Improving embryo quality in assisted reproduction
Mantikou, E.

Citation for published version (APA):
Mantikou, E. (2013). Improving embryo quality in assisted reproduction

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
CHAPTER 8

General discussion and implications for further research
In the thirty-five years since the birth of the first IVF child, about five million children have been conceived worldwide using assisted reproductive technologies [1]. Despite the broad use of these technologies and all the advancements in the field, the two largest data collections available [i.e. of the European Society for Human Reproduction and Embryology (ESHRE) and of the American Society for Reproductive Medicine (ASRM)] report a delivery rate of only 20% and 22% per started cycle of IVF and ICSI respectively [2, 3]. Impaired embryo quality could explain these low success rates after IVF/ICSI treatment. Given the high percentage of human preimplantation embryos that arrest in culture (>50%) or fail to implant upon transfer (>80%, as shown in Chapter 7), it is evident that the human preimplantation embryo, although it has the possibility to adapt to a certain degree, is vulnerable [4].

Early preimplantation embryo development is a highly dynamic process where many important events take place in a short period of time. In humans, after fertilization, the embryo undergoes a series of mitotic divisions (early cleavages) where cells are progressively increasing in number and decreasing in size while the overall embryo size remains the same. These early cleavages rely almost solely on mRNA and proteins provided by the oocyte. Around day three of development, the embryonic genome gets activated. In later developmental stages (day four to six) the embryo compacts to form a morula, and subsequently the first differentiation occurs where two lineages, the trophectoderm and the inner cell mass, are formed in the blastocyst (day six). The molecular mechanisms that drive these events in humans are not fully understood.

The main aim of this thesis was to study the mechanisms that could lead to improved quality of human preimplantation embryos and to investigate factors - with an emphasis on culture media - which can affect the quality of the embryo. A better insight into these factors could help embryologists optimize the in vitro culture conditions. This hopefully will lead to improved quality of embryos and therefore higher success rates of IVF/ICSI treatment.

A basic limitation when studying human embryo quality is the availability of normally fertilized (2PN) human embryos of good morphology. These embryos only become available for research when they are no longer needed in clinical practice, as couples finished their childbearing wish. In the Netherlands, generating human embryos specifically for research is not allowed; something which is possible in other countries such as the United Kingdom. Due to the obvious ethical and practical limitations with working with normally fertilized (2PN) human preimplantation embryos, a model is needed to be able to study human embryo quality from the first day of development onwards. Possible models to consider include animal embryos, trophectoderm and embryonic stem cell lines (ESC) and abnormally fertilized embryos (3PN and 1PN embryos). Supernumerary human embryos after an IVF/ICSI treatment can also be used, which could be left-over lower quality embryos that otherwise will be discarded or cryopreserved good quality embryos that are donated for research.
Animal models are an attractive alternative as they overcome many of the logical, ethical and financial challenges of working with human embryos. Common animal models include mouse, bovine, porcine and nonhuman primate embryos. Nevertheless, there are important differences between animal and human embryos. The timing of embryo development in animals for instance is different compared to human embryos (e.g. the mouse embryo undergoes fast mitotic divisions and reaches the expanded blastocyst stage already on day four [5]). The timing of embryonic genome activation (EGA) also varies among different species (e.g. the mouse EGA happens at day two [6, 7]). Moreover, animal embryos appear less sensitive to mitotic errors than human preimplantation embryos (e.g. the incidence of chromosomal mosaicism in nonhuman primate preimplantation embryos is 31.2%) [8, 9]. Nonhuman primate embryos are the closest to human embryos with similar timing of embryo development and EGA [10, 11].

The use of trophectoderm and embryonic stem cell lines is another alternative but these cells are not representing the entire embryo. They respectively represent just the trophectoderm and the ICM of the blastocyst. Working with supernumerary human embryos or abnormally fertilized embryos after IVF could be a better option. Supernumerary embryos are often of lower quality since the best available embryos were already transferred or cryopreserved. Furthermore, supernumerary embryos are often only available from day four onwards, limiting research on the critical period of the first three days. Abnormally fertilized 3PN embryos are believed to contain a triploid chromosomal content and as such they are never transferred to a woman. Therefore, these embryos can be investigated from the first day onwards. For digynic 3PN embryos, the fact that they are triploid does not make them chromosomally more unstable than normal 2PN embryos as they contain a single bipolar spindle and undergo regular chromosome segregation [12, 13]. Cryopreserved embryos are of good quality and can be available at different developmental stages, from the zygote stage to the blastocyst stage, depending on the cryopreservation policy of the IVF center.

