in at least 20% of cells (Fig. 20), which is concordant to recent

comprehensive validation studies on mosaicism detection using next-generation sequencing (Goodrich et al., 2016; Fragouli et al., 2017).

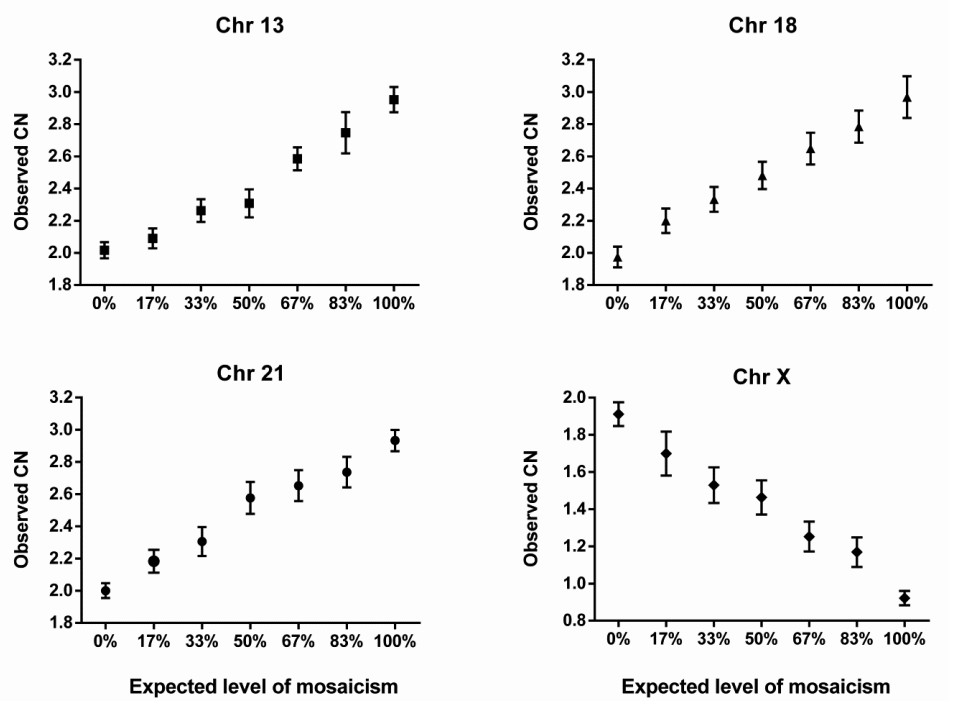

Figure 20. Proof-of-principle mixing experiments to evaluate mosaicism detection via NGS. Mixing experiments were done in at least three replicates and copy number (CN) value was recorded for each event. Geometrical shapes and error bars for each chromosome indicate mean and standard deviation of all independent measurements [Supplementary Fig. S1 in (Tsuiko et al., 2018)].

After validation experiments, 16 cryopreserved embryos were then biopsied with subsequent amplification of ICM, TE and BF-DNA for NGS analysis. After WGA, sufficient amount of DNA was detected in all of ICM and TE samples and in 14 out of 16 BF biopsies (87.5%), which is similar to previous reports. However, following sequencing and initial quality control, BF chromosome profile could not be determined in four embryos. Two embryos also had an inconclusive result for either TE or ICM. In total, BF biopsies were compared to TE and ICM in 10 out of initial 16 (68.8%) embryos, while the comparison of TE and ICM was performed in 14 embryos out of 16 (87.5%). Because low-grade mosaicism detection within an embryo can be influenced in some degree by sampling error and technical artifacts (Munne and Wells, 2017; Treff and Franasiak, 2017), we interpreted the findings in embryos according to the current Preimplantation Genetic Screening International Society (PGDIS) guidelines: embryo, showing mosaicism of <20% were considered to be euploid and

>80% were considered as aneuploid embryos with full chromosome losses or gains, while all the aneuploidies in the range of 20–80% were classified as mosaic. Using these guidelines, aneuploidy screening was performed, with the results summarized in Table 2.

Next, we compared the karyotypes of various biopsy types taken from the same embryo and have classified our results as was performed previously (Magli et al., 2016): (1) full concordance was reported, when the same chromosomes were affected in biopsied samples (including mosaic and/or reciprocal losses and gains); (2) partial concordance was reported, when at least one chromosome corresponded in both biopsies under comparison, but the overall genomic profile did not completely match; and (3) discordance was reported, when none of the affected chromosomes in one biopsy corresponded to other biopsies. Based on the karyotype, a full chromosome concordance between the three embryonic compartments was observed only in four embryos, of which three were uniformly euploid and one had a reciprocal mosaic aneuploidy (Embryo 1; Table 2). The reciprocal nature of chromosomal aberrations indicates that the (mosaic) aneuploidies in the blastocoel DNA are not technical artifacts, but rather true biological findings. Similarly, two embryos had a monosomy, affecting the same chromosome in all three embryonic biopsies, highlighting the meiotic origin of these aneuploidies (Chr 13 and Chr 7 in Embryo 2 and Embryo 5, respectively; Table 2). We have also detected a potentially polyploid embryo with a partially concordant chaotic chromosome profile, characterized by multiple reciprocal losses and gains in all three embryo compartments [see Fig. 2A in (Tsuiko et al., 2018)]. Chaotic genome could be a consequence of chromosome missegregations during first post-zygotic cleavages that accumulated throughout the preimplantation development, resulting in a likely unviable embryo. The reciprocal mosaic aberrations suggest that mitotic errors most likely occurred as a result of chromosome non-disjunction, thus corroborating that the DNA obtained from blastocoel cavity was of embryonic origin. At the same time, two embryos showed multiple aneuploidies in the BF-DNA only, while the corresponding TE and ICM had a euploid karyotype [Embryo 4, and Embryo 7 in Table 2; see also Fig. 2B in (Tsuiko et al., 2018)].

