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The effect of growth factor supplemented ART culture media on whole mouse embryo development

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III Abbreviations

ART	Assisted reproductive technology
CDX2	Caudal type homeobox 2
CO_2	Carbon dioxide
CSF2	Colony stimulating factor 2
DIR	Deutsches IVF Registe
E	Embryonic day
Epi	Epiblast
ET	Embryo transfer
Et al.	*et alii*
GM-CSF	Granulocyte macrophage colony stimulating factor
h	Hour
hCG	Human chorionic gonadotropin
HSA	Human serum albumin
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IU	International units
IUI	Intrauterine insemination
IVC	*in vitro* culture
IVF	*in vitro* fertilization
KSOM(aa)	potassium simplex optimized medium supplemented with amino acids
LIF	Leukemia inhibitor factor
MEA	Mouse embryo assay
mL	Milliliter

mm	Millimeter
NANOG	Homeobox protein NANOG
ng	Nanogram
RNA	Ribonucleic acid
PBS	Phosphate buffered saline
PMSG	Pregnant Mare Serum Gonadotropin
PrE	Primitive endoderm
SOX17	SRY (sex determining region Y)-box 17
TE	Trophectoderm
mRNA	Messenger ribonucleic acid
tRNA	Transcriptome ribonucleic acid

1 Introduction

1.1 The need of assisted reproduction

Since the last decades, unwanted childlessness became an important topic in western countries and different reasons apart from sub- and infertility led to this trend. The postponed family planning is strengthened by the perceived incompatibility of a successful career and managing a family. The accessibility to contraceptives and cultural changes also led to an increased age in men and women before they decide to have children. On the female side, the increasing age leads to a significant decrease in the chance of becoming pregnant naturally and also reduces the effectiveness of possible treatments to overcome sub- and infertility (Dunson *et al.*, 2004). Germany shows huge differences between socials classes. The better the education, the more urban the residence and the younger the women, the higher is the rate of childlessness. Especially women that follow an academic career show a high rate of childlessness (Bujard and Kohorte, 2015). But, numbers also indicate that the fertility and birth rate are increasing in Germany in the last couple of years. This trend may trace back to the better support of childcare which on the other hand supports better the compatibility of career and family (Luci-Greulich and Thévenon, 2013). Still, more couples make use of assisted reproduction every year. This is of course not only due to increasing rates of sub- and infertility or the later wish for a child, but probably because of the higher acceptance receiving help by the reproductive medicine.

1.2 IVF-register and assisted reproductive technologies

In Germany, all *in vitro* fertilizations (IVF) and intracytoplasmic sperm injections (ICSI) procedures are recorded and published annually in the yearbook of the German IVF register (D.I.R.). For example, the records show steadily increasing numbers of artificially initiated cycles (1990: 8,653, 2013: 83,433, 2017: 105,049) or numbers of follicular punctures (1990: 7,343, 2013: 56,092, 2017: 63,321). It also proves the steady average increase of the age of men and women using the help of assisted reproduction (comparison of 1998 to 2017: female: 32.8 to 35.7 years, male: 35.2 to 38.8 years). Since the beginning of the records (1997), 275,452 children have been born using assisted reproduction in Germany. In both, 2015 and 2016 there were over 20,000 children born per year, compared to only 10,116 in 1999 (Deutsches IVF-Register (D·I·R) e. V., 2017). Methods and processes used in reproductive medicine are also known as assisted reproduction technologies (ART). ART includes 1) Intrauterine Insemination (IUI): Purified

ejaculate is transferred by a catheter into the uterus and is intended to fertilize the egg naturally. 2) IVF: Egg and sperm are collected and placed and cultured together in a petri dish. The formed embryo is then transferred back to the uterus. 3) ICSI: A glass pipette is used to inject a selected sperm directly into the egg. The developing embryo is cultured in the laboratory and then transferred back into the uterus. In both IVF and ICSI, embryos are kept artificially in culture media.

1.3 *In vitro* culture

Since more than four decades fertilization and early embryo development can also take place artificially in a petri dish (Steptoe and Edwards, 1978). This means that eggs, sperm, and embryos are handled outside of their natural environment in an artificial environment for a period of time that ranges from hours to days. The environment should be as similar as possible to the natural surrounding (nature) and should provide all factors the embryo requires for healthy development (nurture). All kind of culture media are supposed to offer this near-natural environment, but still, the content and concentration of nutrients are not similar to the natural environment. The first attempts in developing optimized media for *in vitro* culture of mammalian embryos were made in the 1950s and 1960s (Whitten, 1956; Brinster, 1963). Today numerous single step or sequential culture media are available for IVC for pre-implantation stage human embryos. One important factor in embryo culture is the composition of the culture medium. There are two types of embryo culture systems. One type of culture system is the single step culture, where the embryo is cultured in one medium, which supposedly contains every necessary component for the whole pre-implantation phase of development. Together with modern imaging techniques, which allow reliable monitoring, the embryos may be even cultured untouched until they are transferred back into the uterus. The alternative to single step culture media is a sequential culture protocol, where two media are used one after the other. The first medium contains components for the first two to three days of pre-implantation development. As the embryo requirements change over time, the first medium is switched to the second medium which then contains other or additional substances which supports pre-implantation development until day 5. Combined together, the two media are intended to mimic the changing composition of the natural environment of the female genital tract, in which the embryo would migrate from the oviduct to the uterus. In the past, *in vitro* culture media were produced in house, but today the production is commercialized and strict manufacturing and quality assessments are required before they are released to the market. This aspect led to a decreased transparency regarding content, composition, and concentration of the

ingredients of ART media. Although these media are used in a clinical setting, possibly influencing human life in the first days of formation, it is not necessary to be approved by a clinical license, showing that normal embryo development is not affected. Reliability is rather approved by animal studies or clinical trials after the media are already in clinical use. To optimize ART media, high numbers of animal embryos are used in experiments as it is not possible to achieve these numbers with human embryos; additionally also ethical aspects should be considered in this respect. Furthermore, many different factors from the parental side may play a role in the individual success of IVC and embryo quality. For this reason, it is even more important to perform basic research elucidating the influences of ART media on embryo development and to be conscientious when changing media composition or adding new factors to them (Chronopoulou and Harper, 2015). The choice of a culture medium may affect birthweight and long term development of ART children, this has been systematically investigated through consecutive studies in the same cohort of ART children (Dumoulin *et al.*, 2010; Kleijkers *et al.*, 2014; Zandstra *et al.*, 2018). To further improve the quality of embryo culture media and to mimic *in vivo* nutrition, growth factors became attractive candidates for medium supplementation. Growth factors play an important and beneficial role in embryo culture, they can improve blastocyst rates and increase cell numbers (Chronopoulou and Harper, 2015), which might in turn have a benefit for implantation and development.

1.4 Granulocyte-macrophage colony-stimulating factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF), also known as colony-stimulating factor 2 (CSF2), is a glycoprotein secreted by macrophages, T cells, endothelial cells, and fibroblast. The crystal structure of GM-CSF was solved in the mid-1990s (Rozwarski *et al.*, 1996) and it was found that it binds and signals through a heterodimeric cell-surface receptor consisting out of an α-subunit and a dimeric βc-subunit (Stomski *et al.*, 1996). Its main function is the stimulation of stem cells to produce granulocytes and monocytes (Metcalf, 2009), but it is also expressed by epithelial cells of the endometrium under the regulation of estrogens and acts as an embryokine, which supports embryonic development (Hansen *et al.*, 2014). Besides GM-CSF there are other growth factors, like insulin-like growth factor (IGFs) or leukemia inhibitor factor (LIF) which are found in the reproductive tract, where they are involved in the maternal-fetal interface, ovulation, embryo development, embryo implantation and placental growth (Kaye and Harvey, 1995; Robertson *et al.*, 1996; Rahmati *et al.*, 2015). A number of studies reported how GM-CSF influences cell numbers in the trophectoderm or the inner cell mass, depending which animal model was used for the study (Sjoblom, 2002;

Loureiro *et al.*, 2009; Kwak *et al.*, 2012). In human it was shown that embryos cultured in medium containing GM-CSF (2 ng/mL) had an increased number of viable ICM cells and a reduced rate of apoptosis in the TE and the ICM (Sjoblom, 2002) and that GM-CSF affects placental growth, implantation rate and viability of progeny in mice (Sjöblom et al., 2005). High concentrations of GM-CSF (>5 ng/mL) have an impact on blastulation rate (Elaimi et al., 2012). Only a few studies exist, which show the influence of GM-CSF on the clinical outcome. These studies have shown that GM-CSF may be able to improve pregnancy rates in human ART, especially in women who experienced miscarriages before (Zhou et al., 2016; Ziebe et al., 2013).

1.5 Mouse model for experimental embryology

For ethical and legal reasons, it is not possible to use human embryos for research in Germany, which is prohibited by the German law for the protection of the human embryo. Therefore, it is necessary to make use of an animal model. For experimental embryology, there are different animal models possible, such as cattle, pigs and mice. Interpretation of data from mice to human embryos and to the human system must still be done carefully, as results are not transferable on a one-to-one basis. However, mice are easier to maintain in an animal facility, experiments are easier to conduct compared to larger animals and embryos develop *in vitro* in simple and defined media, making mice the preferred model for our study. Despite differences between human and mice (short gestation and large litter sizes), there are also advantages using the mouse model, like low aneuploidy rate in eggs (Pan *et al.*, 2013), similar implantation processes to human (Wang and Dey, 2006) or the use of mice in the ART media toxicity test (Mouse Embryo Assay, MEA). Regarding the application of ART methods, especially of IVC, human and mice are processed almost identical.

1.6 Mouse embryo assay

In IVF it is necessary to work with sterile material and a non-toxic culture medium, in order to safeguard the healthy development of the embryo *in vitro*. Nevertheless, out of ethical reasons, it is not possible to optimize culture media or to test the material directly on human embryos. Therefore, the mouse is used as the pre-eminent experimental model of mammalian embryogenesis and it also serves in the form of the so-called mouse embryo assay (MEA) as quality control in ART. Culture media need to be tested for standard parameters such as pH, osmolality and endotoxin content, but they also have to be tested biologically for toxicity and sterility (Ackerman *et al.*, 1984). In the MEA, one- or two-cell stage mouse embryos are collected from mouse oviducts after mating

and cultured in the test medium until they develop to blastocysts. In order for the approval of a medium, 70-80% of the embryos have to reach the blastocyst stage, in a time that ranges typically from 4 to 7 days. The MEA only provides information about the pre-implantation phase of development and IVC conditions, but gives no information about the post-implantation development, pregnancy rates, and change in gene expression or epigenetic pattern. Concerns exist whether this method is sufficient or sensitive enough to detect really all the effects which an artificial environment may have on the pre-implantation embryo. It was demonstrated in mice that performing functional analysis in addition to morphological analysis, this may improve the sensitivity to detect toxic substances in ART media (Gilbert *et al.*, 2016).

1.7 Embryo development in mouse

Although the prerequisites for embryogenesis are laid down during oogenesis, embryogenesis formally starts with the zygote resulting from the fusion of sperm and egg in the upper part of the female genital tract (oviduct). The zygote (embryonic day 0.5, E0.5) is the first stage of embryo development (Figure 1). The zygote carries one pronucleus from the male and one from the female, two polar bodies and is surrounded by a glycoprotein layer called *zona pellucida*. The zygote contains everything that is necessary for the first cell cycles, e.g. ribosomes, tRNA, mRNA, and proteins. The process of embryo development proceeds by cleavage, whereby the volume of the zygote is divided into smaller cells (blastomeres) through mitotic cell divisions, without changing the total volume and the biomass of the embryo. Protein biosynthesis in the zygote is limited by the amount of maternal mRNA inherited via the egg (Bachvarova, 1985). Soon after the first cleavage, the embryo starts using its own genome for transcription. At E2.0 the mouse embryo reaches the four-cell stage and at E2.5 the eight-cell stage. Further divisions increase the cell number and on E2.5-3.0 the embryo reaches the morula stage. Now the blastomeres undergo their first cell lineage decision; either they form the external part of oligopotent trophectoderm (TE) cells of the later pre-implantation embryo from which the extra-embryonic tissues or annexes arise, or they form the inner part of pluripotent inner cell mass (ICM) cells resulting in the embryonic lineage from which the later fetus arises (Boiani and Schöler, 2005; Wobus and Boheler, 2005). The differences between ICM and TE are based on distinct gene expression pattern with different sets of genes; in particular, the gene *Oct4* is the best-known marker of the ICM while *Cdx2* is the adequate marker for TE (Strumpf *et al.*, 2005). A second cell lineage decision follows when the blastocyst is ready to implant into the uterine endometrium. This second lineage decision separates the ICM into the primitive

ectoderm (marker *Sox17*) and the epiblast (marker *Nanog*) at E4.5 (Boiani and Schöler, 2005; Zernicka-Goetz *et al.*, 2009). It is believed, that this early lineage decision can be influenced by extrinsic factors like culture media or media supplements. Thus, it was already shown that human ART culture media influences early embryo development and prepare embryos differently for post-implantation development (Schwarzer *et al.*, 2012), whereby no developmental effects were detectable after successful implantation in the mouse fetus (Hemkemeyer *et al.*, 2014).

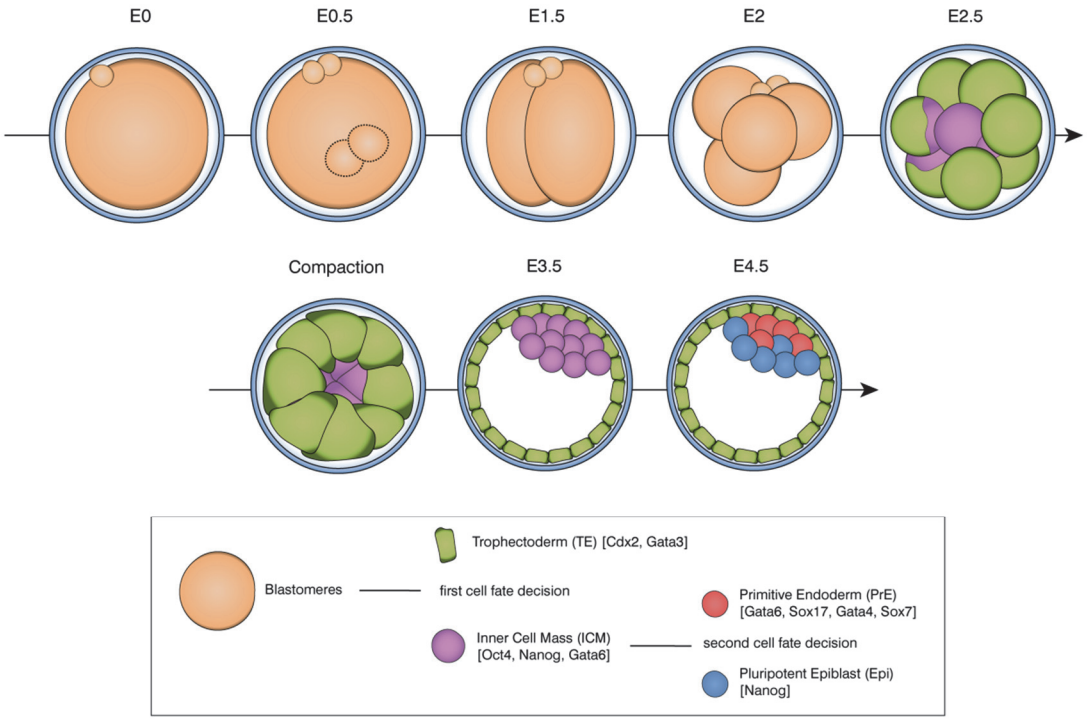

Figure 1 Early mouse embryo development. The zygote (E0.5) undergoes several rounds of cleavages: Two-cell stage (E1.5), four-cell stage (E2.0) and eight-cell stage (E2.5) until it becomes a morula. After the eight-cell stage, the first cell fate decision takes place in the embryo and two types of cells are generated, the trophectoderm (TE), which will later create the extra-embryonic tissue and the inner cell mass (ICM). From E3.5 to E4.0 the second cell fate decision takes place in the embryo and the cells of the ICM either becomes the embryonic epiblast (Epi) or the primitive endoderm (PrE), the latter forming the yolk sac. Totipotent cells are shown in orange, trophectoderm cells in green, ICM in purple, Epi-cells in red and PrE-cells in blue. Adapted from Zernicka-Goetz *et al.* 2009.