The selection of which model to use at a given time depends on the scientific question to be answered and the design of the experiment.

**Culture media and embryo quality**

An essential factor in daily IVF practice is the culture medium used to culture embryos in vitro. Surprisingly, even after 35 years of clinically culturing human embryos in the lab we know very little about the efficacy of culture media and about the optimal composition to achieve the best results not only in numbers of live births but also on the health of children born. Over these years effort has been made to optimize the components of culture media by reducing glucose concentrations [14], adding amino acids [15-18] and supplementing with growth factors [19-21]. Unfortunately, a simple list with essential components of media for human embryo culture is not available since no broad consensus exists. Moreover, a literature search showed that most of the studies on the components of culture media for
human preimplantation embryo culture have been performed in the eighties and nineties and only a couple of such studies have been performed in recent years [22-26].

No broad consensus exist either on whether the choice of culture medium actually matters for obtaining good results. In recent years, there has been a renewed interest in culture media after several studies showed an effect of culture media on human intrauterine growth, neonatal birthweights and epigenetic alterations in the placenta [27-30]. Some studies on both animals and human showed that in vitro culture and culture media in specific can affect embryo quality and pregnancy outcomes [31-38].

The data presented in this thesis show an effect of culture media at the transcriptome level as well as at the level of embryo development and clinical outcomes.

However, other studies showed no effect of culture media either on embryo quality or success rates [39-43]. On neonatal outcomes, in contrast to the one quasi-randomized study that showed an effect, three retrospective studies reported no difference for the compared media [44-46]. All these studies compared different culture media so the different outcomes could be attributed to a general lack of effect of culture media. Alternatively, some culture media might be equally effective and therefore when compared to each other they give similar results. The systematic review described in this thesis (Chapter 6) demonstrated that there is very limited published data on the effect of culture media on IVF/ICSI success rates. Furthermore, the plethora of culture media used currently in IVF laboratories did not allow proper meta-analysis of all these data. This is an important conclusion in itself, and as such is highly relevant for the reproductive community at large. Such an essential component of IVF should be treated with the highest level of scrutiny, before it is introduced in clinical care.

The points raised in the discussion section of our systematic review (Chapter 6) can be used as guidelines for future research and can lead to better designed randomized trials that are urgently needed to develop evidence based laboratory practice [40]. It can be argued that, given the high number of commercially available culture media, even with addition of good designed trials a meta-analysis will not be possible. New analytical methods, such as network meta-analysis that allows for indirect treatment comparisons could potentially overcome this problem [47]. Also, the best trial that would answer this question adequately would be one comparing all available culture media simultaneously. Of course, such a trial is methodologically and practically not feasible given the high number of culture media and the fact that such trial needs to be repeated again upon introduction of any new culture media.

What is feasible though, and should actually go without saying, is conducting properly designed RCTs when new culture media are introduced into clinical care evaluating their (cost-) effectiveness and safety. Surprisingly, this is not a common practice most likely because large-scale RCTs are both time and money consuming. When it comes to
commercial companies the exact components of culture media and the concentrations used are not always revealed, prevailing further research. Moreover, if an RCT indicates equal or even lower success rates than already available media, years of research and development are wasted; thus, from a commercial perspective, an RCT carries a serious risk of harm for a company. In our view, the responsibility for proper introduction of culture media with new formulations lies with companies that should disclose the composition of each medium and should report what studies have been performed and which endpoints were analyzed to confirm effectiveness and safety. But it is not solely a responsibility of companies; embryologists should also aim to properly evaluate any new culture medium before its introduction to the clinic. In order to detect small differences in ongoing pregnancies or live births when comparing two culture media, large numbers of women are necessary. We have demonstrated that with proper collaboration of the reproductive medicine community, in this case in the Netherlands, a large, well-designed multi-center RCT can be performed. With further collaboration at an international level, and utilization of the two reproductive societies in Europe and in America (ESHRE and ASRM) inclusion of high numbers of women should not be an issue.

**Molecular analysis of human preimplantation embryos**

Until recently, molecular analysis of preimplantation embryos was hampered by the inability to analyze small quantities of material. Recent technological advances in mRNA amplification in combination with microarray or sequencing analysis now allow the simultaneous analysis of transcript levels of thousands of genes in a few or even in single cells [48-52]. Given that single cell analysis is an exciting new technology that yields a lot of data and opens up a totally new and undiscovered area of research on preimplantation embryos, this technology has found immediate application.