Table 2. Genomic profiling of blastocoel fluid, trophectoderm and inner cell mass

Embryo	Patient Age	Embryo Morphology	Blastocoel fluid (% of mosaicism)	Trophectoderm (% of mosaicism)	Inner Cell Mass (% of mosaicism)
1	39	3BB	46,XX Mosaic –9 (70%)	46,XX Mosaic +9 (50%)	46,XX Mosaic +9 (20%)
2	39	3–4BB	45,XY, –13 Mosaic +1 (60%) Mosaic –16 (30%) Mosaic –21 (40%)	45,XY, –13	45,XY, –13
3	39	3–4BB	Chaotic, likely polyploid	Chaotic, likely polyploid	Chaotic, likely polyploid
4	33	3–4AB	Chaotic, likely polyploid	46,XY	46,XY
5	33	4BB	45,XY, –7 Mosaic –1 (50%) Mosaic +8 (60%) Mosaic +11 (50%) Mosaic +18 (40%) Mosaic +20 (40%) Mosaic +21 (50%)	45,XY, –7	45,XY, –7
6	37	4–5BB	44, XX,–9,–9 Mosaic –3 (60%) Mosaic –10 (80%) Mosaic –12 (70%) Mosaic –13 (80%) Mosaic +14 (50%) Mosaic –15 (70%) Mosaic +16 (50%) Mosaic +17 (50%) Mosaic +19 (50%) Mosaic +20 (50%) Mosaic –22 (70%)	46,XX Mosaic +3 (20%) Mosaic +9 (50%) Mosaic +10 (30%) Mosaic +12 (20%) Mosaic +13 (30%) Mosaic +15 (20%) Mosaic –20 (30%) Mosaic +22 (30%)	46,XX

Embryo	Patient Age	Embryo Morphology	Blastocoel fluid (% of mosaicism)	Trophectoderm (% of mosaicism)	Inner Cell Mass (% of mosaicism)
7	37	4–5AA	47,XY,+11 Mosaic +2 (80%) Mosaic –9 (30%) Mosaic –10 (30%) Mosaic –12 (30%) Mosaic –13 (40%) Mosaic +19 (50%) Mosaic –21 (50%) Mosaic –X (60%)	46,XY	46,XY
8	32	5BB	ND	46,XY	46,XY
9	23	4BB	46,XX	46,XX	46,XX
10	23	5BB	46,XX	46,XX	46,XX
11	32	4BB	46,XX	46,XX	46,XX
12	32	4BB	ND	46,XX	46,XX
13	32	4BB	ND	46,XX	46,XX
14	42	3BC	ND	46,XY Mosaic –17 (80%)	46,XY Mosaic –17 (70%)

Because of the major differences in the genomic constitution between biopsies, it comes as no surprise that the overall number of affected pairs of chromosomes (22 pairs of autosomes and one pair of sex chromosomes), including the potentially polyploid biopsies, was higher in ten BF samples (79/230), compared to corresponding TE (34/230) or ICM biopsies (26/230) (both $P<0.0001$, Chi-Square test), while no such difference was observed between the available 14 ICM (27/322) and TE biopsies (35/322) (P = ns). Hence, BF-DNA karyotype reached full concordance between either ICM or TE only in 40.0% (4/10) of the embryos. In contrast, full concordance between ICM and TE was observed in 85.7% (12/14) of the embryos, making TE more representative of embryonic chromosomal status than BF ($P<0.03$). The results of our study are similar to previously published report, demonstrating 48% concordance rate between BF and ICM-TE (Tobler et al., 2015), but it also drastically contrasts with the high concordance rates achieved by another group (Gianaroli et al., 2014; Magli et al., 2016). Such contradictory outcome may be explained by different types of material analyzed and in our case also by a different technological approach. The genomic profile of blastocysts may include mosaicism that can be missed by aCGH, which is able to detect only high-degree mosaicism, when >50% of cells are aneuploid (Mamas et al., 2012). Here, by using NGS method with improved resolution and sensitivity we were also able to determine embryos carrying 20-40% of abnormal cells.

4.4.2. Biological and technical challenges associated with BF-DNA analysis

Blastocyst culture has become a milestone in ART and is now widely used for selection of viable embryos for transfer. Steadily, day 5 biopsy is also becoming the preferred option for obtaining embryonic cells for genetic analysis, especially for aneuploidy screening. Apart from the impact of mosaicism on embryo development and implantation potential, another major questions still lingers in the air: how well does the genomic profile of blastocyst trophectoderm layer represent the inner cell mass? The design of our study enabled us to explore that question in greater detail, also taking into account the mosaic nature of the blastocyst stage. As a major outcome, our data indicates that results obtained from BF-DNA may not be comparable to those obtained via standard TE biopsies, because BF-DNA does not adequately predict the status of the rest of the embryo, while TE is quite representative of ICM and no evidence of preferential allocation of aneuploid cells to trophectoderm was observed. Of course, a note of caution should be applied here, because unlike in clinical practice, where only a small number of cells are analyzed, we have screened the whole TE population. Although the impact of BF sampling seems less invasive, functional studies on the effect of BF biopsy on embryo viability may be warranted, as blastocoel may contain proteins crucial for embryonic development (Poli et al., 2015). Thus, contrary to some views that blastocoel