1.8 Cell fate decision

After the morula stage, the embryo starts to form a cavity (E3.5) and the inner cells are pushed to one side of the embryo. At this point two different cell types or cell lineages are visible. The inner cells now termed the inner cell mass (ICM) and the surrounding layer of epithelialized trophoblast cells (trophectoderm). In the second cell fate decision, the ICM will give rise to the epiblast and the primitive endoderm (hypoblast). The TE will later

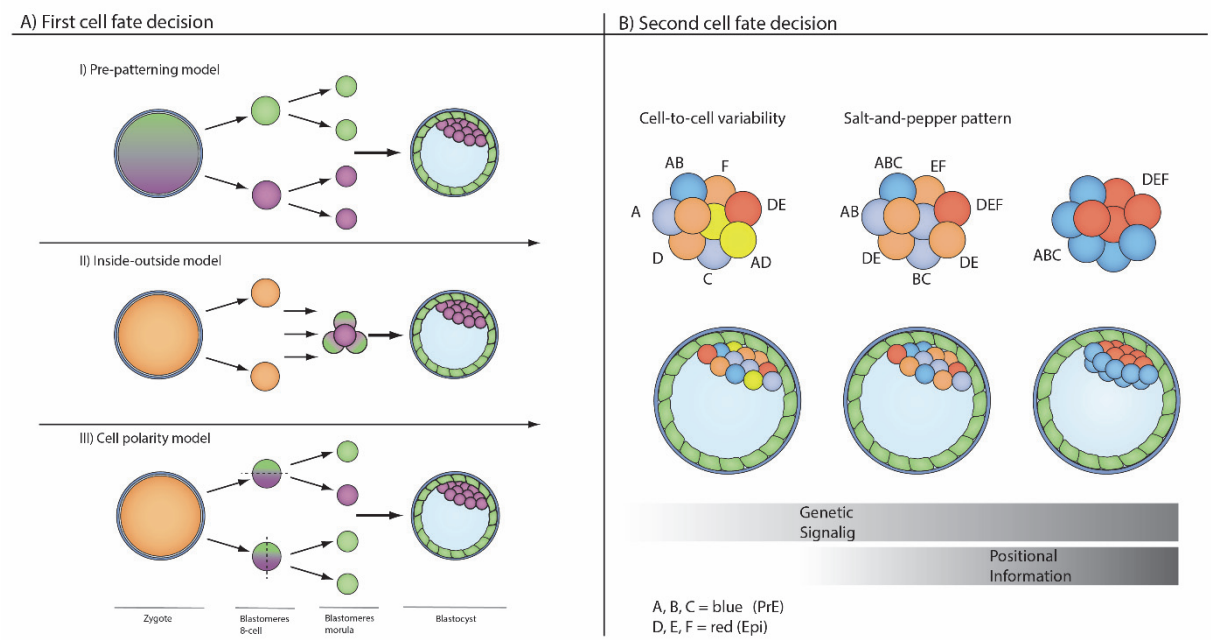

Figure 2 First and second cell fate decision represented as a rough simplification of the classical lineage segregation. A-I) The pre-patterning model proposes that already the egg and zygote is defined by asymmetrically localized molecules. During cleavages, these molecules are segregated between daughter cells and determine cell fate. A-II) The inside-outside model proposes that localization of blastomeres induces cell fate. Whereby inner cells develop to inner cell mass and outer cells develop to trophectoderm cells. A-III) The cell polarity model proposes that trophectoderm cell fate is induced by cleavages of polarized blastomeres. If a blastomere contains an apical polarization and undergoes an asymmetrical cleavage a trophectoderm and an inner cell mass cell is generated. After symmetrical cleavage two trophectoderm cells are generated. B) The second cell fate decision starts with high genetically cell-to-cell variability (genes are represented as A, B, C for PrE and D, E, F for Epi). Cell-to-cell interactions, signaling activities, and feedback mechanisms form a salt-and-pepper pattern, which creates two populations of cells. Gene regulatory networks and positional information may lead to two cell lineages. Undifferentiated cells are shown in orange, trophectoderm cells in green and inner cell mass cells in purple. In the second cell fate decision, unspecific ICM cells are shown in orange, grey, light blue, and light orange. Primitive endoderm cells are shown in blue and epiblast cells are shown in red. Adapted from Zernicka-Goetz et al. 2009 and Wennekamp *et al.* 2013.

contribute to the placenta (Copp, 1978; Dyce *et al.*, 1987). Three classical models have been proposed to explain the first two cell lineages during early embryo development: The pre-patterning model, the inside-outside model and the cell polarity model (Figure 2A). The pre-patterning model is based on an asymmetric patterning of molecular determinants in the egg and the fertilized zygote. These determinants are spread differently in newly developed daughter blastomeres (Dalcq 1957). Until today no asymmetric localized determinant was identified as the decisive factor in the egg or zygote, but in mouse the Hippo pathway is identified as one important regulative factor of polarization (Niakan and Eggan, 2013). However, studies showed that every single blastomere from the 2-cell, 4-cell, and 8-cell stage is able to form an individual blastocyst; however, this is incompatible with the pre-patterning model and suggests a more flexible and dynamic process involved in cell fate decisions. The inside-outside model is based on the presumption that the position of a blastomere in the morula determines its cell fate (Tarkowski and Wróblewska 1967). On the contrary, it was found that there is a molecular heterogeneity between blastomeres in equivalent positions, which is incompatible with the inside-outside model. The last model, the cell polarity model is supported by findings showing that apical membrane domains are distributed specifically on certain cell organelles and are known to play a role in cell polarity. During cell division, these domains are shared among two daughter cells, or only to one daughter cell, which then leads to a conservative or differentiative division (Johnson and Ziomek 1981). Since none of these models is able to explain the asymmetry in early embryos alone, all three were combined and a modified model was created, in which the embryo is a self-organizing system where all these factors play an essential role (Wennekamp et al. 2013). Lineage specification is regulated by complex gene cascades and actions of transcription factors. For trophectoderm specification, prominent examples are Cdx2, Id2, Elf5, and Gata2/3. All blastomeres start with a similar level of CDX2, but later CDX2 has to be upregulated in the TE cells, while CDX2 and other factors in the ICM have to be downregulated. CDX2 is an important factor in the first cell-lineage decision, where asymmetric localization of the CDX2 protein in the cell leads to cell polarization and is responsible together with other transcription factors (e.g. OCT4, NANOG, YAP, TEAD4) for cell differentiation into ICM and TE. NANOG is part of the regulatory network supporting embryonic stem cells, including other factors like OCT4(POU5f1), SOX2, SALL4, KLF2, KLF4, ESRRB, GBX2, and TFCP2L1 and plays an important role in the establishment and maintenance of the ICM and epiblast lineage. In the ICM epiblast and primitive endoderm markers are co-expressed until blastocysts formation takes place during development. Then, expression of cell fate markers occur in a “salt and pepper” pattern which randomly directs cells in

either the epiblast or the primitive endoderm (Figure 2B). Prominent examples are SOX17, GATA4 and SOX7. All differentiation steps are supported and mediated by feedback loops of expression levels and also other factors like FGF4, which plays a role in cell-cell interactions (Pfeffer, 2018). For understanding the processes of early pre-implantation development single-cell analyses became an important topic, as the classical gene expression analysis of the whole blastocyst has the limitation of heterogeneity across the different cells and their increasing specification with ongoing embryo development. These methods are able to confirm and expand findings regarding key marker genes such as OCT4, NANOG, GATA4 and GATA6 and their influence on embryo development. Additionally, these methods allow a temporal observation during differentiation of blastomeres (Durruthy-Durruthy *et al.*, 2016; Petropoulos *et al.*, 2016).

1.9 Aims and hypothesis of the study

In vitro culture should mimic the natural situation and should support embryo development as good as possible. In order to optimize media composition, companies are trying to supplement these media with supposedly supporting factors, like the growth factor GM-CSF. However, the physiological influence on embryo development, fetal development and its possible effect later in life are still not fully understood.

In this study, we aimed to investigate, if the supplementation of GM-CSF in a human ART medium or in a mouse optimized medium leads to a change in cell number and cell lineages in the early pre-implantation mouse embryo.

Thus we propose two working hypotheses:

Hypothesis 1

Mouse embryos cultured in growth factor supplemented media show in comparison to embryos cultured without growth factor an increased overall cell number and a different pattern in cell composition in the blastocysts stage.

Hypothesis 2

Transferred embryos cultured in growth factor supplemented media show in comparison to embryos cultured without growth factor different implantation and fetal rates after embryo transfer.

Aims

1) Analyze the effect of growth factor supplemented medium on pre-implantation mouse embryos by morphometric and cell lineage analysis.

2) Analyze the effect of growth factor supplemented ART medium on implantation and development of mouse fetuses by morphometric and histological analysis.

2 Methods

2.1 Mice

Different strains of mice were used for different experimental approaches (Table 1). C3H male mice and C57Bl/6 mice were mated to generate B6C3F1/N hybrid mice. Female offspring of these hybrids were used as a source of *in-vivo*-fertilized zygotes after mating to C57Bl/6 males. For embryo transfer experiments CD1 female mice were mated to vasectomized CD1 males to produce foster mothers. Mice were kept in the institutional breeding facility (Zentrale Tierexperimentelle Einrichtung).

Table 1 Mouse strains, experimental use and numbers of used animals.

Mouse strain	Experimental use	Company / Supplier
C57Bl/6 female	Mating with C3H male to produce B6C3F1 offspring	CR
C3H male	Mating with C57Bl/6 female to produce B6C3F1 offspring	CR
B6C3F1 female	C57Bl/6 x C3H offspring for egg production	Colony
C57Bl/6 male	Mating with B6C3F1 females for fertilization of eggs	ZTE
CD1 female	Foster mothers for embryo transfers	ZTE
CD1 male	Mating with B6C3F1 females for fertilization	ZTE

All mice were kept in the institutional breeding facility and housed in individually ventilated cages under a 12/12 hours light/dark cycle with food pellets (Altromin, Lage, Germany) and water ad libitum. Housing and exercise conditions were identical for all animals. Experimental procedures were performed in compliance with the German Federal Law on the Care and Use of Laboratory Animals (LANUV NRW, animal license number: 84-

02.04.2016.A255 and according to Federation of European Laboratory Animal Science Associations (FELASA) recommendations.

2.2 Superovulation & recovery of zygotes

Collection of zygotes, embryo culture and immunohistological analysis were conducted with minor modifications as previously described (Balbach *et al.*, 2010; Schwarzer *et al.*, 2012; Schulte *et al.*, 2015). Female C57Bl/6 mice were mated to C3H males to generate B6C3F1/N offspring for the experimental approaches. Male offspring were eliminated 4 weeks after birth. Six to ten weeks old female offspring were superovulated by injection of 5 IU PMSG followed by a single injection of 10 IU hCG 48 hours later. Females were mated to males from the C57BL/6J strain immediately after hCG injection. Both hormones were injected intraperitoneally with a 27 gauge needle. Eggs and zygotes were collected 17 to 18 hours after hCG injection. Female mice were sacrificed by cervical dislocation and the abdominal cavity was opened. The ovaries were separated from the uterus and collected in a petri dish containing HEPES-buffered M2 medium at 37°C. Eggs and zygotes were located in the upper part of the oviduct, the oviductal ampulla. After superovulation the ampulla is swollen and can therefore be easily identified under the stereomicroscope. Ovaries were transferred to a new dish containing M2 medium supplemented with hyaluronidase (10mg/mL). Ovaries were hold in position with watchmaker's forceps and ampulla was torn that cumulus-egg-complexes (COCs) were released to the medium. Ovaries were discarded to the trash afterwards. COCs were incubated in the hyaluronidase solution for 1 to 2 minutes at 37°C until the cumulus cells loosened up. By pipetting the eggs/zygotes up and down with a fine drawn glass pipette, remaining cumulus cells were removed. Eggs/zygotes were washed several times in M2 medium before transferred to embryo culture medium, which was equilibrated at 37°C and 5.5% CO_2 overnight. As an *in vivo* control mice were kept after mating until E3.5 to collect embryos before they transplant into the uterus.

2.3 Embryo culture & media

Eggs and zygotes from different mice were pooled after collection and were randomly distributed to pre-selected media. 30 to 50 eggs/zygotes were transferred to one well in a 4-well plate containing 500µL medium and were incubated at 37°C and 5.5% CO_2. Embryos were cultured for a total of 96 hours, until they reached the blastocysts stage (E4.5) and with a medium refreshment step at E2.5.

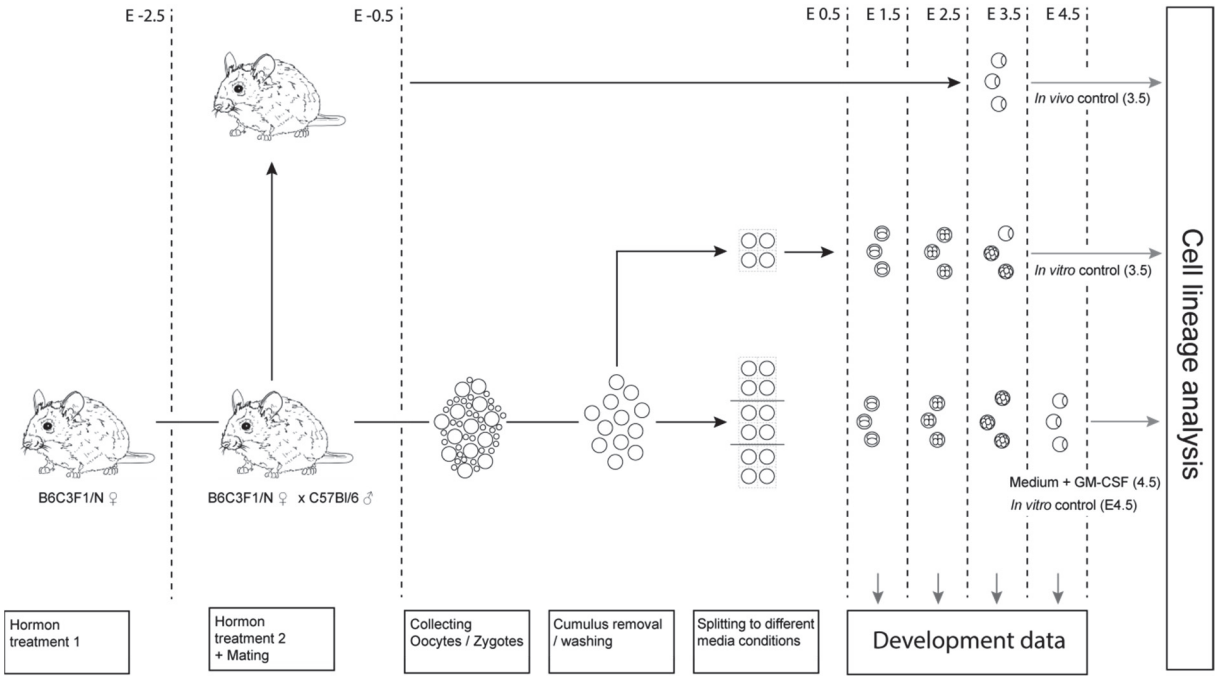

Figure 3 Overview of an embryo culture experiment. Six to ten weeks old female offspring were superovulated by injection of 5 IU PMSG followed by a single injection of 10 IU hCG 48 hours later. Females were mated to males from the C57BL/6J strain immediately after hCG injection. Eggs and zygotes were collected 17 to 18 hours after hCG injection (E.05). The ovary were separated from the uterus and collected in a petri dish at 37°C. Cumulus-eggs (COCs) were released to the medium. COCs were incubated in the hyaluronidase solution until the cumulus cells loosened up. Eggs/zygotes were transferred to embryo culture medium. As an *in vivo* control mice were kept after mating until E3.5 to collect embryos before they transplant into the uterus. Eggs and zygotes from different mice were pooled after collection and were randomly distributed to pre-selected media until E2.5, where medium was refreshed and embryos were cultured further until E4.5. Afterwards blastocysts were used for cell lineage analysis by immunohistochemical staining.

For *in vivo* control, fertilized eggs were not retrieved at E0.5, but instead were allowed to develop to blastocysts *in vivo* until E3.5 before they were flushed from the uterus and are processed in an immunohistochemical staining. After culture in different conditions all other embryos were collected and subjected to immunohistochemical staining. As *in vitro* control an optimized mouse medium, KSOM(aa), was prepared at the Max Planck Institute of Münster by PD Dr. Michele Boiani according to the original recipe (Lawitts and Biggers, 1991). Prior to use, the medium was supplemented with HSA (2 mg/mL), penicillin and streptomycin. At E2.5 4-cell stage embryos were collected and transferred to fresh medium until E4.5. At E4.5 blastocysts were counted for the embryo development rate. Afterwards blastocysts either were fixed for immunohistochemistry or were directly used for embryo transfer experiments.

2.4 Transfer of embryos to pseudo pregnant recipient mice

For the transfer of E4.5 embryos, female CD-1 mice were mated to vasectomized males of the same strain one day before. On the day of transfer only females presenting a vaginal plug were chosen in order to ensure pseudo pregnancy and the receptivity of the oviduct. E4.5 blastocysts from *in vitro* culture were transferred into the uterus of pseudo-pregnant CD1 recipients that had been paired with vasectomized CD1 male mice three days prior and had vaginal plug on the next day. Recipient mice were treated with analgesic (Alvegesic vet. 10 mg/mL) 30 minutes before they were anesthetized with isoflurane. After control for deep anesthesia the mouse was put ventral on a heating plate (37 °C). The skin was disinfected and cut dorsally at the level below the last rib. The reproductive tract was visible through the body wall and a small incision was made right over the ovary and the fat pad. The reproductive tract was pulled out and a serrafine clamp was clipped onto the fat-pad so that the uterus were exposed and extracted surgically after a small incision on the back of the mouse (skin and peritoneum). The embryos were then transferred directly into the uterus by piercing through the outer layer of the uterus close to the oviduct site. The uterus was returned to its natural location with tweezers and the incision was sutured using surgical staples and clips. Embryos were always transferred into the left uterus site, assuming that the side of genital tract does not influence the outcome of implantation. A maximum of eight blastocysts were transferred per CD1 recipient. It was shown that the number of embryos injected per oviduct did not significantly affect the percentage developing into fetuses (McLaren, 1970). The reproductive tract was carefully placed back into the abdomen and the body wall was sutured. Finally the skin was clipped and the mouse was placed in a fresh cage on a heating plate (37 °C) and covered with paper towels until it recovered from anesthesia. Mice were treated with a second analgesic afterwards (Rimadyl 50 mg/mL). The fetal rate was scored at E13.5 for all conditions. Mice were sacrificed by cervical dislocation and fetuses were retrieved by section of the uterus. Mice that had been subjected to ET but were found not to be pregnant were excluded from the fetal rate, as this outcome may be extrinsic to the embryos (e.g. technical problem of ET or unsuccessful induction of pseudo pregnancy in the recipient mouse). Implantation sites without fetal content did not count for the fetal rates but were included in the overall implantation rate.