The implementation of single cell technology in the field of human reproduction has come without proper validation of the reproducibility and accuracy of the protocols used on actual single oocytes and embryos. Prior to the study presented in this thesis where we developed and validated protocols for microarray analysis of single oocytes and embryos (Chapter 4), only one study had validated their protocol to perform sequencing analysis of single mouse blastomeres [50].

Most data on gene expression in preimplantation embryos came from studies on mouse and bovine embryos [53-59]. In humans, few studies investigated overall gene-expression profiles in individual or pooled human preimplantation embryos [60-68]. These studies documented the transcription dynamics as the embryo progresses from the cleavage stage to the morula and blastocyst stage [62-65], the time of embryonic genome activation [62, 64, 68], the molecular mechanisms that govern differentiation of the ICM and TE cell lineages [60, 61, 67] and the heterogeneity in gene expression of embryos that could reflect their developmental competence [66].
The influence of environmental factors, such as culture media and oxygen concentration in the incubator, on embryo quality and pregnancy outcomes in an IVF program is well demonstrated in the literature and, for culture media, becomes more clear in this thesis [40, 69-70]. Several studies tried to find an explanation for these clinical observations based on altered gene expression of *in vitro* cultured animal embryos compared to their *in vivo* counterparts or when cultured in different culture media [33-35, 37, 38, 71-80]. The recognized affected molecular and cellular mechanisms involve metabolism, mitochondrial activity, oxidative stress, cellular integrity, cellular development and proliferation, cell-cell signaling and communication, apoptosis, imprinting, and protein synthesis [34, 37, 69, 76-79, 81-88]. Studies on the effect of *in vitro* culture environment on the transcriptome of human embryos were lacking. The study in this thesis (Chapter 5) presents the first data on the effect of culture media on gene expression of human preimplantation embryos. It was clear that HTF caused up-regulation of expression of genes related to cell death and apoptosis while culture in G5 caused up-regulation of genes involved in regulation of phosphorylation and mitosis, possibly implying that human embryos develop better in G5 compared to HTF between day four and six of development. Indeed, this assumption was substantiated by the RCT presented in this thesis (Chapter 7) that showed that more embryos in the G5 arm were utilized (transferred or cryopreserved) compared to the embryos in the HTF arm. Also, more embryos in the G5 group implanted compared to the HTF group.

Another factor that can influence embryo quality is maternal age. An effect of maternal age on oocyte quality is well established, where oocytes from older women have a high rate of meiotic aneuploidies [89, 90]. Maternal age has also an effect on genomic stability of human preimplantation embryos, with nondisjunction events increasing with maternal age [91-93]. At the level of the transcriptome, few studies have reported differences in gene expression between oocytes from younger and older women. For the first time, in this thesis, an effect of maternal age on gene-expression level in human embryos was shown (Chapter 5). In fact, the number of genes of which transcription was altered with varying maternal ages was higher than the number of genes affected by the culture environment in our experiment. The differences found in embryos of varying maternal ages involved genes associated with cell cycle and cell signaling, similar to the genes in the oocyte found to be affected by maternal age [89, 90]. In addition, genes that were affected by maternal age in embryos included many genes involved in phosphate metabolic processes.

These data, taken together with clinical data that show that pregnancy rates are decreased in women of advanced age [94], could suggest a model of how the effect of advanced maternal age works. During the first days of embryo development, the embryo is dependent on maternally inherited products from the oocyte until activation of the embryonic genome. The pool of maternally inherited products in oocytes of older women would be suboptimal, lacking mRNAs and proteins that are important for correct progression of the cell cycle. That would mean that the embryos derived from such oocytes have diminished cell cycle function and relaxed cell-cycle checkpoints. This could allow nondisjunction
events to occur and the incidence of aneuploidy (both meiotic and mitotic) to increase. Even after activation of the embryonic genome at day three, embryos from older women would have less or a different expression of genes regulating the cell cycle resulting in suboptimal development and for example a lowered potential to deal with aneuploidies in comparison to embryos from younger women. The direct link between decreased gene expression and incidence of aneuploidy, or vice versa, has yet to be confirmed.