biopsy can be a plausible alternative for PGT-A, we argue that blastocentesis as a single source of DNA for PGT-A can potentially lead to an increased rate of false positive findings, at least using current methods and protocols. For the clinic and patients it would mean that a viable embryo with euploid genome can be discarded based only on the results of BF-DNA karyotype analysis, making it diagnostically unacceptable. One must not forget that PGT-A is also a perfect example, where biology compromises technological breakthroughs. The presence of embryonic DNA in BF suggests that potential mechanisms might exist, by which the genetic material is released into the blastocoel cavity, like cell lysis, apoptosis and/or elimination of cellular debris (Hammond et al., 2016). The intact ICM karyotype in the presence of aneuploidy in BF-DNA in some of the embryos also seems to support the idea that aneuploid cells are progressively depleted from the developing embryo through apoptosis, ensuring the genomic integrity of the future foetus (Bolton et al., 2016). This biological phenomenon might also partially explain the high concordance rate previously observed between BF-DNA and blastomeres (Magli et al., 2016), as aberrant cells may be marginalized into the blastocoel cavity at later stages of development, releasing their genetic material that is later picked up again during BF analysis. DNA in blastocoel fluid might also be prone to fragmentation/ degradation and combination of these factors results in chaotic chromosome profiles of BF samples. In turn, this would also have a direct clinical consequence, because when evaluating BF-DNA, one might look at the genomes of cells that were discarded by the embryo, without knowing the real status of ICM. However, given the remarkable genomic plasticity of early embryogenesis, the true origin of genetic material in blastocoel cavity awaits elucidation and advanced genome-wide haplotyping technologies might shed some light on this matter. Taken together, the biology behind BF-DNA data interpretation must be adequately elucidated before implementing it as a diagnostic tool in everyday clinical practice.

In addition to biological challenges, technical limitations can also prevent the use blastocentesis as a first choice biopsy method. Any micromanipulations during BF aspiration can also potentially alter the downstream applications, and in our case aspiration of BF after embryo vitrification and thawing might have potentially interfered with obtaining good quality data. Still, it is important to note that blastocoel fluid contains cell-free DNA with similar profile of fragmented cell-free DNA found in human plasma (Zhang et al., 2016), the quantity and quality of which may also vary from embryo to embryo. It is well established that low amount of good quality DNA may be prone to uneven amplification and allele drop-out (Huang et al., 2015) and subsequent library preparation methods and technical artifacts can result in an altered representation of the genome that will reduce the reliability of chromosome analysis (Goodrich et al., 2016). The overall increased noise ratio can also interfere with low-grade mosaicism evaluation. In the end, the diagnostic accuracy will suffer and the use of BF-DNA as a source of DNA for embryo screening might become restricted (at least using current methods and protocols). Notably,

our data indicates that TE and ICM genomic profiles showed either lower level of mosaicism or absent aneuploidy, compared to corresponding BF-DNA profiles, so one might argue that from the clinical and diagnostic point of view the use of insensitive to mosaicism aCGH platform (that detects only >50% mosaicism) might seem like a more suitable approach for the analysis of BF-DNA, because the potentially biologically irrelevant low-grade mosaicism in BF would not be detected. On the other hand, embryos with normal TE and ICM karyotype also showed high-grade mosaic aneuploidies in BF that would likely be interpreted as true finding using aCGH, leading to misdiagnosis. Such discordance was also reported previously (Perloe, 2013; Poli et al., 2013; Tobler et al., 2015) and taking into account BF-DNA features, none of the methods seem to have any major advantages or disadvantages. In contrast, because karyotype concordance were much higher in TE and ICM cells, it seems that chromosome analysis of TE biopsy remains a more optimal and effective way for predicting the karyotype of an embryo. However, more comprehensive studies on embryonic mosaicism are warranted that would help refine the criteria for embryo selection for transfer without compromising the treatment success rate by excluding mosaic embryo capable of forming viable pregnancies.

4.5. Future perspectives

Over the last decade single-cell technologies paved way to many breakthrough discoveries in the field of reproductive genetics, but as it goes in science, providing an answer to one question only leads to more questions. Because embryonic aneuploidy is not restricted only to human, but can exist also in other mammalian species, in our Study I we first aimed to understand the prevalence of chromosomal imbalances in cattle and to compare it to human. Although chromosomal instability is common in both human and bovine *in vitro* cleavage-stage embryos, as demonstrated by the results, cellular and molecular mechanisms driving this error-prone stage of development are still poorly described. The fact that aberrant cells can persist as far as blastocyst stage suggests that embryos may possess unique cellular regulatory mechanisms that make them more vulnerable to chromosome segregation errors. First, studies on mouse and porcine embryos suggest that DNA damage checkpoint and repair may be insufficient in embryos prior to EGA due to limited ATM kinase activity, which is the major upstream mediator of the DNA damage response and repair pathway (Adiga et al., 2007; Wang et al., 2015). The loss of ATM activity in early mouse embryos and embryonic stem cells compromises phosphorylation of downstream targets necessary for the repair of DNA double-strand breaks, consequently leading to proliferation defects and genomic instability due to chromatid breaks (Yamamoto et al., 2012). Second, upon massive DNA damage somatic cells triggers apoptosis to eliminate the damaged cells, however functional apoptotic pathways seem to be suppressed in early

human and bovine cleavage-stage embryos to facilitate embryo survival until later stages of development (Fear and Hansen, 2011; Bazrgar et al., 2014). Finally, embryonic cell cycle checkpoints and regulators may be more permissive to chromosome missegregations, allowing rapid cleavage divisions. Indeed, some of the essential G1 and G2 cell checkpoint proteins, like RB and WEE1, are silenced in 8-cell human embryos and are only activated at later stages of development (Kiessling et al., 2009; Kiessling, 2010; Kiessling et al., 2010). Taken together, these observations suggest that, unlike somatic cells, blastomeres with damaged or incompletely replicated DNA and/or improper chromosome alignment can still proceed to mitosis, bypass SAC-mediated arrest and apoptosis, and continue cell division, leading to increased rates of chromosomal aberrations and aneuploidy upon post-zygotic divisions. Intriguingly, late embryonic genome activation may trigger apoptotic response in the embryo to eliminate aneuploid cells from further development, so even mosaic embryos can still result in live birth, challenging the hypothesis that aneuploid embryos are developmentally incompetent. In turn, this raises important questions regarding the burden and nature of genomic imbalances that can be tolerated by the embryo without compromising its survival. With this in mind, further research also needs to address the putative role of CIN in subsequent post-implantation and prenatal development to understand, which genomic anomalies can be compatible with normal foetal development.