2.5 Immunohistochemical staining

E4.5 blastocysts were fixed in 1.5 % paraformaldehyde in PBS, following permeabilization in 0.1% Triton X-100, PBS and H_2O each for 15 minutes at room temperature (RT). Embryos were then put in blocking buffer to block unspecific binding sites (0.1%Tween in 1xPBS, 2% BSA, 2% glycine, 5 % donkey serum) for 2h at 4°C. After blocking the embryos were transferred to Tyrode's acidic solution to remove the *Zona pellucida* for 20 to 30 seconds at RT. Blastocysts were transferred to fresh blocking buffer and incubated up to a maximum of 3 days until the antibody staining was performed. Embryos were stained with primary antibodies (anti-CDX2 mouse IgG, abcam; anti-SOX17 goat IgG, R&D Systems; anti-Nanog rabbit IgG, Cosmo Bio Co.) in antibody solution (0.5% BSA, 0.5% glycine, 1.25% donkey serum, 0.1% Tween-20 in 1x PBS). Embryos were incubated in a dilution of the secondary antibodies anti-rabbit, -goat, -mouse IgG donkey IgG, conjugated with Alexa Fluor 488, 568, 647, respectively (dilution 1:2000) in antibody buffer for 1h at RT. After washing in wash buffer for 10 min at RT, embryos were mounted in microdrops of PBS covered with mineral oil on a thin bottom glass dish. Blastocysts were analyzed using a fluorescence microscopy to identify cell lineages. Markers of the three cell lineages trophectoderm (CDX2), primitive endoderm (SOX17) and epiblast (NANOG) enabled selective scoring of absolute cell numbers using Fiji (Schindelin *et al.*, 2012).

2.6 Image Analysis

Acquired pictures were stacked and combined to a max intensity Z-projection using Fiji. Number of stained cell nuclei in the three different channels (emission wavelengths: 488 for CDX2, 568 for SOX17 and 647 for NANOG) were counted using the cell counter analysis plugin offered by Fiji.

2.7 Statistical analysis

Absolute numbers of the different cell lineages were analyzed by one-way ANOVA followed by Bonferroni post-hoc test. Significant differences are marked with * for $P<0.05$, ** for $P<0.01$ and *** for $P<0.001$. Data were either presented as box plots showing median and quartiles and the whiskers extend for 1.5x the inter quartile distance, or only mean values were plotted for direct comparison of different experimental groups.

2.8 Project design

Basis of our project is built by a set of experiments, in which zygotes were allocated into different experimental groups: 1) ART media with and without GM-CSF 2) KSOM(aa) with different concentrations and without mouse GM-CSF (mGM-CSF) 3) KSOM(aa) with different concentrations and without human GM-CSF (hGM-CSF). Blastocysts at E4.5 were analyzed regarding cell lineage composition by immunohistochemical staining. In the ART experiment a day 3.5 *in vivo* and *in vitro* control group was conducted but not integrated into the statistical analysis due to the different developmental stages. Blastocysts cultured in mGM-CSF were also used to perform embryo transfer experiments (Figure 4). Further experiments shown in this study are controls to exclude mouse strain or laboratory specific findings.

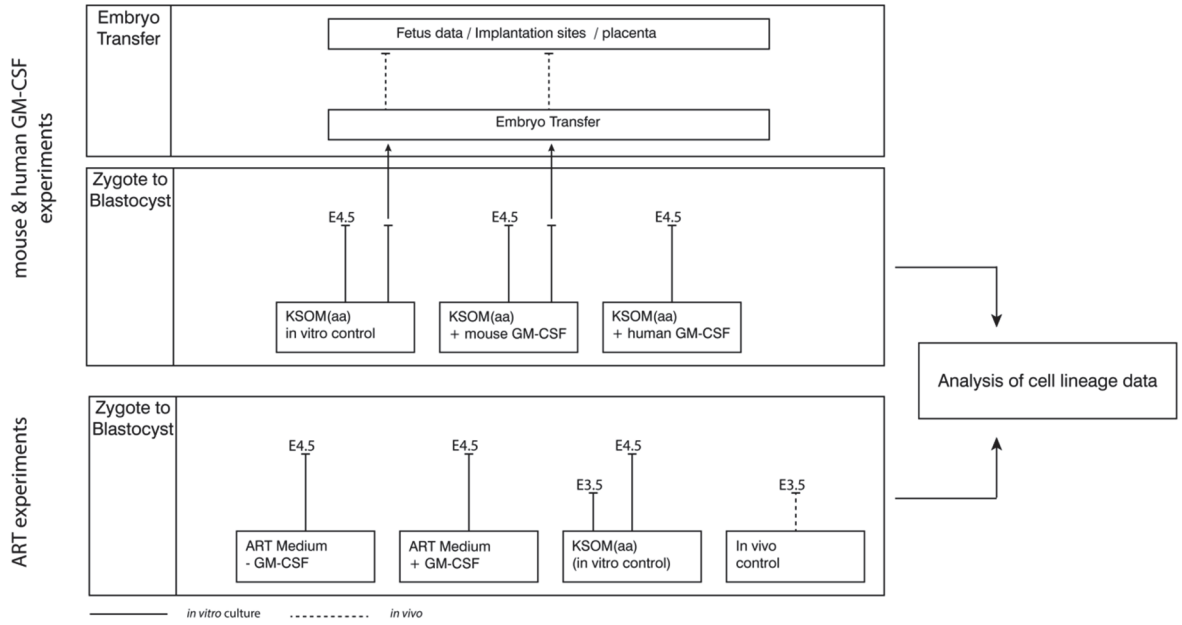

Figure 4 Study design for culture media experiments. ART experiments were conducted with media with and without GM-CSF and the in vitro control medium KSOM(aa). Additionally, an vivo control was generated. Afterwards culture experiments were repeated with different concentrations of mouse and human GM-CSF supplemented in KSOM(aa). Embryos cultured in KSOM(aa)+mGM-CSF were also used for embryo transfer experiments. All embryos collected in the culture experiments were fixed at E4.5 and were stained by immunohistochemical staining to analyze cell lineage composition.

3 Results

The aim of this thesis was to assess the effect of the growth factor supplemented ART medium on pre-implantation mouse embryo development. For a better understanding and to expand the range of new findings during the course of the thesis the aim had to be extended. The extension aimed at investigating the effect of different concentrations of the growth factor, supplemented into an optimized mouse embryo culture medium. To address these aims it was necessary to use mice as an animal model to produce fertilized zygotes (zygotes) for embryo culture experiments. The study was divided into different experiments: The ART experiment, where the effect of human ART culture media with and without supplemented growth factor was analyzed. This followed by the mGM-CSF experiment, in which the effect of different concentrations of the mouse growth factor on mouse embryo development was assessed. We also included a cross species approach using the human growth factor, termed the hGM-CSF experiment. We included these cross-species combinations of mouse embryos and human ART medium and mouse embryos and human growth factor to mimic the situation which occurs when culture media are released to the market. A so-called mouse embryo assay (MEA) is routinely used to assess human culture media before they are released for the human ART market. Every human culture medium passes the test if it supports mouse embryo development by reaching an 80% blastocyst formation rate. We extended the study and repeated our experiment at the Max Planck Institute for Molecular Biomedicine in Münster and additionally used a strain of mice for egg fertilization (CD1) to exclude laboratory- and mouse strain-specific results, respectively. Finally, E4.5 blastocysts were transferred back into foster mothers to analyze the effect of GM-CSF on subsequent placental and fetus development.

3.1 Zygote collection

During this study different strains of mice were used to generate zygotes for embryo culture (Table 2). All retrieved eggs and zygotes were pooled together from different mice and were randomly allocated to the experimental groups (e.g. different culture media). They all were cultured together for 2.5 days. But only embryos which developed to the 4-cell stage were cultured further until E4.5 in fresh medium. Embryos which then developed into blastocysts were used for immunohistochemical staining or embryo transfers.

Table 2 Numbers of collected eggs and zygotes, 4-cell stage embryos, developed blastocysts and analyzed blastocysts.

Experiment	Retrieved eggs/zygotes (n)	4-cell stage Embryos (n)	Blastocysts (n)	Analyzed blastocysts (n)
ART experiment	530	385	312	185
mGM-CSF experiment	1157	670	375*	375
hGM-CSF experiment	1117	655	356	287
CD1 experiment	376	320	252	205
Embryo transfer	721	474	385	/

3.2 ART experiment

The ART experiment was performed to assess the effect of ART culture media with and without the growth factor GM-CSF on embryo development.

3.2.1 Development rates in the ART experiment

For the ART experiment a total of 530 eggs and zygotes were retrieved on E0.5. 385 4-cell stages embryos were cultured further until E4.5 from which 312 developed into blastocysts (81%). In the *in vitro* control group (KSOM(aa)) the mean development rate was 78%, in the human ART medium with GM-CSF 76% and in the human ART medium without GM-CSF 76% (Figure 5 & Table S1).

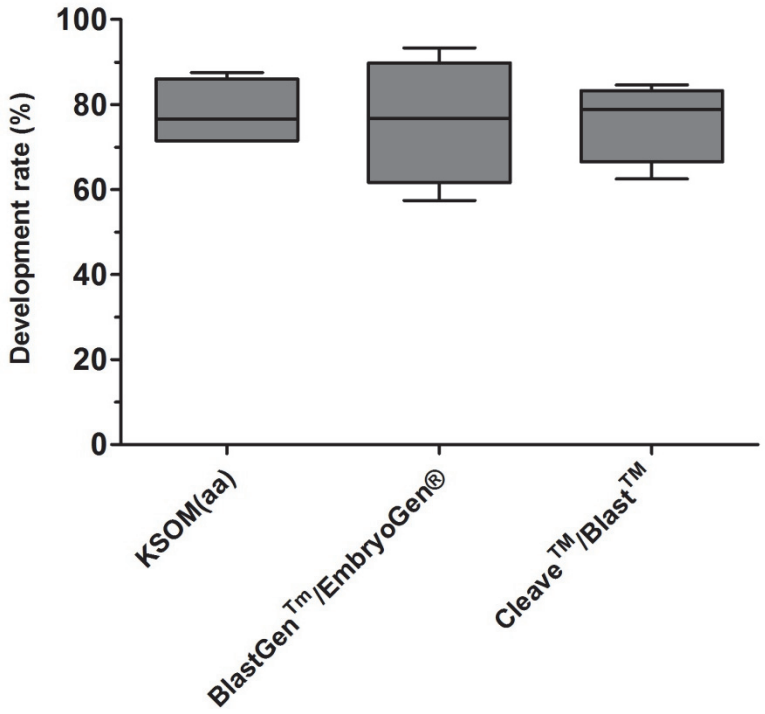

Figure 5 Development rates for the ART experiment. . Shown is the percentage of 4-cell stage embryos, which developed into blastocysts (E2.5-E4.5). Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance.

3.2.2 Cell numbers of different cell lineages in the ART experiment

Absolute cell numbers of all cultivated blastocysts and the respective cell numbers of the three lineages, trophectoderm (CDX2 positive), primitive endoderm (SOX17 positive) and epiblast (NANOG positive) in KSOM(aa), the *in vitro* control and the commercially available human ART medium Cleave™/Blast™ without GM-CSF (Origio) and the same medium supplemented with 2ng/mL GM-CSF, EmbryoGen®/BlastGen™(Origio) were counted. A total of 185 blastocysts were analyzed after immunohistochemical treatment. Cell lineages showed equal distribution among the three different media, both in absolute and proportional cell numbers (Figure 6, 7 and Figure S1).

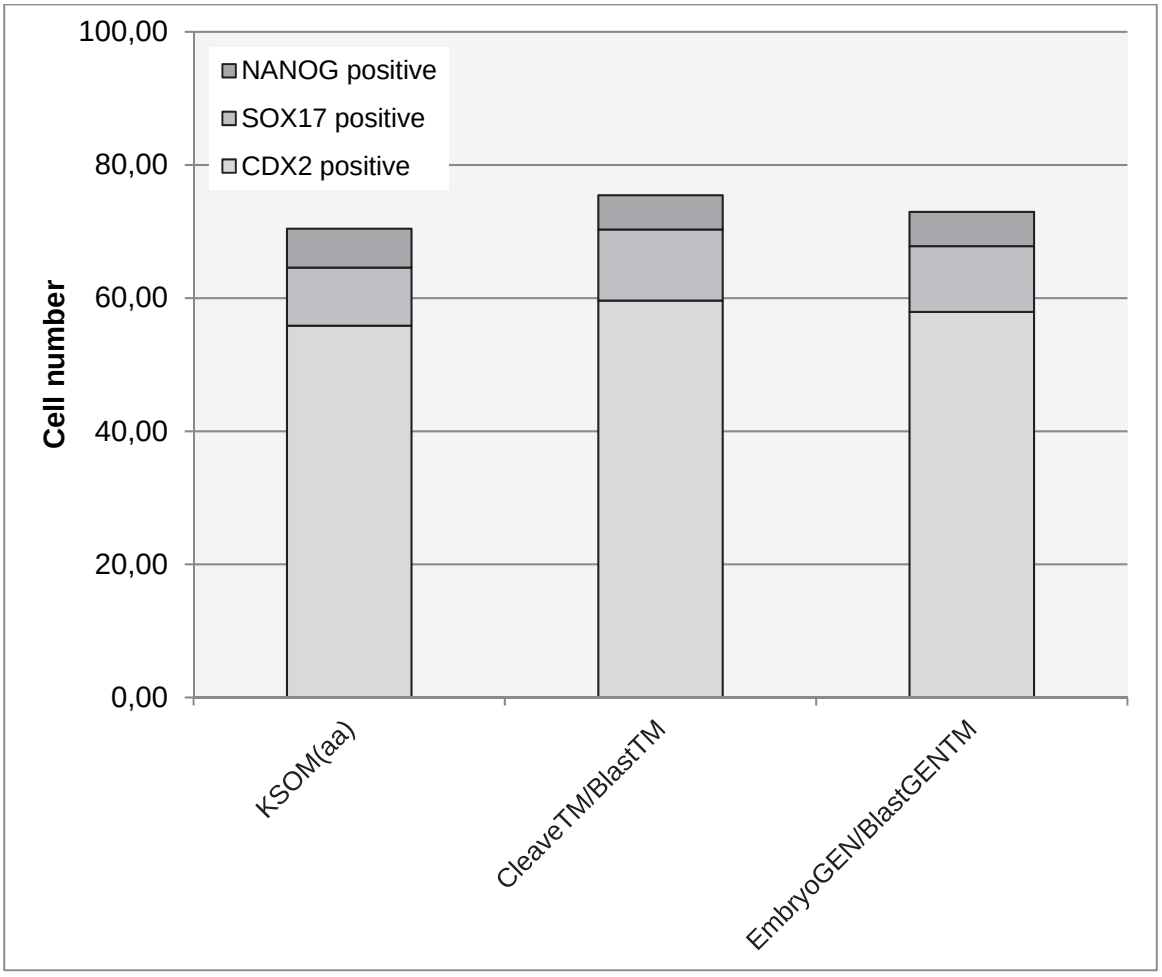

Figure 6 Comparison of mean absolute numbers of different cell lineages. Lineages are identified by specific cell lineage markers (CDX2: trophectoderm; SOX17: primitive endoderm; NANOG: epiblast) in ART media and the in vitro control medium, 0ng/mL (KSOM(aa)).

A significant change was only found in the number of SOX17 positive cells between KSOM(aa) (n = 46) and the ART medium without growth factor (n = 48; mean numbers: KSOM(aa): 8.72, SD: 2.83; Cleave™/Blast™: 10.67, SD: 3.66; P<0.05, One-Way ANOVA followed by Bonferroni post-hoc test). No changes were found regarding total cell number, CDX2 positive cells or NANOG positive cells.