It would be worthwhile to conduct an additional analysis on the occurrence of mitotic errors in relation to maternal age. The data reported in Chapter 2 could potentially be used for such an analysis. Furthermore, genes involved in phosphate metabolism appear to be less active in embryos of older women as compared to embryos of younger women. Phosphate metabolism is essential for phosphorylation of many proteins, enzymes and transcriptional factors thereby turning them on and off. Embryos of older women could be diminished in this ability, disturbing further the presence of essential active proteins for embryo development. Genes associated with mitochondrial energy production and oxidative phosphorylation were also found to be less expressed in embryos of older women compared to younger women. Since embryo development requires a high amount of energy, diminished mitochondrial function could affect the rate of cell division and the correct formation of the morula and the blastocyst.

Another interesting finding is the interaction between maternal age, culture medium, oxygen concentration and developmental stage (Chapter 5). These interactions suggest that embryos of different maternal age or developmental stage respond differently to various culture conditions. Theoretically, this could be translated into personalized IVF/ICSI treatments where some embryos and/or the embryos of some women require different culture conditions.

In this thesis, several factors (maternal age, culture media and oxygen concentration) have been investigated for their effect on embryo quality and emphasis was put on culture media. Other factors that could influence embryo quality are culture associated factors like CO₂ levels, culture medium volume, number of embryos per drop of culture medium, pH, stability of the incubator, temperature, and embryo produced factors [95-100], but also physiological factors such as ovarian reserve and maternal body mass index (BMI) and exogenous factors such as hormonal stimulation [101-105].

All molecular work presented in this thesis, has been performed using microarray technology, which has been the method of choice for many years for gene-expression analysis. Meanwhile, RNA sequencing techniques (RNA-seq), which allow DNA fragments to be repeatedly sequenced in a very short time, have been developed and validated for whole transcriptome analysis of single cells albeit not of single human embryos or oocytes [50, 106-108]. Since microarray probes are designed on the basis of prior genomic data, microarrays potentially miss novel expressed regions while these should be detectable using RNA-seq [109]. The main advantage of microarrays over sequencing is that they
are better validated and mature analysis strategies and experimental designs have been developed [109, 110]. Additionally, microarrays currently still have relative low costs compared with sequencing [109].

**How to assess embryo quality**

Embryo quality can be assessed from two different perspectives. The first one is the ability of an embryo to develop to a blastocyst, to implant and to lead to a live birth, i.e. the embryo’s developmental potential. The second perspective is the embryo’s genomic and epigenetic integrity that is necessary to lead to a delivery of a healthy offspring with lifelong health. A proper outcome to assess embryo quality from both perspectives is thus the delivery of a healthy baby. However, in the laboratory, other tests must be used to evaluate embryo quality and assume its developmental potential. Morphological evaluation by embryologists is currently the preferred method of choice [111]. It is however hampered by the lack of parameters with perfect sensitivity and specificity, and has inter- and intra-observer variability that is inherent to any imaging technique. Embryos with the expected cell number according to their time in development, and low percentage of fragmentation are generally considered of good quality [111]. However, this evaluation is considered suboptimal as less than 20% of embryos selected for transfer implant while some embryos considered of less good quality and therefore less suitable for transfer on day 2 - 3 are able to develop to blastocysts and implant following transfer to the uterus [112]. Time-lapse imaging is an emerging tool that allows the identification of additional morphological parameters indicative of good embryo quality and may allow more accurate prediction of embryonic developmental and implantation potential. Predictive parameters include duration of the first cytokinesis, the time between the first and second cytokinesis and the time between second and third cytokinesis [113-116]. Time-lapse imaging is a non-invasive, computer-automated test and as such it could be able to overcome the above mentioned disadvantages of morphological evaluation by the embryologists. Validation of the efficacy of time-lapse imaging with clinical trials is currently underway, and is needed before it can routinely be applied into clinical practice [113]. Given the high expense of both the equipment and the disposables necessary for time-lapse imaging, a cost-effectiveness analysis would be necessary as well.

Other methods have emerged producing a comprehensive analysis of the embryo’s genome, transcriptome, proteome, epigenome and metabolome [117]. These procedures can be applied at different stages during preimplantation embryo development.