As a major breakthrough of Study I, we have incidentally discovered that zygotes can spontaneously segregate entire parental genomes to distinct cellular lineages, following first cytokinesis. The discovery of this phenomenon, termed heterogoneic division, was possible due to the use of a novel computational method, enabling concurrent genome-wide haplotyping and copy number profiling of single-cells. Importantly, heterogoneic cell lineages were observed not only in dispermic embryos, but such event can also spontaneously arise from normally fertilized zygotes. These findings provide novel conceptual framework for the emergence of embryonic mixoploidy that may also potentially underlie the pathogenesis of gestational trophoblast diseases (GTD) – rare pregnancy-related disorders, characterized by the abnormal tissue growth inside a woman's uterus. For example, complete hydatidiform moles (CHMs) of androgenetic origin is the most common form of GTD that occurs in human in about 1 in every 1500 pregnancies (Moein-Vaziri et al., 2018). In human CHMs, the foetus and amniotic membranes are usually absent and the product of conception resembles a grape-like structure that can become invasive, developing into malignant trophoblastic cancer, known as choriocarcinoma. CHMs have also been reported in bovine with tissue structures similar to human, but the exact aetiology of bovine hydatidiform moles is unclear (Meinecke et al., 2002; Morris et al., 2008). The current dogma states that the origin of CHMs comes from fertilization of an anuclear egg by a haploid sperm with subsequent endoreplication (Jacobs et al., 1980). Another hypothesis states that CHMs are a result of premature independent mitotic division of a male haploid pronucleus with subsequent fusion (diploidization) of these two post-mitotic products and

disappearance of the egg pronucleus (Golubovsky, 2003). It is not clear why and how the female pronucleus would be eliminated. Hence, the observed persistence and independent proliferation of distinct androgenetic and gynogenetic cell lineages in our study may provide a novel and more plausible mechanistic basis for the formation of some types of GTDs. Due to high proliferative capacity, these distinct cell lineages can outgrow other blastomeres and upon implantation such embryo can potentially give rise to abnormal pregnancy formation. However, the frequency of this phenomenon, as well as fate of mixoploid embryos remains to be investigated. In addition, the existence of heterogoneic cell division in zygotes prompts an investigation into the regulatory mechanisms of cell divisions during preimplantation embryo development. Different pathways have been proposed recently, as to how and why the symmetric propagation of parental genomes during embryonic development would get perturbed, and these hypothesis include the formation of a gonomeric spindle driven by asymmetric k-MT dynamics between parental genomes and asynchronous parental cell cycles in the zygote, driving the nucleation of parental-specific "private" spindles (Destouni and Vermeesch, 2017). Recently, an elegant microscopy study in mice has demonstrated that during the first post-zygotic division, male and female chromosomes are assembled on separate mitotic spindles, challenging the old model of nuclear envelope breakdown (Reichmann et al., 2018). Hence, failure to properly align two zygotic spindles would provide a mechanistic basis for parental genome segregation. Further fundamental research in the field of cellular biology combined with confocal live-cell imaging technologies will greatly contribute to our understanding of zygotic chromatin, kinetochore and spindle dynamics that underlie successful maternal-to-zygotic transition.

Because maternal factors regulate first zygotic and post-zygotic processes, it is important to expand our knowledge on molecular mechanisms of oocyte nuclear and cytoplasmic maturation and determine factors that influence oocyte competence. In human IVF, it is relatively hard to acquire oocytes for research. Instead, easily accessible granulosa/cumulus cells can shed some light on the biology of the oocyte, because they shape its intrinsic parameters during oogenesis, therefore they can potentially reflect the characteristics of the oocyte and future embryo. Indeed, altered gene expression in granulosa cells was associated with oocyte aneuploidy (Fragouli et al., 2012), so it was hypothesized that impaired somatic cell function and altered follicular microenvironment can lead to reduced oocyte and embryo quality. Transcriptome profiling of cumulus and granulosa cells has also been investigated as a novel non-invasive approach to evaluate oocyte and embryo quality (Uyar et al., 2013), but the practicality of this type of testing was questioned recently (Green et al., 2018). Still, the information on how cumulus cells determine or reflect oocyte and embryo quality is scarce and detailed characterization of molecular transcriptome profiles of cumulus cells in relation to oocyte and embryonic aneuploidy awaits further investigation. From translational point of view, oocyte and granulosa/

cumulus cells-targeted research might potentially be quite beneficial in the development of IVM protocols.

In light of our research, it is also important to understand how *in vitro* conditions influence oocytes and early embryo development. Ultimately, IVF embryos must survive a rough artificial environment very different to that in natural reproduction. In our Study II, we compared the genomic architecture of *in vivo* and *in vitro* bovine embryos and we have demonstrated the negative impact of *in vitro* conditions on genomic stability of early bovine embryos. Another profound aspect of IVF that deserves further investigation is the potential influence of *in vitro* culture systems on embryonic epigenome. During early embryogenesis major epigenetic reprogramming takes place that is essential for proper embryo development, but this process can be vulnerable to *in vitro* techniques and culture environment. These effects have mostly been studied in bovine, where calves born from *in vitro* produced embryos often exhibit increased birth weight, known as large offspring syndrome, which is similar to Beckwith-Wiedemann syndrome in human (Chen et al., 2015; Sirard, 2017). Altered phenotype observed in these calves was likely the result of failure to properly establish or maintain DNA methylation levels in early embryogenesis. The regulation of embryonic epigenome may also pose safety concerns regarding IVF, because of the worry that it might have detrimental long-term health consequences in IVF newborns (Pinborg et al., 2015; Ventura-Junca et al., 2015; Jiang et al., 2017). In the future, the upcoming sophisticated single-cell multiomics approaches, which allow screening both the (epi)genome and transcriptome of a single cell, can fill in the missing gaps on the regulation of preimplantation development and pinpoint to essential biological pathways, dysfunction of which can also predispose the embryo to aneuploidy. In addition, with the translation of the uterine lavage technique (which has been used in cattle for over 40 years) to human (Pagidas et al., 2014), the ability to analyse naturally conceived human embryos will soon become a reality. The obtained knowledge on the molecular control of oocyte maturation that determines subsequent embryonic developmental potential, as well as IVF factors that compromise embryonic survival will help to refine and improve assisted reproductive technologies and *in vitro* culture conditions in human.