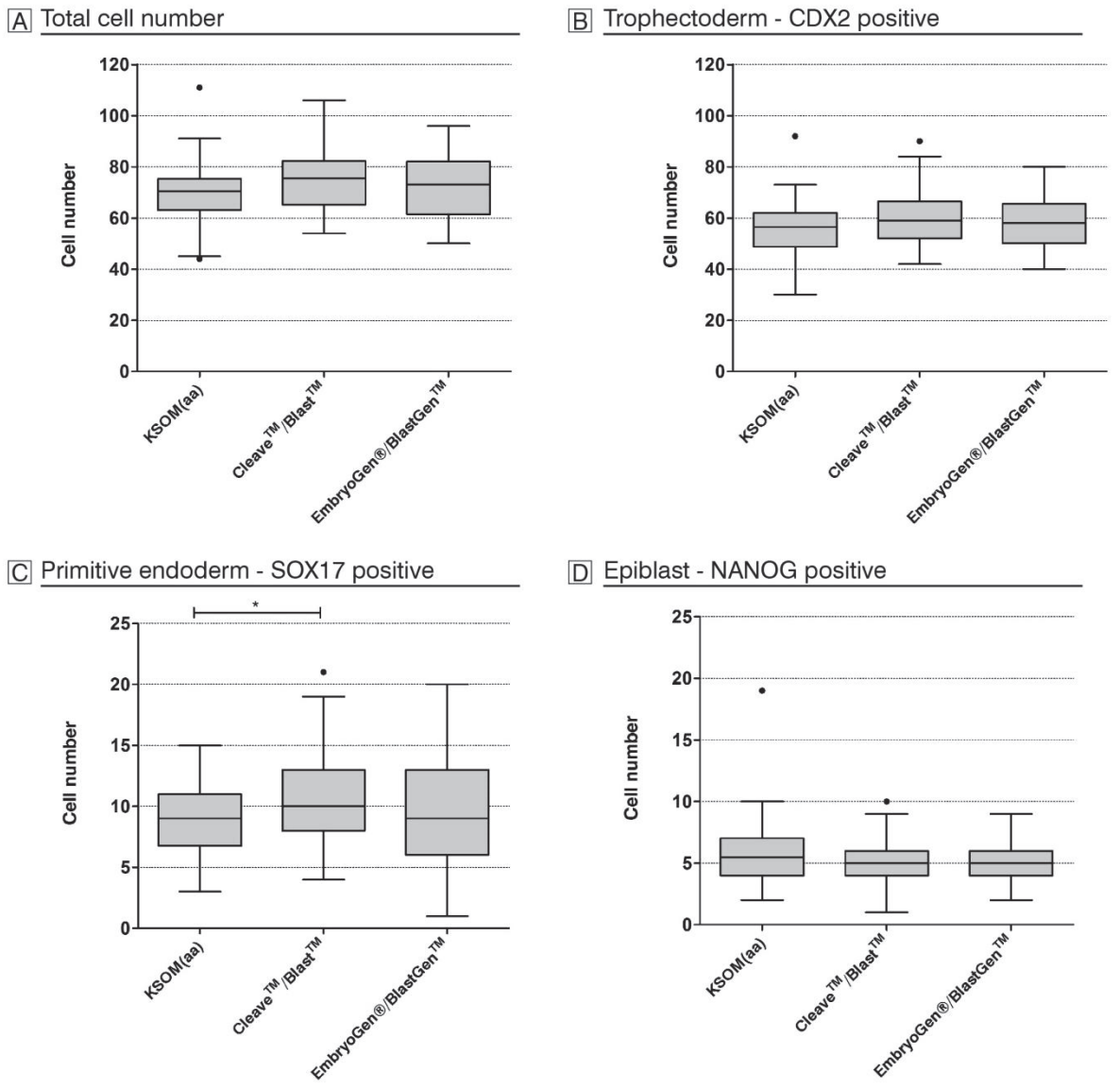

Figure 7 Effect of ART media: comparison of absolute cell numbers. A) total cells, B) CDX2 positive cells, C) SOX17 positive cells and D) NANOG positive cells in three different media settings (KSOM(aa): *in vitro* control; Cleave™/Blast™: ART medium without GM-CSF (Origio); EmbryoGen®/BlastGen™: ART medium containing 2ng/mL GM-CSF (Origio)). Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance; dots not included between the whiskers indicate outliers; P<0.05 (*) significant. Number of embryos: KSOM(aa) n = 46; Cleave™/Blast™ n = 48; EmbryoGen®/BlastGen™ n = 45.

3.3 mGM-CSF/hGM-CSF experiment

In the mGM-CSF and hGM-CSF experiments different concentrations of human and mouse GM-CSF were analyzed regarding the effect on embryo development and cell lineage distribution.

3.3.1 Development rates in the m- and hGM-CSF experiment

For the mGM-CSF experiment a total of 1157 eggs and zygotes were retrieved on E0.5. A total of 670 4-cell stages embryos were cultured further until E4.5 from which 375 developed into blastocysts (56%). The mean development rates are: (KSOM(aa))=47%, 1 ng/mL=40%, 2ng/mL=51%, 5ng/mL=51%, 10ng/mL=52% and 20ng/mL=49%; Figure 8, supplementary Table SX).

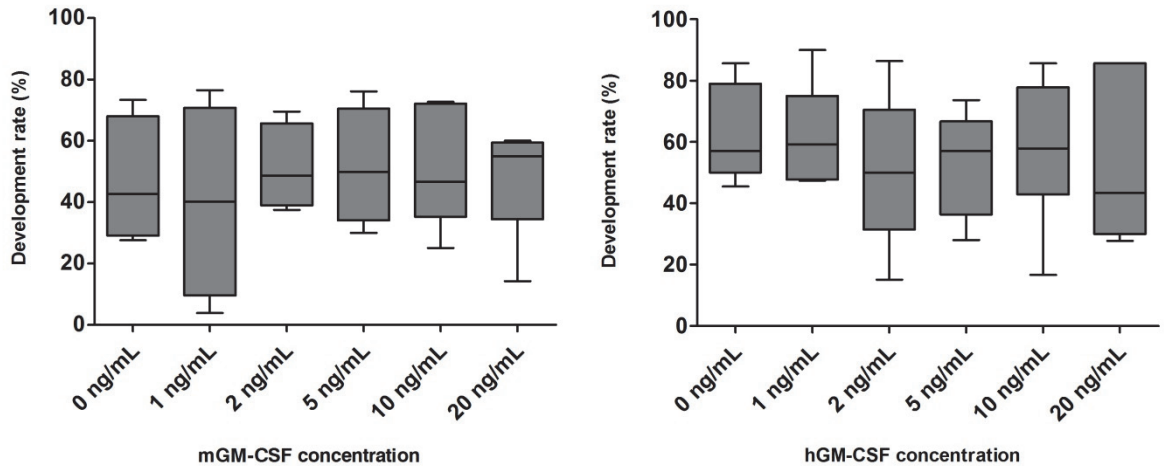

Figure 8 Development rates for the ART experiment. Shown is the percentage of 4-cell stage embryos, which developed into blastocysts (E2.5-E4.5). Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance.

For the hGM-CSF experiment a total of 1117 eggs and zygotes were retrieved on E0.5. A total of 655 4-cell stages embryos were cultured further until E4.5 from which 356 developed into blastocysts (54%). The mean development rates are: (KSOM(aa))=62%, 1 ng/mL=61%, 2ng/mL=51%, 5ng/mL=54%, 10ng/mL=57% and 20ng/mL=54; Figure 8, supplementary Table SX).

3.3.2 Cell numbers of different cell lineages in the mGM-CSF experiment

In the mGM-CSF experiment mouse embryos were cultivated in the optimized KSOM(aa) medium with different concentrations of mGM-CSF (1, 2, 5, 10, 20 ng/mL) and cell numbers of the three cell lineages were evaluated. Cell lineages showed equal proportional distribution among different concentrations of mGM-CSF (Figure S2), whereas absolute numbers showed an increase of cell numbers with increasing concentration (Figure 9 & 10).

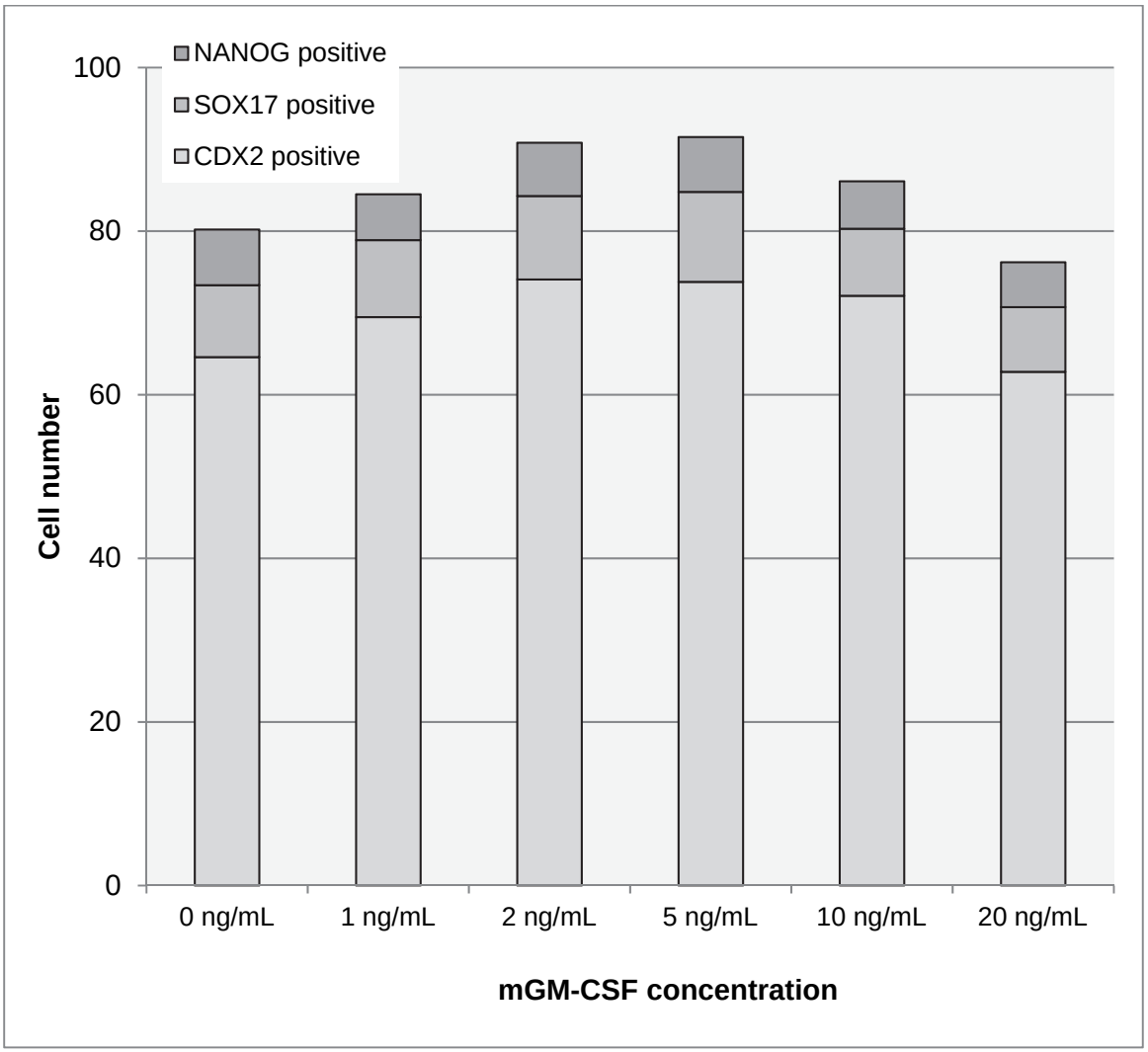

Figure 9 Comparison of mean absolute numbers of different cell lineages. Lineages are identified by specific cell lineage markers (CDX2: trophectoderm; SOX17: primitive endoderm; NANOG: epiblast) in different mouse GM-CSF concentrations (1, 2, 5, 10 and 20 ng/mL) and the *in vitro* control medium, 0ng/mL (KSOM(aa)).

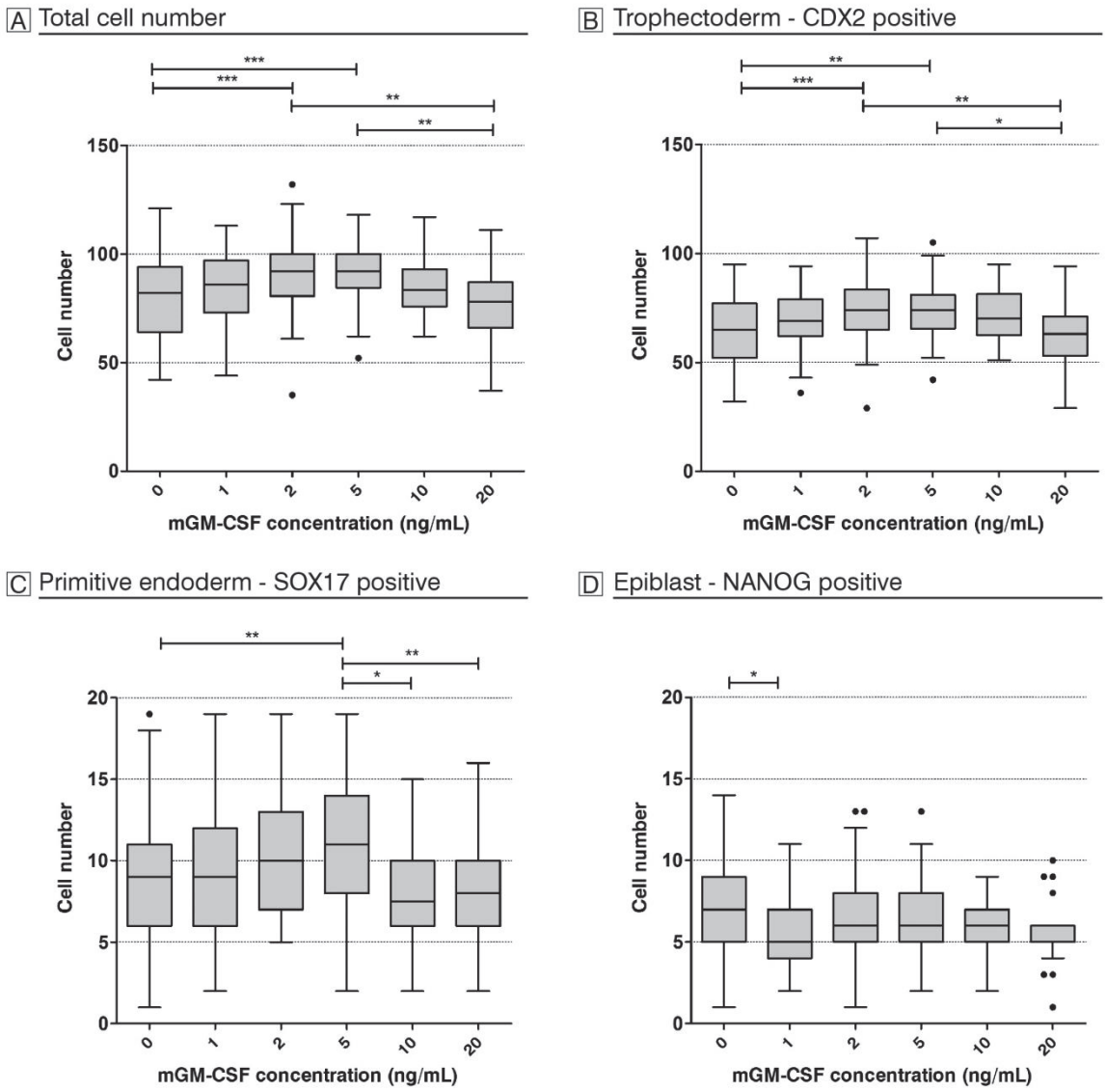

Figure 10 Effect of mGM-CSF: comparison of absolute cell numbers. A) total cells, B) CDX2 positive cells, C) SOX17 positive cells and D) NANOG positive cells in five different mGM-CSF concentrations (1, 2, 5, 10 and 20 ng/mL) and the *in vitro* control medium, 0ng/mL (KSOM(aa)). Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance; dots not included between the whiskers indicate outliers. Significance values: P<0.05 (*), P<0.001 (**) and P<0.0001 (***): Number of embryos: 0 ng/mL n = 95; 1 ng/mL n = 63; 2 ng/mL n = 69; 5 ng/mL n = 61; 10 ng/mL n= 26; 20 ng/mL n = 27).

Embryos cultivated in medium containing the mouse growth factor showed significant differences in total cell numbers and CDX2 positive cells between the KSOM(aa) control and 2 and 5 ng/mL mGM-CSF respectively, and between 2 and 20 ng/mL and 5 and 20 ng/mL mGM-CSF respectively (mean values total cells: 0 ng/mL mGM-CSF: 80.2±18.8; 1ng/mL: 84.5±15.7; 2ng/mL: 90.9±16.7; 5ng/mL: 91.4±13.5; 10ng/mL: 86.1±14.9; 20ng/mL: 76.2±17.1; P<0.0001 One-Way ANOVA followed by Bonferroni post-hoc test). Differences were also

found in SOX17 positive cells between the KSOM(aa) control and 5ng/mL mGM-CSF respectively, and between 5ng/mL and 10 and 20 ng/mL mGM-CSF respectively (Figure 10 and Table 3). The change in total cell numbers was mainly due to higher mean number of the trophectoderm cells (CDX2) and not due to changes in the inner cell mass cells, namely the primitive endoderm (SOX17) or epiblast cells (NANOG).