Preimplantation genetic screening (PGS) has been proposed as an alternative method to assess embryo quality at the genomic level by screening for embryos that are aneuploid. However, the clinical trials performing PGS using FISH showed that PGS not only did not increase but instead significantly reduced IVF success rates [118, 119]. New technologies that enable in depth assessment of the embryonic genome (comparative genomic hybridization arrays or single nucleotide polymorphism arrays) have recently
been developed [120-122] but whether performing PGS using these techniques results in better outcomes remains to be tested. No matter which technique is used to assess the ploidy status of an embryo, the high percentages of mitotic errors occurring during the first three cleavages (Chapter 2) resulting in mosaicism, especially diploid-aneuploid mosaicism, hinders the efficacy of PGS on day 3 [123]. Biopsy of an aneuploid blastomere from a diploid-aneuploid embryo will lead to the discarding of a potentially viable embryo containing normal blastomeres. Alternatively, biopsy of a diploid blastomere will lead to transfer or cryopreservation of an embryo that has one less diploid blastomere, thereby potentially hampering its developmental potential. Performing the biopsy on day 5 might reduce these risks since more blastomeres can be biopsied. Further, it is possible to remove only blastomeres from the trophectoderm leaving the inner cell mass intact. However, whether PGS on day 5 is better than no biopsy should be evaluated.

Transcriptomic analysis reveals the cell’s phenotype and could yield information on how the embryo’s transcriptome reflects its quality and provide candidate genes as markers of embryo quality. Assessment of proteomics and metabolomics is the next step to find biomarkers of human embryo developmental potential through investigating proteins and metabolites that are secreted by the embryo in the culture medium [124-128]. However, these methods are all still at the very early stage of development. The first randomized controlled trials on selection of embryos by metabolomic profiling as an adjunct to morphology showed no significant difference in success rates when selection of embryos by metabolomic profiling and morphology was compared to embryo selection by morphology alone [129, 130].

Some first attempts have been made to assess epigenetic regulation in the preimplantation embryo, also in regards to the effect of in vitro culture [131-135]. There are still important limitations with this technique that prohibits its current application as a method to assess embryo quality. Firstly, the required input does not allow for global DNA methylation and histone modification studies in single cells. Secondly, the studies have thus far investigated epigenetic regulation in relation to ART only in animal embryos and little is known about epigenetics in the human preimplantation embryo.

Very recently, the presence of both genomic and mitochondrial DNA (mtDNA) in used culture media of human embryos was demonstrated in 99% of the 800 cases examined [136]. Moreover, a positive correlation between DNA release and embryo fragmentation on days 2 and 3 of development was found [136]. An increase of mtDNA release into the culture media was also found in correlation to advance maternal age. Such results may provide a new non-invasive model to assess embryo quality.

Despite all these technological advancements, which easily excite the human brain that constantly strives for innovation, a critical note has to be made on the usefulness of any form of embryo assessment and subsequent embryo selection. Assessment of embryo quality and selection of the best embryo for transfer was deemed highly relevant when
embryo transfer policies were implemented to decrease the rate of multiple pregnancies and cryopreservation protocols of supernumerary embryos were far from optimal. In this situation the best embryo clearly had to be transferred first. Recent developments in embryo cryopreservation however, challenged the current role of embryo selection in IVF [137]. An RCT in a selected population, that is, women with a high response to ovarian hyperstimulation showed that vitrification of all embryos followed by a transfer in a subsequent non-hyperstimulated cycle resulted in significantly improved ongoing pregnancy rates compared with a fresh transfer in a cycle with ovarian hyperstimulation [138]. In a scenario where all available embryos can be cryopreserved and transferred in subsequent cycles without impairment of embryo quality, no selection method will ever lead to improved live birth rates, as, by definition, the live birth rate per stimulated IVF cycle can never be improved when all embryos are serially transferred. The only parameter that could possibly be improved by embryo selection would be time to pregnancy, if embryos with the highest implantation potential are transferred first [137]. Despite these potential limitations for the use of assays of embryo quality in a clinical setting, investigating embryo quality at the (epi) genetic, transcriptomic, proteomic and metabolomic levels could lead to interesting observations and provide ideas on how to enhance embryo quality in general.

Another assessment of embryo quality should be performed at the level of the health of the offspring born after IVF/ICSI treatment. There are currently data suggesting that culture medium can affect intrauterine growth as early as in the second trimester as well as neonatal birthweight [27-29]. Data on the effect of prenatal undernutrition on the health of the offspring born are also striking. These data showed that men and women who were conceived during the Dutch famine during the Second World War have an increased risk for coronary heart disease, diabetes, an atherosclerotic lipid profile, obesity and breast cancer compared to people that were born before or conceived after the famine [139-143]. This risk was greater in those who were exposed to Dutch famine during early gestation compared to those exposed during mid and late gestation. Moreover, a trans generational effect was found in offspring of prenatally undernourished fathers that were heavier and more obese than offspring of fathers that had not been undernourished prenatally [144]. Even though these data do not assess the effect of undernutrition directly on preimplantation embryos the fact that greater effects were found during the first trimester might be indicative that undernutrition of the early embryo by culturing it in a suboptimal culture medium could lead to similar findings. This needs to be investigated.