Finally, nowadays, numerous attempts are made to find the best way to predict embryo aneuploidy, including blastocoel fluid biopsy and evaluation of embryo spent culture medium. In Study III, we have analysed the genomic profiles of blastocoel fluid and showed that BF-DNA demonstrated low concordance rates with TE and ICM of an embryo. This result is in line with some of the previously published reports, suggesting that due to dynamic nature of early embryogenesis, indirect embryo testing, in which the embryonic genome is not analyzed directly from the embryonic cells, is not yet a reliable approach for diagnostic purposes. Notably, in recent years, the increasingly popular PGT-A has created a predominant view that only genetically normal embryos are required to attain pregnancy. However, we can also look at CIN from a different angle that completely differs from the black-and-white clinical perspective: the

evolutionary angle. Chromosomal mutations may, albeit rarely, provide unexpected evolutionary advantage to an organism [reviewed in (Carbone and Chavez, 2015)]. Intriguingly, many inversions, translocations and centric fissions, which drive genome evolution, can already take their origin during cleavage-stage of development. Although the elevated levels of genomic aberrations in oocytes and IVF embryos are most likely a by-product of adverse *in vitro* conditions and manipulations, both *in vitro* and *in vivo* embryo genome dynamics can underlie mosaicism, chimerism and mixoploidy in live-born individuals. Hence, one might speculate that chromosomal rearrangements in natural conception may also potentially drive the evolution by speciation (e.g. the formation of new and distinct species in the course of evolution), as extensive chromosomal rearrangements distinguish the karyotype of more distant relatives. In addition, transposable elements, activation of which can occur already during preimplantation development (Gerdes et al., 2016), are at the core of genetic transformation, establishing novel transcriptional and species-specific gene regulatory network by disrupting genome integrity (Chuong et al., 2017). Unfortunately, the full extent of aneuploidy and CIN in human oocytes and embryos in natural conception is not currently known. Nevertheless, the possible elevated rate of CIN in human early reproduction and its potential role in genome evolution itself may potentially be an intriguing topic for evolutionary biologists that awaits to be discussed, at least at a theoretical level.

CONCLUSIONS

The uprising era of single-cell research has expanded our knowledge on early embryogenesis, highlighting the alarming fact that cleavage-stage embryos have high prevalence of chromosomal instability (CIN), which represents one of the most serious challenges in IVF. The current thesis addresses the topic of CIN in early embryos, focusing on important fundamental and translational questions:

1. Embryo-related research is ethically one of the most complex areas of reproductive science that greatly relies on the use of appropriate animal models, so the first aim of the thesis was to investigate the genomic profile of bovine *in vitro* cleavage-stage embryos. By using novel single-cell genomics approaches, the current thesis demonstrates that the nature and frequency of genomic abnormalities in *in vitro* cleavage-stage embryos is highly conserved between cattle and human, making bovine embryo a suitable model for elucidating the origins and molecular mechanisms of CIN in early preimplantation development.

2. The second aim of the thesis was to evaluate the nature and prevalence of CIN in naturally conceived embryos, in comparison to *in vitro* embryos. By using the established bovine model the current thesis demonstrates that oocyte/embryo micromanipulations and *in vitro* culture exacerbate CIN in early cleavage-stage bovine embryos, compromising their survival rate. If this is the same for human, then *in vitro* environment can greatly reduce the number of viable embryos for transfer. In turn, this should encourage scientific and medical communities not only to refine and improve *in vitro* culture conditions, but also raise awareness on fertility issues among men and women of reproductive age.

3. Based on the unexpected results of bovine single blastomere analysis, the current thesis also demonstrates that zygotes can segregate entire parental genomes into separate blastomeres, creating androgenetic and gynogenetic cell lineages. Intriguingly, it seems that *in vivo* embryos may also undergo heterogoneic division, suggesting that parental genome segregation can happen regardless of *in vitro* or *in vivo* environment. This observation opens up new horizons into investigating the mechanistic origin of mixoploidy and chimaerism in humans.

4. The final third aim of the study was to investigate the utility of blastocoel fluid analysis for preimplantation genetic testing for aneuploidy (PGT-A). The use of blastocoel fluid biopsy, or blastocentesis, for PGT-A has gained much controversial attention. The data presented in the current thesis demonstrates that DNA from blastocoel fluid can be successfully amplified and sequenced, but due to biological and technical challenges, as well as increased discordance rate between inner cell mass and trophectoderm, blastocentesis is not suitable for diagnostic purposes, at least using current protocols and methods. Therefore, trophectoderm biopsy remains the safest option with the most reliable results for comprehensive chromosome screening.

In summary, the current thesis raises scientific excellence and brings new and unique knowledge on genomic instability in mammalian embryos. The novel insight obtained here opens up new conceptual frameworks to study important questions on the origin and fate of embryonic CIN. The data demonstrated here is also critical for reproductive medicine and raises the need to revise IVF as we know it. However, one must always remember that although the use of animal models is sometimes inevitable due to ethical reasons, caution must be applied when extrapolating animal data to humans.