3.3.3 Cell numbers of different cell lineages in the hGM-CSF experiment

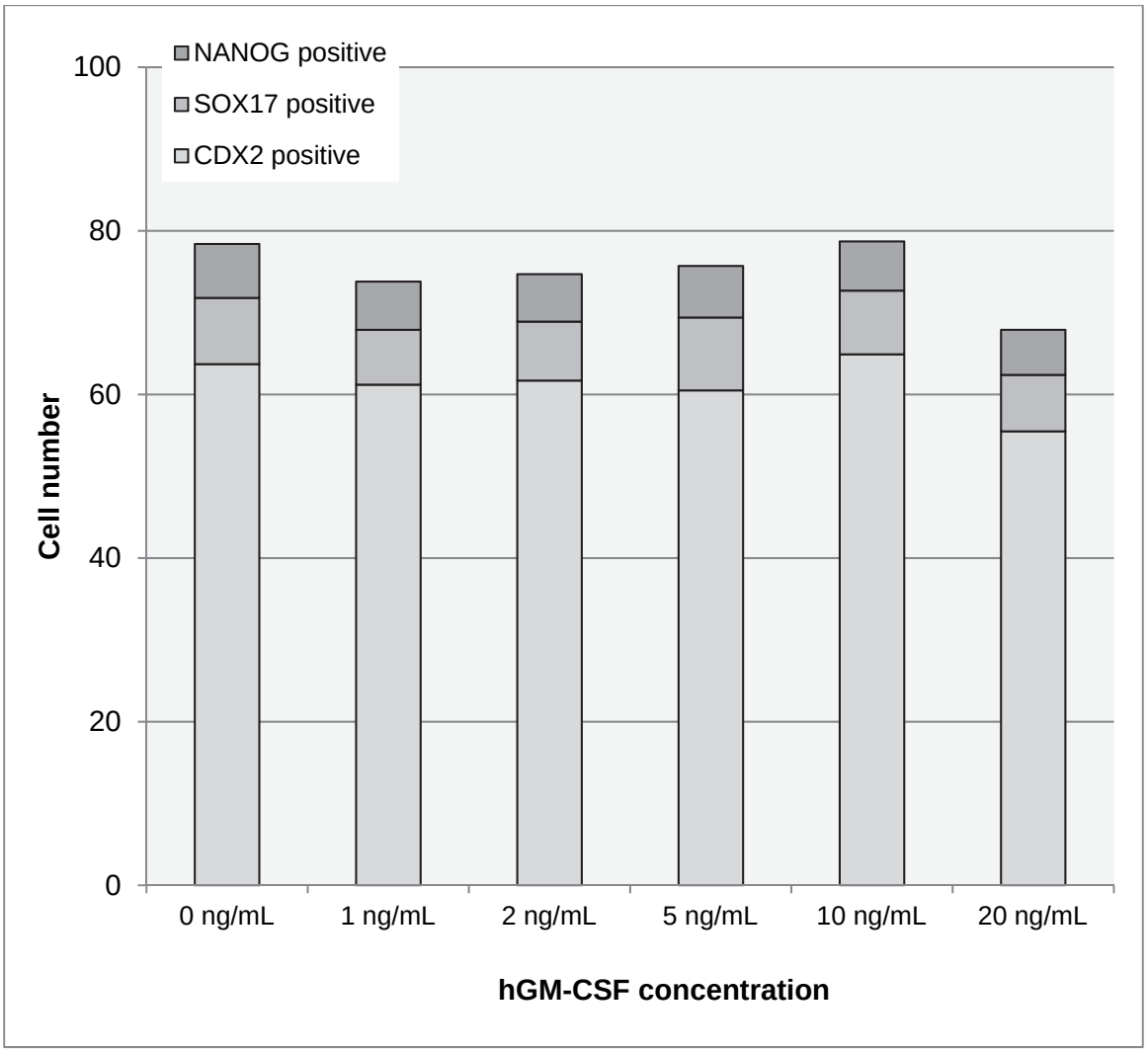

Figure 11 Comparison of mean absolute numbers of different cell lineages. Lineages are identified by specific cell lineage markers (CDX2: trophectoderm; SOX17: primitive endoderm; NANOG: epiblast) in different human GM-CSF concentrations (1, 2, 5, 10 and 20 ng/mL) and the *in vitro* control medium, 0ng/mL (KSOM(aa)).

In the hGM-CSF experiment mouse embryos were cultivated in KSOM(aa) medium with different concentrations of human GM-CSF (1, 2, 5, 10, 20 ng/mL) and cell numbers of the three cell lineages were evaluated (Table 3). Cell lineages showed equal distribution among

different hGM-CSF concentrations, regarding absolute and proportional cell numbers (Figure 11, 12 Figure S3).

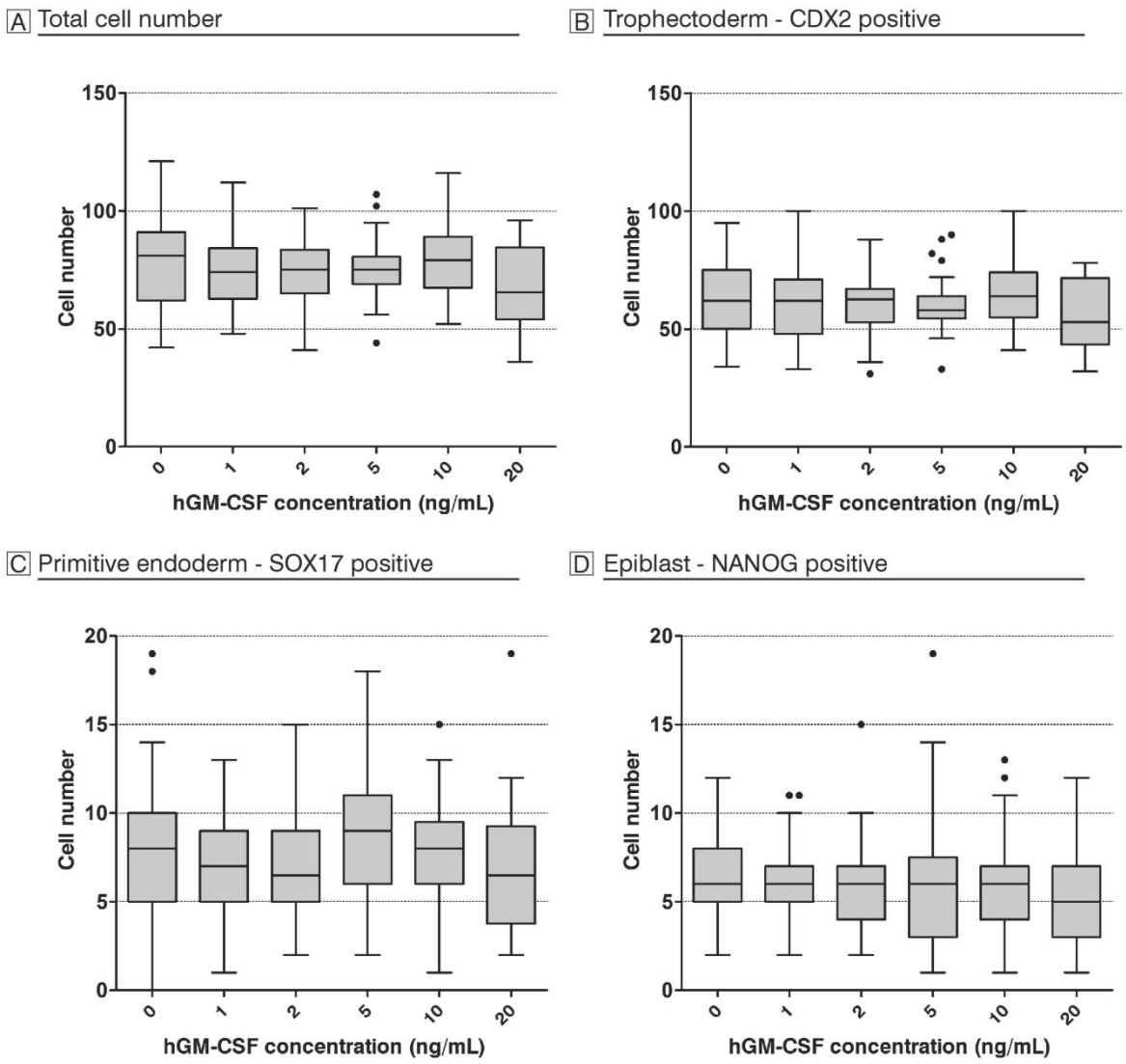

Figure 12 Effect of hGM-CSF: comparison of absolute cell numbers. A) total cells, B) CDX2 positive cells, C) SOX17 positive cells and D) NANOG positive cells in five different hGM-CSF concentrations (1, 2, 5, 10 and 20 ng/mL) and the *in vitro* control medium, 0ng/mL (KSOM(aa)). Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance; dots not included between the whiskers indicate outliers. Numbers of embryos: 0 ng/mL n = 54; 1 ng/mL n = 58; 2 ng/mL n = 34; 5 ng/mL n = 28; 10 ng/mL n= 37; 20 ng/mL n = 26).

Total cell numbers did not change in the different human GM-CSF groups in comparison to the non-supplemented KSOM(aa) control group (mean values: 0 ng/mL hGM-CSF: 78.4±18.7; 1ng/mL: 73.9±14.8; 2ng/mL: 74.7±14.5; 5ng/mL: 75.7±13.4; 10ng/mL: 78.7±15.6; 20ng/mL: 68.0±17.7) and no differences were found in the three specific cell lineages among different concentrations.

Table 3 Mean values of cell lineages, standard deviation and n-values of all experimental groups.

Group		CDX2 positive		SOX17 positive		NANOG positive		n
		Mean	SD	Mean	SD	Mean	SD	
ART media	**KSOM(aa)**	55.9	11.0	8.7	2.8	5.8	2.7	46
	Cleave™/Blast™	59.6	10.5	10.67[i]	3.6	5.2	2.1	48
	EmbryoGEN®/BlastGEN™	57.9	9.9	9.87[i]	4.2	5.2	1.7	45
mGM-CSF concentration (ng/mL)	**0 ng/mL**	64.6[a,b]	15.1	8.8[e]	3.8	6.8[h]	2.6	95
	1 ng/mL	69.5	13.3	9.4	3.9	5.6[h]	2.1	63
	2 ng/mL	74.1[a,c]	14.2	10.2	3.3	6.5	2.4	69
	5 ng/mL	73.8[b,d]	11.6	11[e,f,g]	3.8	6.7	2.5	61
	10 ng/mL	72.1	12.4	8.2[f]	3.2	5.8	1.7	26
	20 ng/mL	62.8[c,d]	14.4	7.9[g]	3.4	5.5	1.9	27
hGM-CSF concentration (ng/mL)	**0 ng/mL**	63.7[a]	14.7	8.1	4.1	6.6	2.4	54
	1 ng/mL	61.2[a]	13.5	6.7	2.8	5.9	2.1	58
	2 ng/mL	61.7	13.1	7.2	2.9	5.8	2.6	34
	5 ng/mL	60.5	12.3	8.9	3.6	6.3	4.0	28
	10 ng/mL	64.9	13.3	7.8	2.9	6.0	3.0	37
	20 ng/mL	55.5	14.8	6.9	3.9	5.5	2.9	26

Significance values: a: P<0.0001; b, c, e, f: P<0.001; d, g, h, i: P<0.05; One-Way ANOVA followed by Bonferroni post-hoc test

3.4 Blastocysts with ectopic expression of NANOG

During the analysis, several blastocysts were found exhibiting a different expression pattern than the expected one. This anomaly occurred in different media and different concentrations of GM-CSF. Additionally to the normal CDX2 pattern in trophectoderm cells, a simultaneous expression of NANOG was detected in some cells (Figure 13).

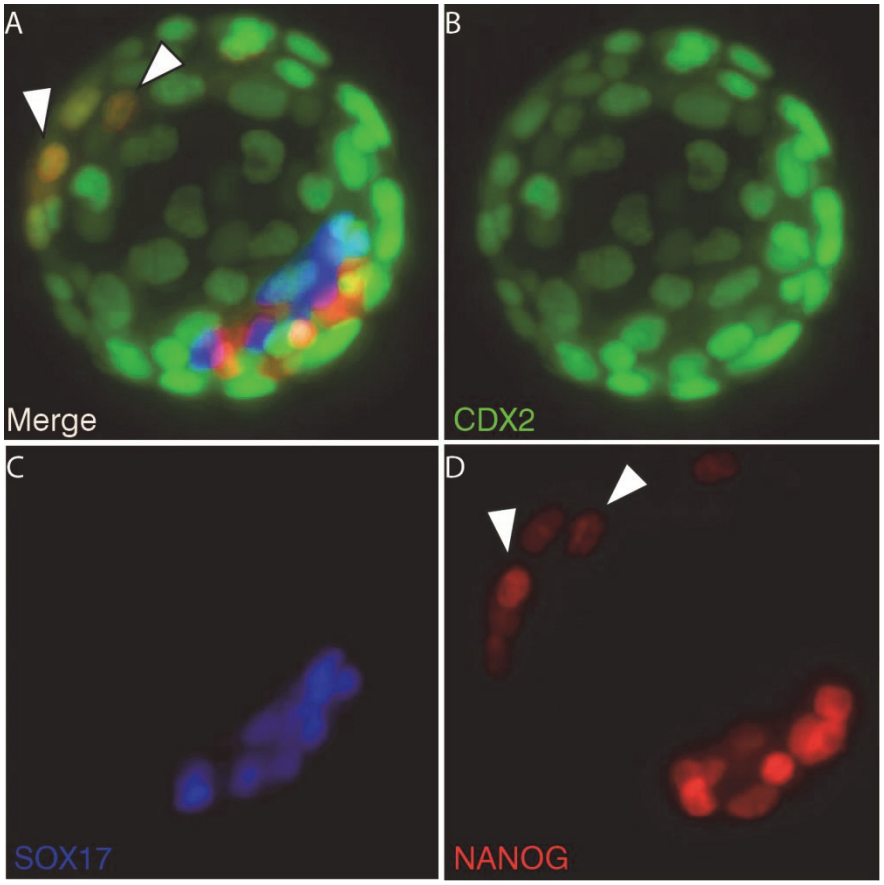

Figure 13 Representative picture of a blastocyst (E4.5) with ectopic expression of NANOG. A) Merged picture of all cell lineages B) trophectoderm cells (CDX2) C) primitive endoderm cells (SOX17) D) epiblast cells (NANOG). White arrows are highlighting NANOG positive trophectoderm cells in the merged and NANOG picture.

3.4.1 Amount of blastocysts with ectopic NANOG expression among different growth factor concentrations

Of 185 analyzed blastocysts in the ART experiment 46 blastocysts (25%) showed an ectopic expression of NANOG among CDX2 positive trophectoderm cells (Table 4).

Table 4 Numbers of blastocysts with normal and ectopic expression of NANOG in trophectoderm cells.

Experiment	Total blastocysts (n)	Normal expression of markers (n/%)	Ectopic expression of NANOG in TE cells (n/%)
ART	185	139 (75)	46 (25)
mGM-CSF	375	341 (91)	34 (9)
hGM-CSF	287	236 (82)	51 (18)
Total	847	716 (85)	131 (15)

Of a total of 185 blastocysts in the ART experiment 46 showed an atypical expression pattern (14 in the ART medium without GM-CSF, 32 in the medium with GM-CSF) and 139 embryos were found without ectopic expression. No ectopic expression was found in the control and mouse optimized medium (KSOM(aa)). In the m- and hGM-CSF experiments a total of 664 blastocysts were analyzed and 85 (13%) had an ectopic expression of NANOG among CDX2 positive trophectoderm cells; 34 in the mouse and 51 in the human GM-CSF experiment. With increasing concentrations of either mouse or human GM-CSF supplemented media also the number of blastocysts with ectopic expression increased. While embryos cultured in medium supplemented with mGM-CSF showed ectopic expression only in the highest concentrations (KSOM(aa) +10ng/mL: 40%; +20ng/mL: 39%), embryos cultured in hGM-CSF showed ectopic expression already from the lowest concentration onwards (0 ng/mL: 0%; 1ng/mL: 11%; 2ng/mL: 15%; 5ng/mL: 29%; 10ng/mL: 24%; 20ng/mL: 35%).

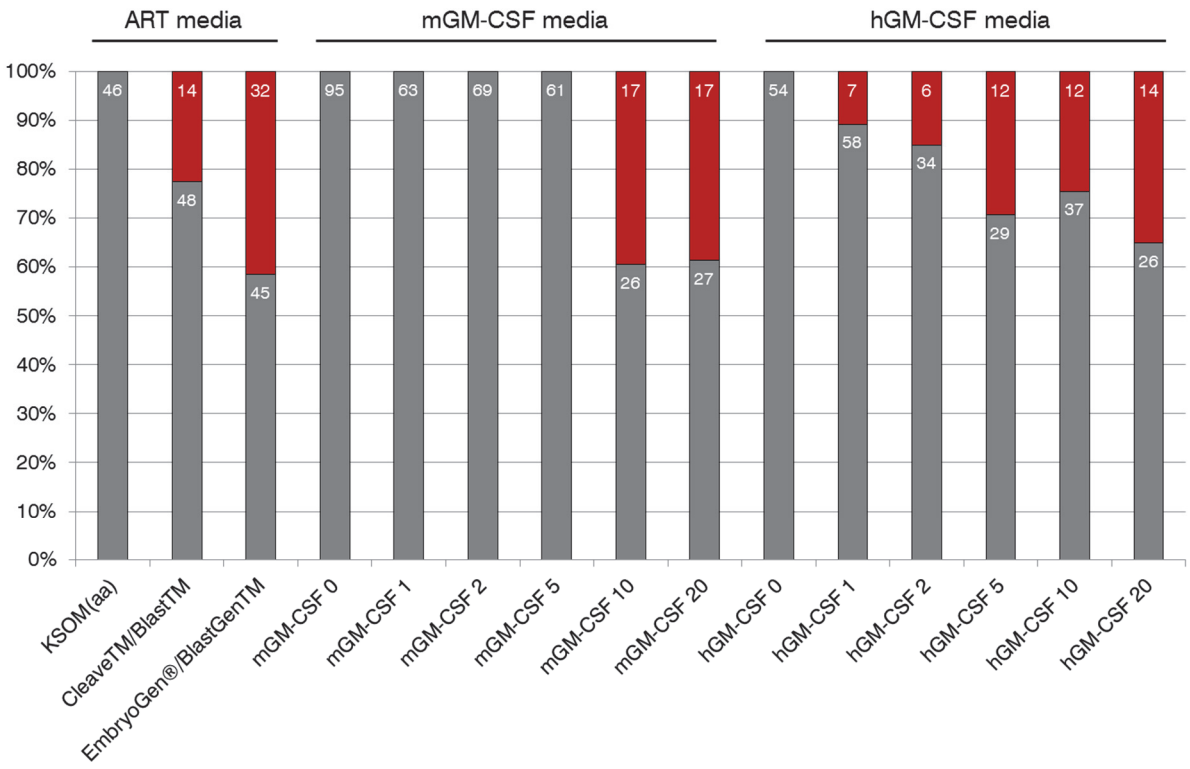

Figure 14 Percentage of occurrence of blastocysts exhibiting a double staining of the cell lineage markers CDX2 and NANOG in trophectoderm cells. Shown are KSOM(aa) and both ART media (KSOM(aa): in vitro control; Cleave™/Blast™: ART medium without GM-CSF (Origio); EmbryoGen®/BlastGenTM: ART medium containing 2ng/mL GM-CSF (Origio) and all concentrations of mouse and human GM-CSF (1, 2, 5, 10 and 20 ng/mL). Grey: blastocysts without double staining; Red: blastocysts with CDX2 and NANOG positive trophectoderm cells. Bars indicate the percentage occurrence and numbers inside bars indicate absolute number of blastocysts.