**How to enhance embryo quality**

This thesis clearly shows that the choice of culture medium can improve embryo quality. Even though this is an important finding and research on culture media should continue, there is a limit on how much culture media can improve embryo quality. For example, if the embryo derives from an old oocyte that has impaired mRNA and protein pools, has acquired meiotic errors and lacks control mechanisms, the embryo quality most probably will not fully improve just by embedding it in the best culture medium.
Investigation of the molecular machinery of good and bad quality oocytes and embryos could identify the mRNA and proteins that are necessary for successful embryo development. Theoretically this could open up ways for manipulation of the oocyte and/or embryo, where important mRNA or proteins for cell-cycle checkpoints, DNA repair and chromosome cohesion that help maintain genomic integrity could be injected into the cytoplasm of oocytes and embryos to guide embryos through the first mitotic divisions. Of course, such forms of oocyte or embryo optimization should be treated with the greatest level of scrutiny and would involve a significant amount of preclinical work to assess effectiveness and safety. Even so, being able this way to ‘repair’ old oocytes would overcome the greatest threat to reproductive potential, i.e. advanced maternal age.

The same could hold for mitochondria. Mitochondrial activity is important for the developing embryo since it provides the embryo with the necessary energy [145]. Following fertilization, the embryo depends on the function of existing mitochondria that were provided by the oocyte. The number of mitochondrial DNA (mtDNA) copies in metaphase II oocytes varies from $2 \times 10^4$ to $9 \times 10^5$ [146, 147]. This variability in mtDNA copy number is thought to reflect oocyte quality, with oocytes containing higher copy numbers displaying higher fertilization rates [148, 149]. A reduction in mitochondrial copy number affects further the embryonic developmental potential [150]. Moreover, uncleaved embryos in women who were older than 40 years of age showed a significantly lower mtDNA copy number compared to uncleaved embryos of women of a younger age [151]. As the cells within an embryo divide, the total amount of mitochondria within each cell decreases due to the absence of mitochondrial synthesis until genomic activation. mtDNA replication is strictly down-regulated from the fertilized oocyte through the preimplantation embryo in those mammalian species so far investigated (mouse and porcine embryos) [152]. At the blastocyst stage, the onset of mtDNA replication is specific to the trophectodermal cells. The inner cell mass cells restrict mtDNA replication until they receive the key signals to commit to specific cell types [152]. Mitochondrial mutations and subsequent reduced energy production are increased in women of advanced age [153]. Therefore, another possibility for oocyte and/or embryo optimization will be the transfer of mitochondria from healthy young oocyte donors to support the development of embryos from older women. Such transfers have already been performed in human oocytes and led to the birth of normal healthy children [154-156]. A critical point of mitochondrial transfer is the occurrence of mitochondrial heteroplasmy in the offspring where mitochondria from young donors will co-exist with the suboptimal mitochondria of older donors. Another option that avoids the use of donor oocytes is the synthetic generation of mitochondrial DNA and its subsequent microinjection into the ooplasm. Commercial gene-synthesis services are already available from numerous companies worldwide.

The concept of oocyte or embryo optimization is still in its infancy and evidence of clinical usefulness is lacking. The idea of oocyte/embryo optimization requires manipulation of the embryo and injection of molecules (genes, mRNA or proteins) that are not intrinsically present. As such it raises important ethical issues. The technology should only be used to
avoid transmission of genetic diseases and improve embryo quality to yield higher success rates in cases of subfertility.

**Implications for clinical practice and further research**

The results described in this thesis have significant clinical relevance. We clearly demonstrate lack of evidence-based practice when it comes to selecting the medium for *in vitro* culture of human preimplantation embryos. After 35 years of IVF there is no consensus whether culture media do have an effect on IVF/ICSI success rates and what the magnitude of such an effect is. We believe that there is enough evidence to suggest an effect of culture media on pregnancy rates and that such an important part of daily IVF practice should be treated with the highest scrutiny. Further, the introduction of new culture media in the IVF laboratory should be accompanied by properly designed studies evaluating both readily available commercial media as well as individual components. When G5 culture media was compared to HTF culture medium in a prospective randomized trial, G5 culture media resulted in better embryo development, increased clinical pregnancy rate and increased ongoing pregnancy rate (although borderline non-significant). The primary outcome for this RCT was the number of women with an ongoing pregnancy after a year of treatment. Data on the number of women who achieved a live birth where still not complete at the time of the writing of this thesis. It remains therefore to be seen what the effect of the two compared culture media on live births will be. Based on the currently available data we suggested using G5 instead of HTF.