SUMMARY IN ESTONIAN

Kromosomaalne ebastabiilsus imetajate varajastes embrüotes

Viljatus on kogu maailmas kiiresti kasvavaks probleemiks ning iga kuues paar seisab silmitsi olukorraga, et soovitud rasedust ei teki. Esmasünnitajate keskmine vanus kasvab samuti, kuid naise vananedes tema viljakus langeb ning seetõttu pöördub tänapäeval aina rohkem paare viljatusravi poole, et leida oma probleemile lahendus. Kehaväline viljastamine (*in vitro fertilization*, IVF), mille korral toimub munarakkude viljastamine ning embrüote areng katseklaasis, on maailmas kõige sagedamini kasutatav lastetusravi meetod. IVF protseduuri tulemusena sünnib Eestis rohkem kui 600 last aastas, mis moodustab 4–5% kõikidest vastsündinutest. Samas annab IVF ravi erakordse võimaluse analüüsida inimese varajase embrüo arengut väljaspool naise organismi. Viimastel aastatel on üksikraku tehnoloogiate areng laiendanud meie teadmisi varajase embrüogeneesi osas. Embrüote üksikute blastomeeride genoomi uuringud on näidanud, et varajaste embrüote tähelepanuväärseks iseärasuseks on väga sagedased kromosomaalsed aberratsioonid, mis võivad esineda kuni 80% embrüotes. Paraku on embrüo aneuploidsus kõige suurem väljakutse IVF ravil, sest see on üks peamisi raseduse varajase katkemise (kuni 75%) ja IVF ebaõnnestumise põhjuseid. See on ajendanud teadlasi uurima nii embrüo kromosoomide aberratsioonide tekkemehhanisme, kui ka nende esinemissagedust mõjutavaid faktoreid. Paralleelselt sellele on hakanud märgatavalt parenema ka embrüo diagnostilised meetodid, mis aitavad hinnata IVF protseduuril saadud embrüote eluvõimelisust, tuvastada pärilikke haigusi põhjustavaid geenialleele ja kromosoomi muutusi embrüotes ning võimaldavad selekteerida geneetiliselt „terveid“ embrüoid emakasse siirdamiseks. Käesoleva doktoritöö eesmärgiks oli kaasaegseid üksikraku genoomi tehnoloogiaid kasutades uurida embrüote genoomi ebastabiilsust, keskendudes olulistele fundamentaalsetele ja rakenduslikele küsimustele:

1. Embrüotega seotud teadustöö on väga piiratud eetiliste põhjuste tõttu, seega sõltuvad inimese embrüote uuringud suurel määral sobivatest loommudelitest. Kasutades uudset üksikraku kogu-genoomi analüüsi metoodikat, näidati antud doktoritöös, et ka veise IVF embrüotes esinevad väga sagedased kromosomaalsed aberratsioonid, mille tulemusena on suurem osa varajasi embrüoid mosaiiksed. Seega on veis hea mudel inimese embrüogeneesi uurimiseks ning genoomi ebastabiilsuse päritolu selgitamiseks varajases preimplantatsioonilises arengufaasis.

2. Bioloogia fundamentaalne dogma ütleb, et raku jagunemisel saavad mõlemad tütarrakud kaks genoomi koopiat: üks pärineb isalt ja üks emalt. Käesolevas doktoritöös näidati embrüo genoomi erakordset plastilisust, mis lükkab antud dogma ümber. Nimelt avastati genoomi ebastabiilsuse uuringute käigus, et embrüo on võimeline segregeerima ema- ja isapoolse genoomi eraldi rakuliinidesse. Selline unikaalne nähtus avastati tänu kogu-genoomi DNA analüüsi metoodikale, mis võimaldab samaaegselt määrata nii haplotüüpe, kui koopia-

arvu muutusi üksikutes rakkudes. Tuvastatud heterogoneeiline (*heterogoneic*, kreeka keeles „erinev vanemate päritolu“) sügoodi jagunemine avab uusi teadussuundi, et uurida varajaste preimplantatsiooni embrüote raku jagunemise regulatoorseid mehhanisme. Samuti võib heterogoneeiline sügoodi jagunemine olla aluseks eriploidsuse (*mixoploidy*) ja kimääride tekkele, kuid selle nähtuse esinemissagedus on hetkel teadmata.

3. Kromosoomide aneuploidsus on iseloomulik inimese IVF embrüotele, kuid arvatakse, et kromosomaalne ebastabiilsus ei piirdu ainult *in vitro* embrüotega vaid esineb ka *in vivo* embrüotes. Sellist hüpoteesi toetab ka asjaolu, et embrüote aneuploidsus on peamine raseduse varajase katkemise põhjus. Lisaks sellele on kirjanduses avaldatud andmeid harva esinevate juhtumite kohta, kus indiviidil on tuvastatud eriploidne ja/või mosaiikne karüotüüp. Kasutades veise loommudelit, näidati käesolevas doktoritöös, et kromosomaalne ebastabiilsus eksisteerib samuti loomulikul teel saadud embrüotes ning ka *in vivo* embrüod võivad segregeeruda vanemata genoomi, mis viitab sellele, et see protsess toimub sõltumata *in vivo* või *in vitro* keskkonnast. Heterogoneeiline jagunemine *in vivo* embrüotes võib seletada eriploidsuse esinemist looduses. Samas suurendab munarakkude ja embrüote *in vitro* manipuleerimine kromosomaalse ebastabiilsuse sagedust veise varajastes embrüotes, vähendades nende elujõulisust. Kui sama trend kehtib ka inimese embrüote puhul, tähendab see, et *in vitro* keskkond võib drastiliselt kahandada „tervete“ embrüote arvu emakasse siirdamiseks, sest embrüod peavad ellu jääma karmis kunstlikus keskkonnas, mis erineb suurel määral loomulikest tingimustest. Omakorda peaks see julgustama teadus- ja meditsiinikogukonda mitte ainult parandama *in vitro* kultuuri tingimusi, vaid ka tõstma reproduktiivses eas olevate inimeste teadlikkust viljakuse ja IVF ravi osas.