3.4.2 Cell lineage distribution of normal blastocysts in the ART experiment compared to blastocysts with ectopic expression of NANOG.

All cell lineages taken together showed a higher additive cell number in the atypical blastocysts compared to the normal blastocysts in the ART medium without GM-CSF and that proportions of cell lineages did not differ among the groups in both ART media (Figure 15 and supplement Table S4).

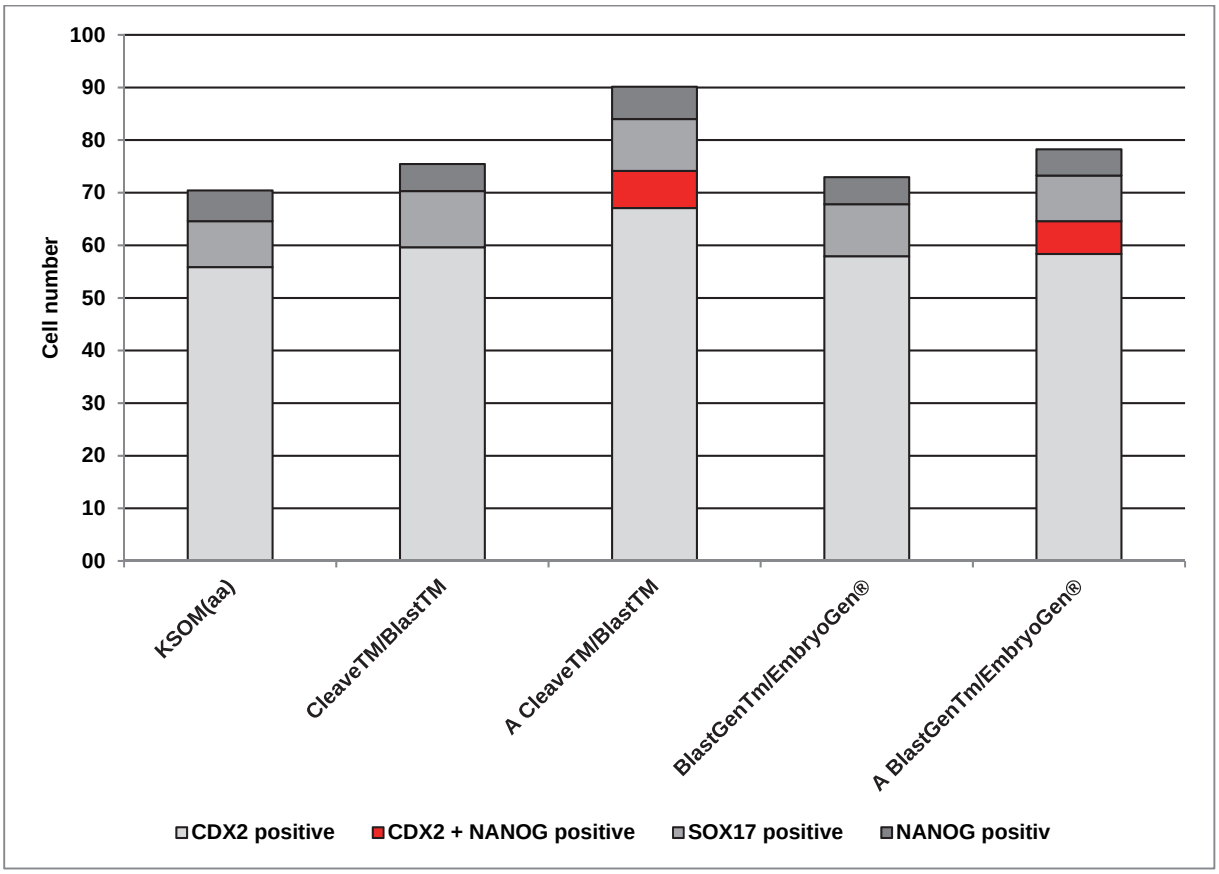

Figure 15 Mean absolute numbers of different cell lineages in normal blastocysts and blastocysts with ectopic expression of NANOG. Cells are identified by their specific cell lineage marker (CDX2: trophectoderm; SOX17: primitive endoderm; NANOG: epiblast) and the number of trophectoderm cells identified with a double staining of CDX2 and NANOG in the ART media which contained atypical blastocysts with ectopic NANOG expression.

Analysis of total cell numbers between the normal group and the group of blastocysts with atypical expression of NANOG compared for each medium showed significant difference between the Cleave™/Blast™ groups, the ART medium without GM-CSF (Figure 16 and Table 5, mean values: Cleave™/Blast™: 75.4±12.2; A Cleave™/Blast™: 83.1±12.34; non-parametric t-test: P<0.05). No change was found between the ART media containing GM-CSF.

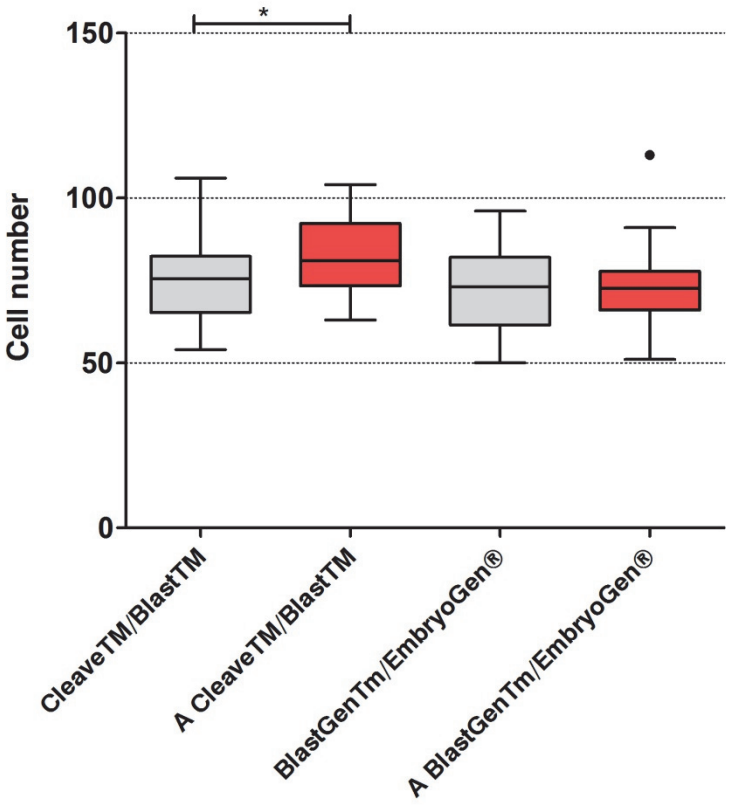

Figure 16 Effect of ART media: comparison of total cell numbers between normal blastocysts and blastocysts with ectopic NANOG expression. Total cells in two different media settings (Cleave™/Blast™: ART medium without GM-CSF (Origio); EmbryoGen®/BlastGen™: ART medium containing 2ng/mL GM-CSF (Origio)). Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance; dots not included between the whiskers indicate outliers; Check for significant differences between cell numbers of one medium group by non-parametric t-test: P<0.05 (*) significant. Number of blastocysts ("A" indicates the group of blastocysts with atypical NANOG expression): Cleave™/Blast™ n = 48; A Cleave™/Blast™ n = 14;EmbryoGen®/BlastGen™ n = 45; A EmbryoGen®/BlastGen™ n = 32.

3.4.3 Cell lineage distribution of normal blastocysts in the m- and hGM-CSF experiment compared to blastocysts with ectopic expression of NANOG.

Cell lineage distribution showed a decrease in absolute number of all cells between the normal group of 10 ng/mL mGM-CSF, 2 and 5ng/mL hGM-CSF and their groups with ectopic expression of NANOG. The other mouse and human concentrations showed equal distribution of cell lineages, regarding absolute and proportional cell numbers (Figure 17, 18 & Table S5).

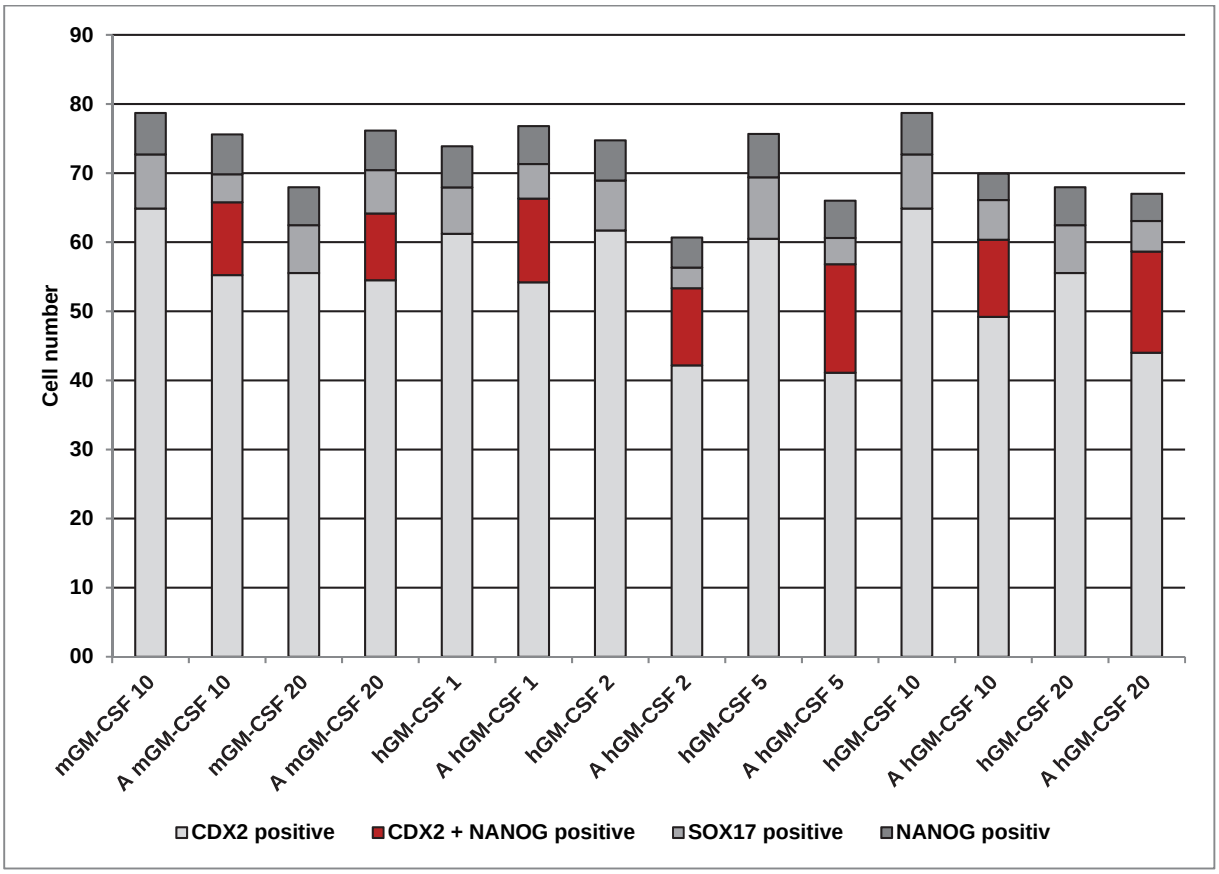

Figure 17 Mean absolute numbers of different cell lineages in normal blastocysts and blastocysts with ectopic expression of NANOG. Lineages are identified by specific cell lineage markers (CDX2: trophectoderm; SOX17: primitive endoderm; NANOG: epiblast) and the number of trophectoderm cells identified with a double staining of CDX2 and NANOG in different mouse and human GM-CSF concentrations (1, 2, 5, 10 and 20 ng/mL).

Analysis of total cell numbers between the normal group and the group of blastocysts with atypical expression of NANOG compared for each medium showed significant differences in the 10ng/mL mGM-CSF, 2ng/mL hGM-CSF and the 5ng/mL hGM-CSF group (Figure 18; mean values: 10ng/mL mGM-CSF: 91.4±13.5, A-10ng/mL mGM-CSF 75.6±15.5; 2ng hGM-CSF: 74.7±15.5, A-2ng/mL hGM-CSF: 60.7±13.4; 5ng/mL hGM-CSF: 75.7±13.4, A-5ng/mL hGM-CSF: 66.0±7.8).

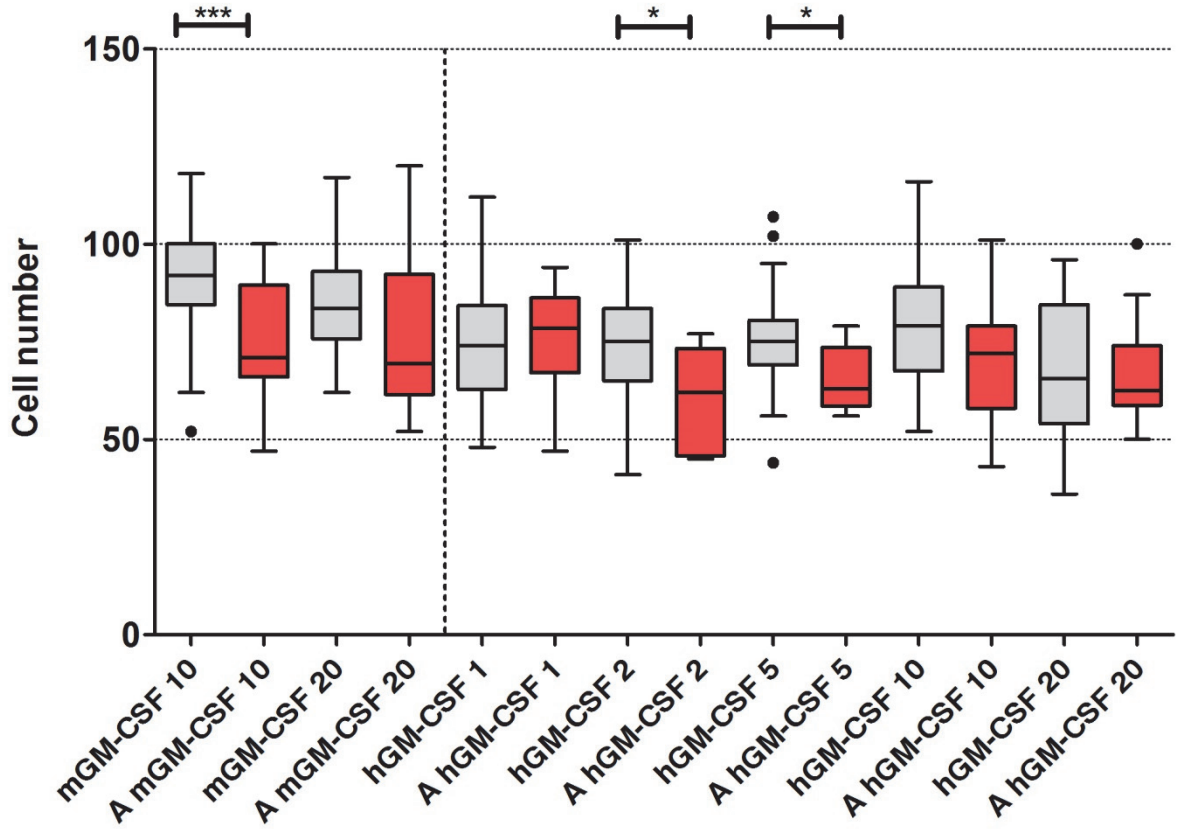

Figure 18 Effect of ART media: comparison of total cell numbers between normal blastocysts and blastocysts with ectopic NANOG expression. Total cell number of blastocysts in all media with supplemented m- or hGM-CSF and atypical expression pattern. Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance; dots not included between the whiskers indicate outliers; Check for significant differences between cell numbers of one medium group by non-parametric t-test: P<0.05 (*) significant. “A” indicates the group of blastocysts with atypical NANOG expression.

Table 5 Mean values of all cell lineages, standard deviation and n-values of blastocysts with ectopic NANOG expression in all experimental groups.

Group		CDX2 positive		SOX17 positive		NANOG positive		CDX2+NANOG positive		n
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	
ART media	**Cleave™/Blast™**	47.1	8.1	9.9	4.0	6.1	2.4	7.1	6.1	14
	EmbryoGEN®/BlastGEN™	58.3	11.0	8.7	2.8	5.0	1.5	6.3	5.5	32
mGM-CSF concentration (ng/mL)	**10 ng/mL**	55.2	12.5	4.1	3.6	5.8	3.0	10.5	5.4	17
	20 ng/mL	54.5	14.1	6.3	4.8	5.7	3.0	9.6	6.1	14
hGM-CSF concentration (ng/mL)	**1 ng/mL**	54.2	7.5	5.0	4.0	5.5	3.2	12.1	6.0	10
	2 ng/mL	42.2	9.0	3.0	3.5	4.3	2.9	11.2	3.7	6
	5 ng/mL	42.9	8.5	5.2	4.8	4.8	4.5	13.2	9.0	13
	10 ng/mL	49.2	11.2	5.7	4.1	3.8	3.1	11.2	5.5	11
	20 ng/mL	44.0	11.9	4.4	3.9	3.9	3.4	14.6	7.6	14

3.5 Repeating experiments in a second laboratory at the MPI Muenster

To exclude a laboratory specific result, regarding the ectopic expression of NANOG, the embryo culture experiments with GM-CSF were repeated two times at the MPI in Muenster.