Moreover, given the described effects of culture media on neonatal birthweight [28], it is highly recommended that every study investigating the effect of treatment interventions during IVF/ICSI treatment also provides data on the follow-up of the children born. Reproductive technologies should aim not for more children alone but for more and healthier children. For the RCT presented in this thesis, follow-up data of the children born are currently being gathered and will be presented separately in the near future. The effect of culture media even later in life should be also examined.

The results of the RCT are especially relevant for the Dutch IVF centers that have been changing their culture systems from HTF to G5 over the last years. Very recently, some of these centers have moved to Quinn's advantage (SAGE) as the culture medium of choice. In the meantime, the next multi-center RCT comparing G5 to Quinn's advantage is being set up. The Dutch reproductive community is well organized via the professional organizations of embryologists and gynecologists [society for clinical embryology (KLEM) and the Dutch association for obstetrics and gynecology (NVOG), respectively], and the research consortium for studies in women's health and reproduction (ObsGyn consortium). These forums provide a unique environment to conduct good designed, multi-center RCTs with high number of participants in a cost- and time-effective way.
The data presented in this thesis also suggest that the decision as to which culture medium to use could depend on the maternal age and developmental stage of the embryos. Such suggestion could have important implications for clinical practice leading to personalized treatment but needs further investigation before it could be implemented. A sub analysis of the RCT data presented in this thesis (Chapter 7) based on maternal age can be the first step to investigate this relationship. If the direction of effect between the two culture media is different in young versus old women then a new randomized trial should be performed. The results of an RCT in a given population (e.g. women below 35 years of age with a medical indication) are often extrapolated to other populations (e.g. older women or women with unexplained infertility). Such conclusions are not substantiated and might even harm women undergoing IVF/ICSI treatments.

From a research perspective, this thesis brings forward new interesting research questions. In regards to culture media, the clinical trial provided a unique randomized source of materials, in this case supernumerary frozen embryos and placentas, that enables further research on the effect of culture media on the gene-expression profile of these embryos and placentas. Many placentas from the pregnancies derived from the RCT have been collected and the effect of GS and HTF culture media on expression, methylation, and maternal/paternal expression of specific imprinted genes will be investigated. This research is relevant as the placenta is important for proper development of the fetus in the uterus.

Supernumerary embryos that were cultured during the RCT in GS or HTF in the IVF center of Maastricht University Medical Center have also been collected to investigate the effect of culture media on embryonic gene expression. This study resembles the study described in this thesis (Chapter 5) but utilizes embryos of supposedly lower quality, but with the advantage that they have been cultured in the experimental conditions from day one onwards. Moreover, supernumerary embryos cultured in two different oxygen concentrations (20% and 5%) were collected in the IVF center of University Medical Center Groningen and they will be analyzed in a similar design. Comparing these datasets will give more insight into the regulation of embryonic transcription and could establish an overall embryonic behavior, irrespective of embryo quality or duration of culture. It could also be that differences in the results are found, indicating that good quality embryos are better equipped to culture in suboptimal conditions compared to embryos of lower quality. All of these studies are underway.

The epigenetic regulation in preimplantation embryos cultured in different media should be studied especially since this thesis suggests an environmental role on early embryo quality. For this purpose, supernumerary human preimplantation embryos that reach the blastocyst stage in different culture media can be collected to allow investigation of the effect of culture on epigenetic regulation. This effect can be studied in blastomeres of arrested or normally developed embryos and in TE and ICM samples. Such study is currently challenged by the above mentioned technical limitations. Also, analysis of single
or few blastomeres might not be representative of the entire embryo, especially since human embryos have a high rate of mosaicism as described in this thesis.