4. Tänapäeval kasutatakse preimplantatiivses embrüo sõeluuringus (PGT-A) kas kolmanda päeva biopsiat (analüüsitakse ühte embrüo rakku) või viienda päeva biopsiat (analüüsitakse 5–6 rakku). Viimasel ajal on aga hakatud pöörama suuremat tähelepanu ka blastotsööli vedeliku (blastotsüsti sees olev vedelik) analüüsile, kuid selle analüüsi tulemused on olnud seni vastuolulised. Käesoleva doktoritöö raames analüüsiti viienda päeva embrüoid, millest oli eraldatud kolm erinevat blastotsüsti osa: sisemine rakumass (*inner cell mass*, ICM), trofektodermi rakud ning blastotsööli vedelik. Tulemuste analüüsil selgus, et blastotsööli vedelik ei peegelda alati kromosoomide olukorda ICM-s või trofektodermi rakkudes ning seda meetodit ei saa hetkel kasutada embrüote kromosoomide analüüsis.

Kokkuvõttes annab käesolev doktoritöö uut ja praktilist informatsiooni embrüote genoomi ebastabiilsuse kohta. Antud töö püstitab samuti olulisi küsimusi embrüo arengut mõjutavate molekulaarsete faktorite kohta, mis väärivad edaspidist uurimist ja tähelepanu. Antud teadmisi saab tulevikus rakendada reproduktiivmeditsiinis, mis on paljudele peredele ainukeseks võimaluseks saada järglasi.

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Thanks to all the people from the diagnostics lab for letting me process my occasional cow samples on your grounds: again Efty and also Cindy (and thanks for tolerating all the project management related questions), Nathalie B. and Natalie S., Kris and Hilde. A very big thank you also goes to Veerle Mattheus, Annemie Puttemans and Narcisse Opdekamp for administrative support and for making sure that things run smoothly and according to the rules.

All this incredible cow work was a true effort of prof. Ann Van Soom's group, including Katrien and Maaike, so thank you for this heroic act! Maaike – keeping up with those cows that throw poop at you – it is a work of blood, sweat and tears! Too bad we could not get our cows for BBQ. By the way, thanks for not leaving me behind when I ran into trouble with the security during terrorist threat level 4; from that moment I knew we are a real team! Good luck at your new job, boss lady!

Big thanks also goes to prof. Thierry Voet and the amazing people of his lab: Niels, Koen, Parveen, Masoud, Elia, Daniel (with Justine and her cakes on the side) and the rest of the group for pushing the boundaries of single-cell technologies to a whole new level. Of course, all the credit for computational work goes to Masoud – thanks for putting on our detective hats during sample mix-ups and unexpected inbreeding (you can't fool genetics). Parveen – thanks for our spontaneous drinks and random conversations, they were one of a kind! After you left, the dynamics were never the same, but luckily I still had my Fresita! Elia, I might have finally earned a badge of my own, but I will always raccoon food from you, just so you know. Thanks for all the "fresh" air breaks and no-drama moments.

A very special place in my acknowledgments goes to my favorite people in the world: Tanya, Romain and Kurt. Tanya – you are the best colleague one can wish for and an even better friend! You are always there to help people, putting their needs ahead of yours. Even when your own deadlines pressure you, you still find time to make a graph for someone or check their documents! Thanks for all the support and encouragements throughout the years and for all the crazy adventures. Meow! Romain and Kurt – unfortunately I cannot make inappropriate jokes, so I will have to keep it boring☺. It was much harder for me to leave home for the second time to return back to Belgium (just to lock myself out of the apartment), but you made it so easy, by turning my frown upside down (or maybe it was just the effect of G&T, masterly prepared by Kurt). Wednesday is my favorite day, because I know I will be standing in my little kitchen corner, occasionally peeling and cutting and chopping (herb lady as I am). Thank you for your friendship and hospitality and for always making me feel at home. Go Estolgium!

Дорогие родители!
Этот долгий путь наконец-то достиг конца, и весь этот непонятный тезис с набором английских слов посвящён вам! Спасибо за вашу нескончаемую поддержку и веру, а самое главное – за доверие. Дорогая мама, я уехала в Бельгию с самым тяжёлым сердцем, но ты доказала, что можно преодолеть все жизненные барьеры, главное не сдаваться! Твой дух и твоя стойкость были моими самыми большими источниками сил. Дорогой папа – спасибо, что был нашим главным телемостом, который помогал держать связь (а то мама за компьютером… ☺), и, конечно же, Шмель – спасибо, что на протяжении университетских дней всегда катал меня с поезда на поезд в любое время суток.

Дорогой Злат – пусть эта книга служит тебе примером, что нам всё по зубам, главное захотеть и работать в нужном направлении! Кто сказал, что мы хуже, чем супергерои?

PUBLICATIONS

CURRICULUM VITAE

Name: Olga Tšuiko
Date of birth: 18/07/1988
Address: Competence Center on Health Technologies,
Tiigi 61b, Tartu 50410
E-mail: olga.tsuiko@ut.ee

Education

2012–… University of Tartu, Faculty of Medicine, Institute of Bio- and Translational Medicine; PhD student in reproductive genetics

2010–2012 University of Tartu, Faculty of Science and Technology, Institute of Molecular and Cell Biology, Department of Biotechnology; MSc in molecular diagnostics

2007–2010 University of Tartu, Faculty of Science and Technology, Institute of Molecular and Cell Biology, Department of Biotechnology; BSc in molecular diagnostics

Working experience

2018–… KU Leuven, Laboratory of Cytogenetics and Genome Research (Belgium); associate researcher/project coordinator

2014–2018 Competence Centre on Health Technologies; associate researcher

2014–2016 KU Leuven, Laboratory of Cytogenetics and Genome Research (Belgium); visiting PhD student at prof. Joris Robert Vermeesch lab

2013 – 2014 Competence Centre on Health Technologies; lab technician

Fields of research

- Reproductive genetics and biology
- Developmental biology and embryology; preimplantation embryo development
- Molecular diagnostics; preimplantation genetic testing

Publications

Tran Quoc, T.; Jatsenko, T.; Poolamets, O.; **Tšuiko, O.**; Lubenets, D.; Reimand, T.; Punab, M.; Peters, M.; Salumets, A. Chromosomal scan of single sperm cells by combining fluorescence-activated cell sorting and next-generation sequencing. *Journal of Assisted Reproduction and Genetics*, 2018, under review.