3.5.1 Cell numbers of different cell lineages in the MPI experiment

In the previous experiments, media supplemented with hGM-CSF showed atypical blastocysts from the lowest concentration onwards. To challenge these results, 2 and 10 ng/mL hGM-CSF was used for the embryo culture experiment at the MPI. Cell lineage distribution showed an equal distribution among different GM-CSF concentrations, regarding absolute and proportional cell numbers (Figure 19 and supplement Table S6**)**.

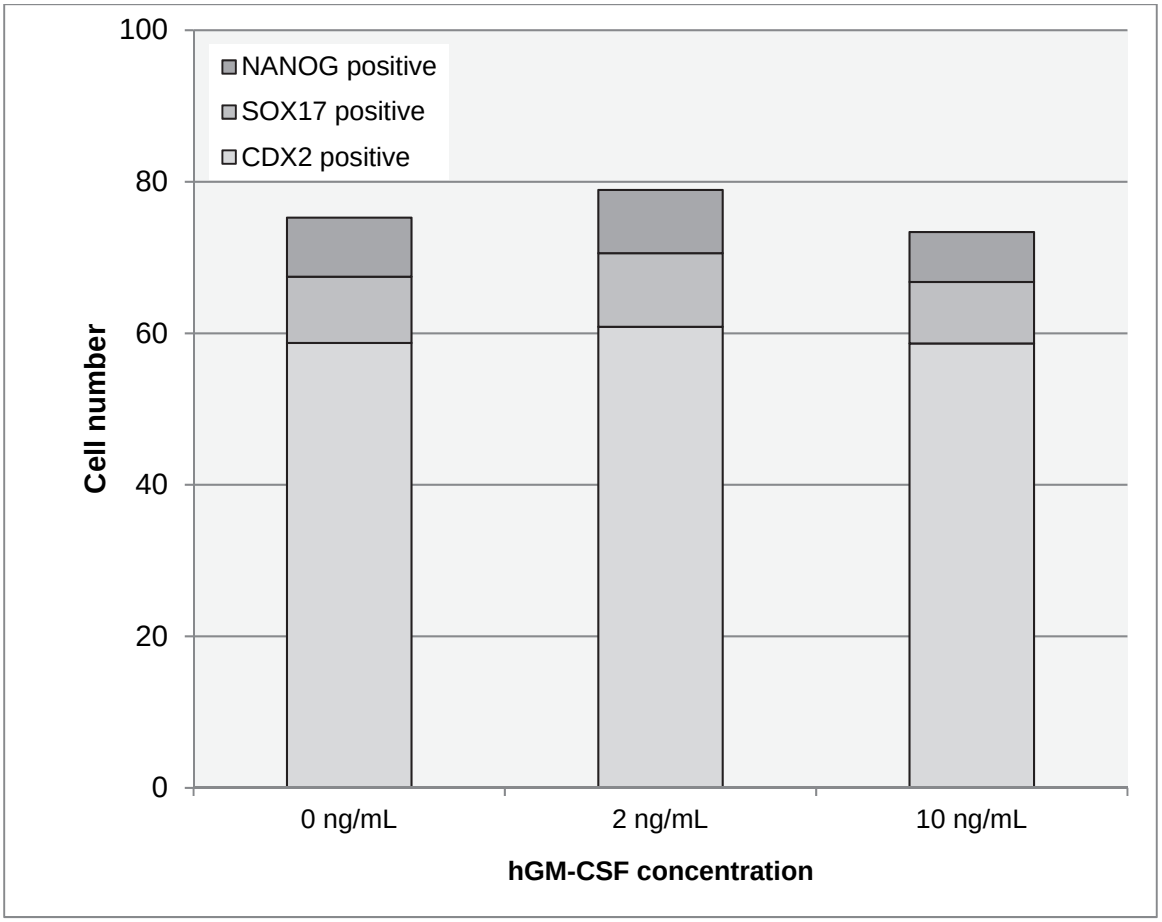

Figure 19 Comparison of mean absolute numbers of different cell lineages in the MPI experiment. Lineages are identified by specific cell lineage markers (CDX2: trophectoderm; SOX17: primitive endoderm; NANOG: epiblast) in different human GM-CSF concentrations (2, 10 ng/mL) and the *in vitro* control medium, 0ng/mL (KSOM(aa)).

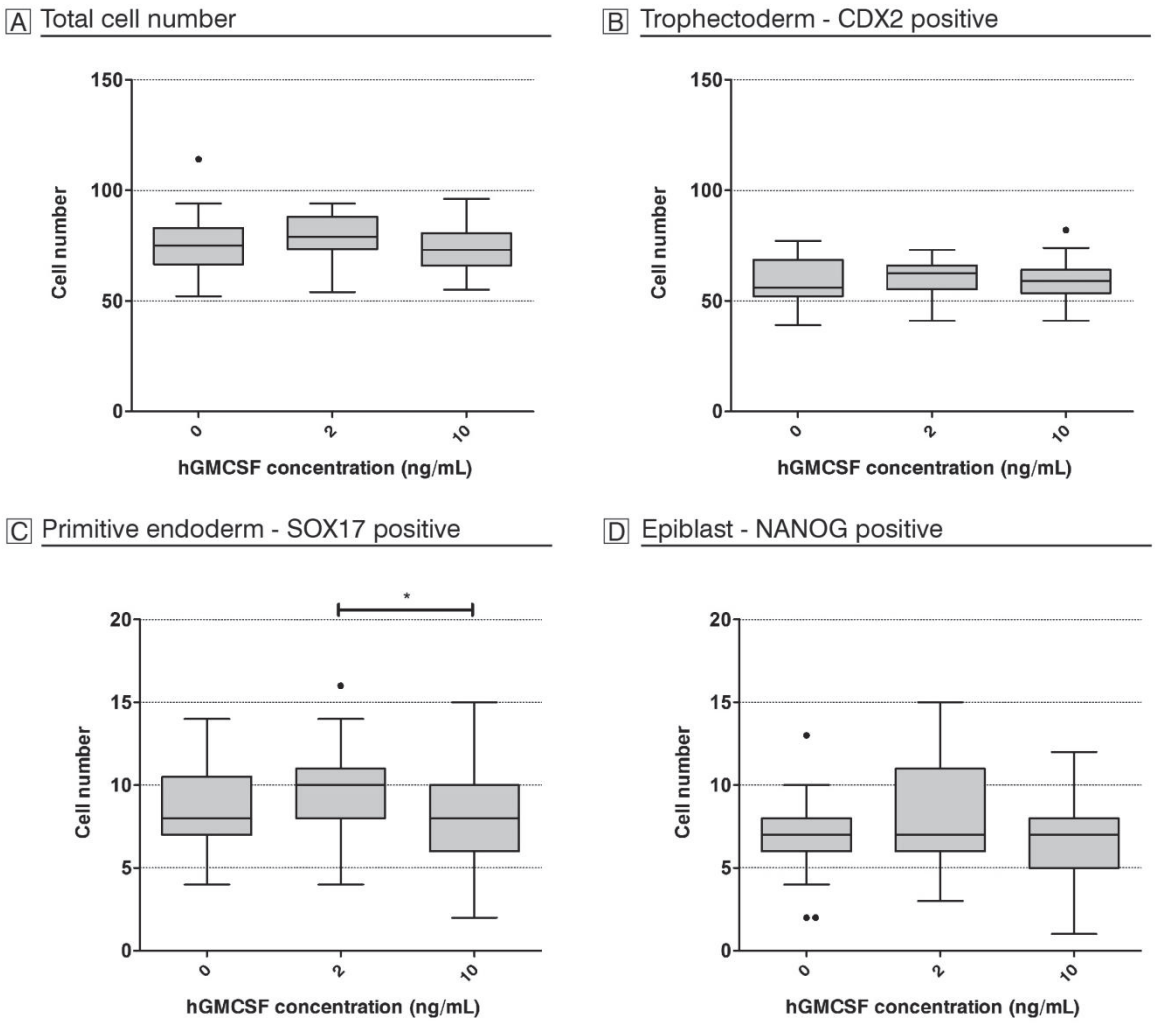

Figure 20 Effect of hGM-CSF media: comparison of absolute cell numbers in the MPI experiment. A) total cells, B) CDX2 positive cells, C) SOX17 positive cells and D) NANOG positive cells in two different hGM-CSF concentrations (2, 10 ng/mL) and the *in vitro* control medium, 0ng/mL (KSOM(aa)). Blastocysts with ectopic NANOG expression are excluded. Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance; dots not included between the whiskers indicate outliers. Significance values: $P<0.05$ (*).

A total of 164 blastocysts could be analyzed after immunohistochemical treatment. 50 (30%) of these blastocysts had an ectopic expression of NANOG in trophectoderm cells. The 114 blastocysts without ectopic NANOG expression were analyzed separately (Figure 20 and Table 6). Total cells did not change in the different concentrations of hGM-CSF or in the non-supplemented KSOM(aa) control group (mean values: 0 ng/mL hGM-CSF: 75.3±12; 2ng/mL: 78.9±9.7 and 10ng/mL: 73±9.4). A significant change was found between 2 and 10 ng/mL in the number of SOX17 positive cells (mean values: 2ng/mL: 9.7±2.6 and 10ng/mL: 8.1±2.7; $P<0.05$ One-Way ANOVA followed by Bonferroni post-hoc test). No changes were found for CDX2 and NANOG positive cells.

3.5.2 Amount of blastocysts with ectopic NANOG expression among different growth factor concentrations in the MPI experiment.

Cell lineages showed equal distribution among different GM-CSF concentrations, regarding absolute and proportional cell numbers (Figure 21 and Table S7).

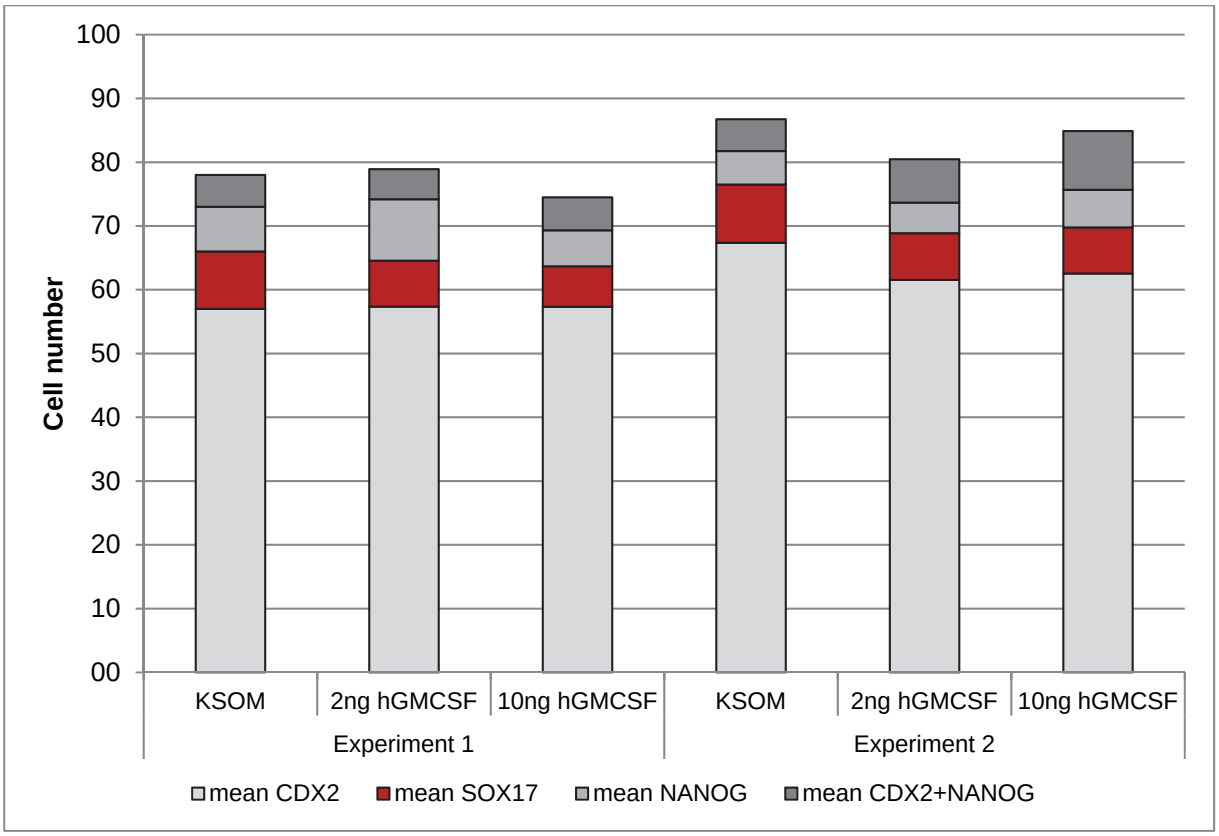

Figure 21 Mean absolute numbers of different cell lineages in blastocysts with ectopic expression of NANOG in the MPI experiment. Cells are identified by their specific cell lineage marker (CDX2: trophectoderm; SOX17: primitive endoderm; NANOG: epiblast) and the number of trophectoderm cells identified with a double staining of CDX2 and NANOG in two different hGM-CSF concentrations (2 and 10 ng/mL).

Of 164 analyzed blastocysts in the two MPI experiments 50 (30%) showed the ectopic expression of NANOG in trophectoderm cells. In contrast to the experiments which were performed in house blastocysts with atypical expression were also found in the KSOM(aa) *in vitro* control groups (Figure 22). In the second experiment, 79% of the blastocysts cultured in 2ng/mL hGM-CSF showed an atypical expression of NANOG, which was the highest amount in the whole study. In both experiments less blastocysts with atypical expression were found in the higher concentrations of hGM-CSF.

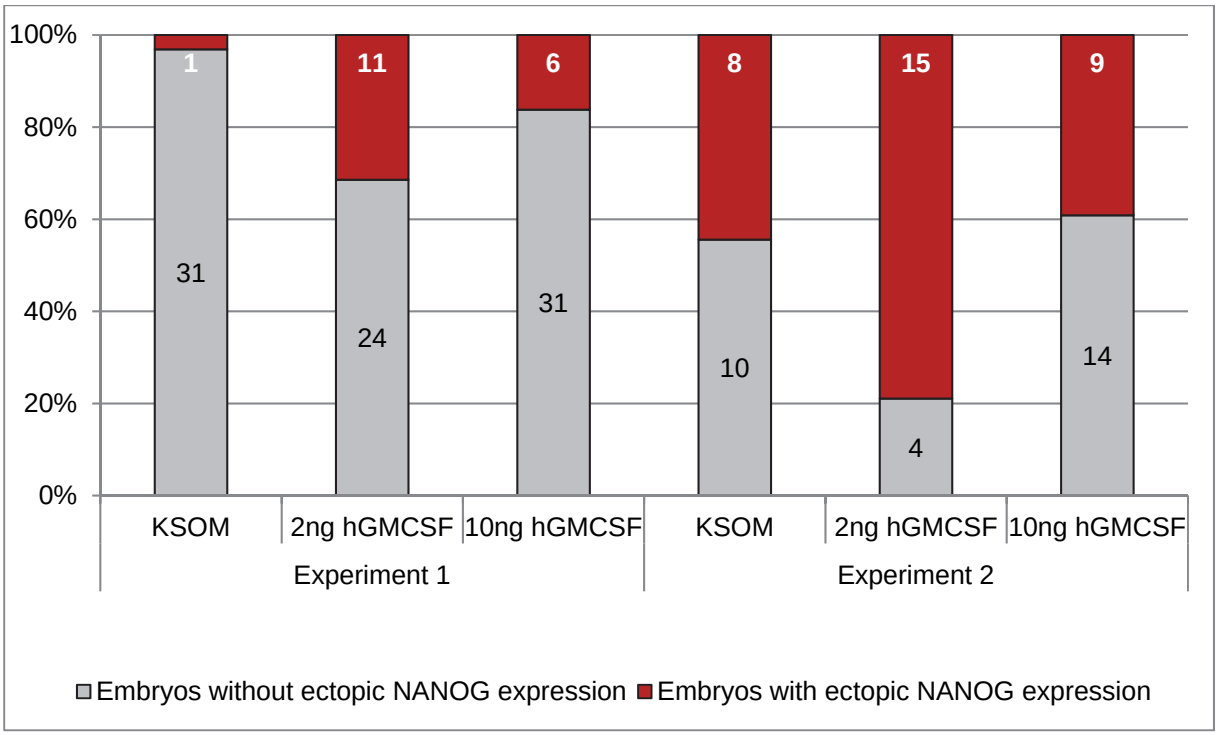

Figure 22 Percentage of occurrence of blastocysts exhibiting a double staining of the cell lineage markers CDX2 and NANOG in trophectoderm cells in the MPI experiment. Shown are KSOM(aa) and two different concentrations of human GM-CSF (2 and 10 ng/mL) in two separated experimental repeats. Grey: blastocysts without double staining; Red: blastocysts with CDX2 and NANOG positive trophectoderm cells. Bars indicate the percentage occurrence and numbers inside bars indicate absolute number of blastocysts.