As mitotic errors were found most likely to occur during the first three cleavages, more research is required on early cleavage embryos to assess the mechanisms causing these errors. Also, the role of suboptimal environmental conditions or increased maternal age in those early developmental stages needs further investigation. For example, do culture conditions (embryo culture medium or oxygen concentration) also affect the first three cleavage divisions? If the embryos of earlier developmental stages (day 1 - 4) are more vulnerable to their environment one could argue that use of suboptimal culture conditions could aggregate the problem. Another possibility could be that embryos of women of advanced age that have already a disadvantage from compromised maternal mRNA and protein pool acquire additional disadvantage from growing in suboptimal culture conditions during these first crucial days of development before the embryonic genome gets activated. To investigate the mechanisms leading to mitotic errors during the first cleavages, supernumerary human embryos cryopreserved in the zygote stage or 3PN embryos could be analyzed for their chromosomal content using array CGH. Embryos could also be created for this kind of research, using donated gametes. However, this is currently in the Netherlands not allowed by law. If all blastomeres of a given embryo are analyzed the causative events of the aneuploidies found can be assessed (e.g. monosomies and trisomies of the same chromosome within one embryo are indicative of nondisjunction events). Similar experiments have been performed for later developmental stages mainly using FISH [157-159]. If this approach is repeated, using more advanced and accurate analysis techniques, with embryos of women of different ages and in different culture conditions, the specific mechanisms and relationships can be further investigated. Such experiments are hampered by the limited availability of human embryos. Moreover, transcription analysis of blastomeres from early cleavage embryos will shed more light into which molecular mechanisms cause these events and whether they are similar to the mechanisms found in the later developmental stages. Finally, a simultaneous analysis of the genome and transcriptome of a given cell will provide insight into the correlation of ploidy status and transcription. This is however not yet technically possible in single cells.

The data on gene expression of human preimplantation embryos presented in this thesis are a valuable addition to the few available studies investigating gene-expression changes at different developmental stages [62-65]. For the first time, data on gene-expression changes in regards to maternal age and culture conditions were described for human embryos. Given the limited availability of human preimplantation embryos, scientists working on reproductive biology have to work with a limited, heterogeneous embryo population that does not always allow matching for maternal age, fertilization method, developmental stage and culture conditions. This heterogeneity is difficult to avoid, but our results showed that strong interactions exist among these factors, stressing the importance of correction for confounders in such analyses. With this in mind, the findings of previous studies evaluating gene expression of human preimplantation embryos should
be re-assessed. Studies on single embryos are in this way different from studies analyzing expression changes in an experimental setting in, for example, cell lines, where all other factors can be accounted and controlled for.

Our data on gene-expression changes in regards to maternal age and culture conditions are suggestive of new and interesting questions for further research. For example, what are the specific transcripts and proteins that are necessary for proper embryo development? Should we provide oocytes/embryos from older women with these proteins to improve IVF/ICSI success rates? Would that really result in improved success rates? Should the intervention target the oocyte, the embryo or both? In regards to culture media, is there a correlation between the components of the media and gene expression? If this correlation exists, could we drive gene expression just by altering the composition of the culture medium? What culture conditions are better for women of older ages? Further, since culture media affect apoptosis, what is the effect of culture media in number of cells in the two cell lineages at the blastocyst level? Would changes in cell number affect the proper formation of these lineages? And how do culture media affect cell fate gene expression and cell lineage allocation?

To answer these questions, the specific genes affected per maternal age and culture conditions should be further investigated. The transcriptome data that are published on human embryos of different developmental stages could be re-analyzed in regards to maternal age and/or culture conditions to identify genes that are commonly found affected irrespective of study population (women of different ethnic backgrounds and subfertility indication), materials (supernumerary embryos, 3PN embryos, zygotes, good quality embryos) and techniques applied (microarrays and microarray platforms and sequencing). When a shorter list of candidate genes is identified, further in depth experiments can be performed on interesting genes and pathways. Such experiments could involve knockout or over expression studies using animal models or perhaps even human embryo models (as long as it does not involve transfer to the uterus) with outcomes such as embryo quality, embryo development, and for the experiments with animal models implantation and generation of healthy offspring. Alternatively, mRNA for the candidate genes could be microinjected into the cytoplasm of oocytes or early zygotes from women of advanced age to check whether the resulting embryo will develop similarly to embryos of younger women (for example in regards to the number of mitotic errors) especially during the first days of development. Moreover, human embryos can be cultured using culture media other than HTF and G5, different pH values of the culture media and different temperatures of the incubator. At the end of the culture, PCR analysis of the candidate genes could help in deciding the general conditions that are best for embryo culture.

Eventually, by combining the best culture conditions and the right interventions to ‘correct’ or ‘avoid’ the effects of maternal age on oocyte and embryo competence, better success rates during IVF/ICSI could be obtained.
References


112. Rijnders PM, Jansen CA. The predictive value of day 3 embryo morphology regarding blastocyst formation, pregnancy and implantation rate after day 5 transfer following in-vitro fertilization or intracytoplasmic sperm injection. Hum Reprod 1998; 13:2869-2873.