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ELULOOKIRJELDUS

Nimi: Olga Tšuiko
Sünniaeg: 18/07/1988
Aadress: Tervisetehnoloogiate Arenduskeskus AS, Tiigi 61b, Tartu 50410
E-mail: olga.tsuiko@ut.ee

Haridus

2012–… Tartu Ülikool, Bio-ja Siirdemeditsiini Instituut, doktoriõpe, arstiteaduse õppekava
2010–2012 Tartu Ülikool, Molekulaar ja rakubioloogia instituut, magistriõpe, biomeditsiini õppekava
2007–2010 Tartu Ülikool, Molekulaar ja rakubioloogia instituut, bakalauruseõpe, geneetika õppekava

Teenistuskäik

2018–… KU Leuven, Laboratory of Cytogenetics and Genome Research (Belgia); järeldoktorantuur
2014–2018 Tervisetehnoloogiate Arenduskeskus AS; nooremteadur
2014–2016 KU Leuven, Laboratory of Cytogenetics and Genome Research (Belgia); külalisdoktorant
2013–2014 Tervisetehnoloogiate Arenduskeskus AS; laborant

Teadustöö põhisuunad

- Reproduktiivgeneetika-, bioloogia ja meditsiin
- Arengubioloogia ja embrüoloogia; varajaste embrüote areng
- Molekulaarne diagnostika; embrüo siirdamiseelne diagnostika

Publikatsioonide loetelu:

Tran Quoc, T.; Jatsenko, T.; Poolamets, O.; **Tšuiko, O.**; Lubenets, D.; Reimand, T.; Punab, M.; Peters, M.; Salumets, A. Chromosomal scan of single sperm cells by combining fluorescence-activated cell sorting and next-generation sequencing. *Journal of Assisted Reproduction and Genetics*, 2018, under review.

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DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

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51. **Ants Kask**. Behavioural studies on neuropeptide Y. Tartu, 1999.
52. **Ello-Rahel Karelson**. Modulation of adenylate cyclase activity in the rat hippocampus by neuropeptide galanin and its chimeric analogs. Tartu, 1999.
53. **Tanel Laisaar**. Treatment of pleural empyema — special reference to intrapleural therapy with streptokinase and surgical treatment modalities. Tartu, 1999.
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59. **Anneli Beilmann**. Epidemiology of epilepsy in children and adolescents in Estonia. Prevalence, incidence, and clinical characteristics. Tartu, 1999.
60. **Vallo Volke**. Pharmacological and biochemical studies on nitric oxide in the regulation of behaviour. Tartu, 1999.
61. **Pilvi Ilves**. Hypoxic-ischaemic encephalopathy in asphyxiated term infants. A prospective clinical, biochemical, ultrasonographical study. Tartu, 1999.
62. **Anti Kalda**. Oxygen-glucose deprivation-induced neuronal death and its pharmacological prevention in cerebellar granule cells. Tartu, 1999.
63. **Eve-Irene Lepist**. Oral peptide prodrugs – studies on stability and absorption. Tartu, 2000.

64. **Jana Kivastik**. Lung function in Estonian schoolchildren: relationship with anthropometric indices and respiratory symptomas, reference values for dynamic spirometry. Tartu, 2000.
65. **Karin Kull**. Inflammatory bowel disease: an immunogenetic study. Tartu, 2000.
66. **Kaire Innos**. Epidemiological resources in Estonia: data sources, their quality and feasibility of cohort studies. Tartu, 2000.
67. **Tamara Vorobjova**. Immune response to *Helicobacter pylori* and its association with dynamics of chronic gastritis and epithelial cell turnover in antrum and corpus. Tartu, 2001.
68. **Ruth Kalda**. Structure and outcome of family practice quality in the changing health care system of Estonia. Tartu, 2001.
69. **Annika Krüüner**. *Mycobacterium tuberculosis* – spread and drug resistance in Estonia. Tartu, 2001.
70. **Marlit Veldi**. Obstructive Sleep Apnoea: Computerized Endopharyngeal Myotonometry of the Soft Palate and Lingual Musculature. Tartu, 2001.
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81. **Leena Puksa**. Late responses in motor nerve conduction studies. F and A waves in normal subjects and patients with neuropathies. Tartu, 2003.
82. **Krista Lõivukene**. *Helicobacter pylori* in gastric microbial ecology and its antimicrobial susceptibility pattern. Tartu, 2003.

83. **Helgi Kolk**. Dyspepsia and *Helicobacter pylori* infection: the diagnostic value of symptoms, treatment and follow-up of patients referred for upper gastrointestinal endoscopy by family physicians. Tartu, 2003.
84. **Helena Soomer**. Validation of identification and age estimation methods in forensic odontology. Tartu, 2003.
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86. **Jaan Soplepmann**. Peptic ulcer haemorrhage in Estonia: epidemiology, prognostic factors, treatment and outcome. Tartu, 2003.
87. **Margot Peetsalu**. Long-term follow-up after vagotomy in duodenal ulcer disease: recurrent ulcer, changes in the function, morphology and *Helicobacter pylori* colonisation of the gastric mucosa. Tartu, 2003.
88. **Kersti Klaamas**. Humoral immune response to *Helicobacter pylori* a study of host-dependent and microbial factors. Tartu, 2003.
89. **Pille Taba**. Epidemiology of Parkinson's disease in Tartu, Estonia. Prevalence, incidence, clinical characteristics, and pharmacoepidemiology. Tartu, 2003.
90. **Alar Veraksitš**. Characterization of behavioural and biochemical phenotype of cholecystokinin-2 receptor deficient mice: changes in the function of the dopamine and endopioidergic system. Tartu, 2003.
91. **Ingrid Kalev**. CC-chemokine receptor 5 (CCR5) gene polymorphism in Estonians and in patients with Type I and Type II diabetes mellitus. Tartu, 2003.
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