Table 6 Mean values of all cell lineages, standard deviation and n-values of blastocysts with and without ectopic NANOG expression in all experimental groups.

Group		CDX2 positive		SOX17 positive		NANOG positive		CDX2+NANOG positive		n
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	
MPI normal expression	0 ng/mL GM-CSF	58.7	10.0	8.7	2.8	7.8	6.3	/	/	41
	2 ng/mL hGM-CSF	60.9	8.3	9.7	2.6	8.4	3.0	/	/	28
	10 ng/mL hGM-CSF	58.7	8.3	8.1	2.7	6.6	2.4	/	/	45
MPI atypical expression	0 ng/mL GM-CSF	66.2	10.8	9.1	2.7	5.4	2.5	5.0	1.7	9
	2 ng/mL hGM-CSF	59.8	11.4	7.3	2.5	6.8	3.4	5.9	4.2	26
	10 ng/mL hGM-CSF	60.5	13.4	6.9	3.2	5.8	2.3	7.6	4.3	15

3.6 CD1 strain experiments

To exclude a mouse strain specific effect, regarding ectopic expression of NANOG, the experiments were repeated with a different mouse strain (CD1) for egg fertilization.

3.6.1 Development rates in the CD1 experiment

For the CD1 experiment a total of 376 eggs and zygotes were retrieved on E0.5. A total of 320 4-cell stages embryos were cultured further until E4.5 from which 252 developed into blastocysts (79%). In the *in vitro* control group (KSOM(aa)) the mean development rate was 68%, in the hGM-CSF group with 2 and 10 ng/mL growth factor 80%% and 67%, respectively. In the mGM-CSF group with 2 and 10 ng/mL growth factor 86% and 75%, respectively (Figure 23).

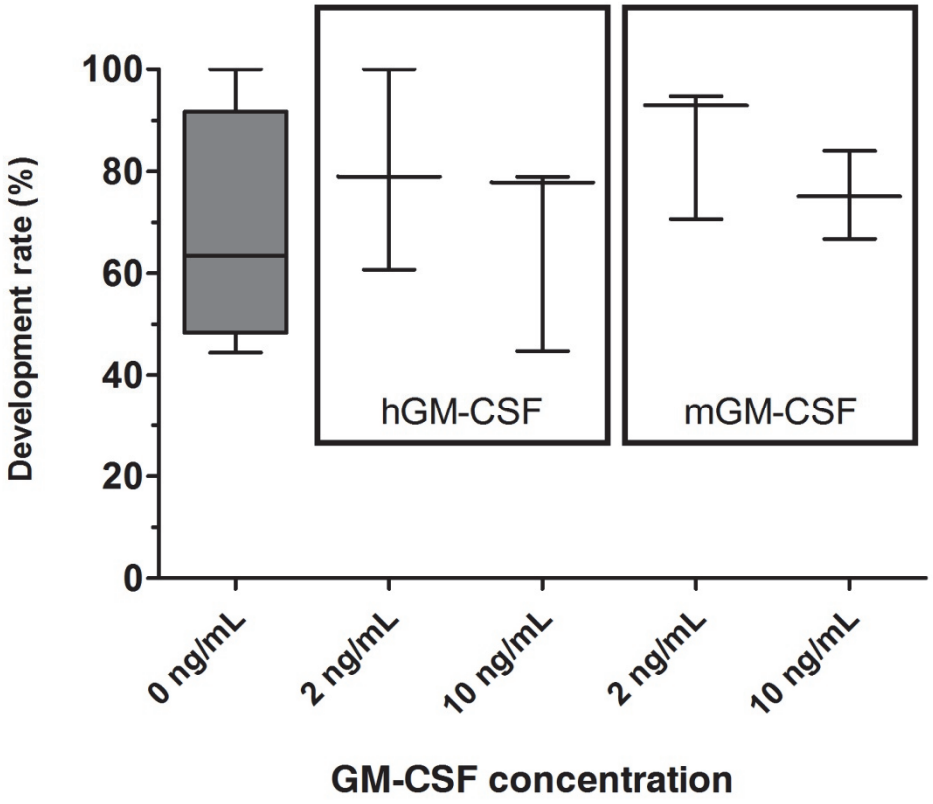

Figure 23 Development rates for the CD1 experiment. . Shown is the percentage of 4-cell stage embryos, which developed into blastocysts (E2.5-E4.5). Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance.

3.6.2 Cell number of different cell lineages in the CD1 experiment

Cell lineages showed a decrease of cell numbers between KSOM(aa) and the 10ng/mL hGM-CSF groups. 2ng/mL hGM-CSF and the mGM-CSF groups showed an equal proportion of absolute and proportional cell numbers (Figure 24, 25 and Figure S8).

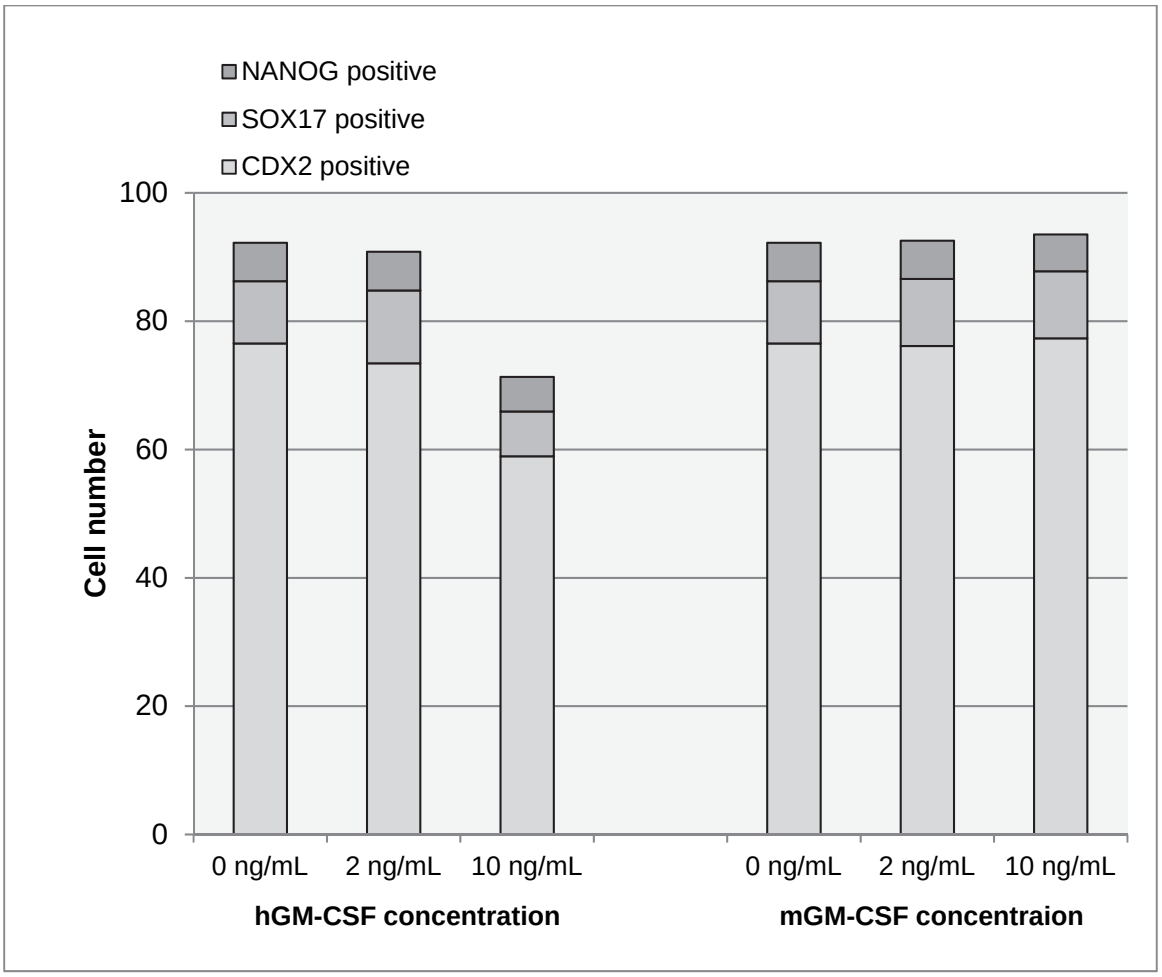

Figure 24 Comparison of mean absolute numbers of different cell lineages in the CD1 experiment. Lineages are identified by specific cell lineage markers (CDX2: trophectoderm; SOX17: primitive endoderm; NANOG: epiblast) in different mouse and human GM-CSF concentrations (2, 10 ng/mL) and the *in vitro* control medium, 0ng/mL (KSOM(aa)).

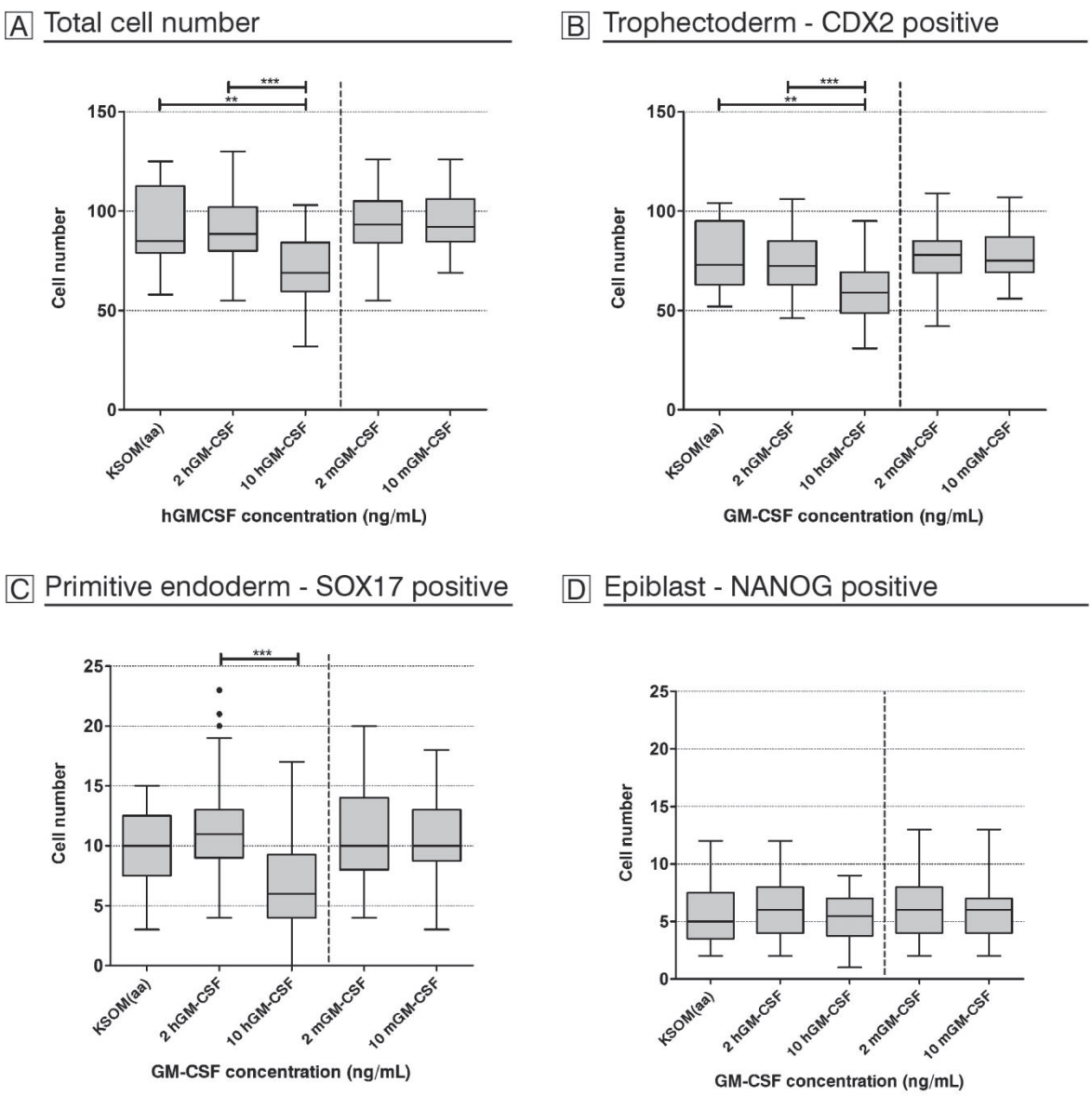

Figure 25 Effect of m- and hGM-CSF: comparison of absolute cell numbers in the CD1 experiment. A) total cells, B) CDX2 positive cells, C) SOX17 positive cells and D) NANOG positive cells in two different m- and hGM-CSF concentrations (2 and 10 ng/mL) and the *in vitro* control medium, 0 ng/mL (KSOM(aa)). Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance; dots not included between the whiskers indicate outliers. Significance values: P<0.001 (**) and P<0.0001 (***): Numbers of blastocysts: 0 ng/mL n = 13; 2 ng/mL hGM-CSF n = 48; 10 ng/mL hGM-CSF n = 42; 2 ng/mL mGM-CSF n = 46; 10 ng/mL mGM-CSF n = 38;

A total of 205 blastocysts were analyzed regarding cell lineages. 16 of these blastocysts were found with atypical expression. Analyzing the 189 blastocysts without ectopic NANOG expression showed significant differences in the total cell number and CDX2 positive cells between the *in vitro* control and 10ng/mL hGM-CSF and between 2ng/mL hGM-CSF and 10ng/mL hGM-CSF (Figure 25 & Table 7; mean values total cells: 0 ng/mL hGM-CSF: 92.2±22.7; 2ng/mL: 90.8±16.9; 10ng/mL: 71.3±17.25; P<0.001 One-Way ANOVA followed by

Bonferroni post-hoc test). Differences were also found in SOX17 positive cells between 2ng/mL hGM-CSF and 10ng/mL hGM-CSF. No differences were found in the mGM-CSF groups.

3.6.3 Amount of blastocysts with ectopic NANOG expression among different growth factor concentrations in the CD1 experiment.

Of 205 analyzed blastocysts a total of 16 (7.8%) had an ectopic expression of NANOG in trophectoderm cells (Figure 26).

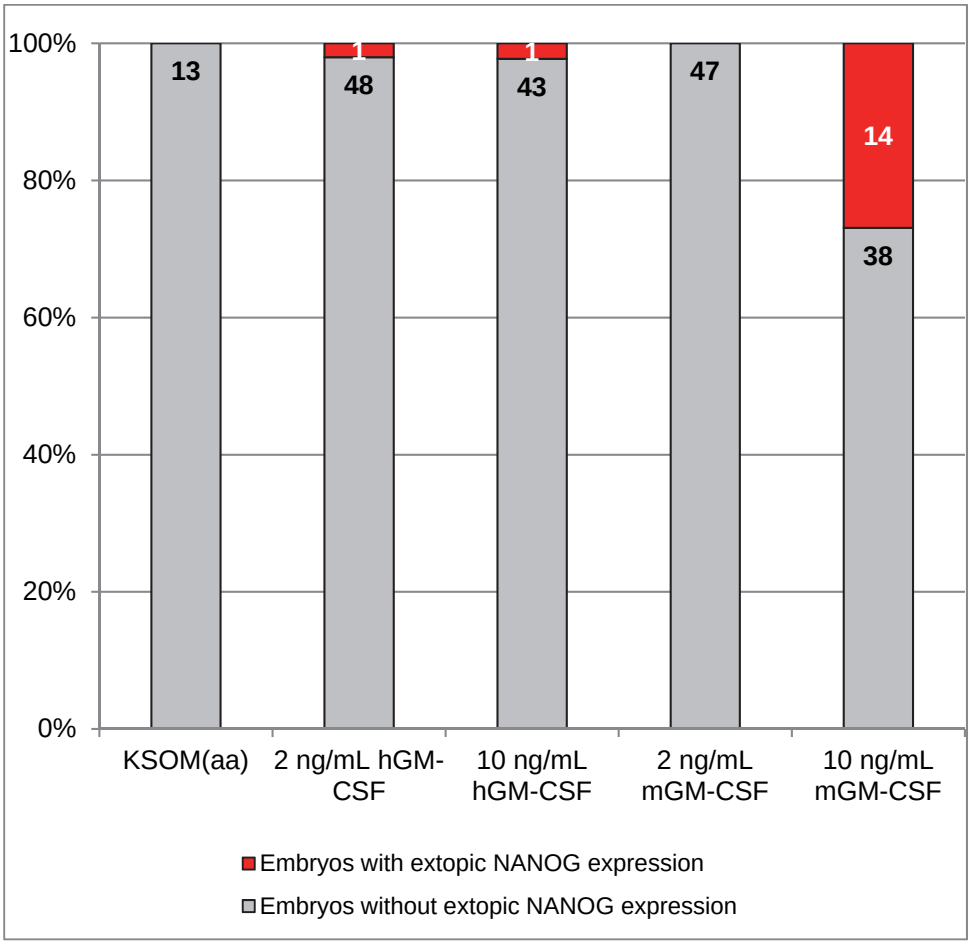

Figure 26 Percentage of occurrence of blastocysts exhibiting a double staining of the cell lineage markers CDX2 and NANOG in trophectoderm cells in the CD1 experiment. Shown are KSOM(aa) and two different concentrations of human GM-CSF (2 and 10 ng/mL) in two separated experimental repeats. Grey: blastocysts without double staining; Red: blastocysts with CDX2 and NANOG positive trophectoderm cells. Bars indicate the percentage occurrence and numbers inside bars indicate absolute numbers of blastocysts.

In the hGM-CSF groups only 1 atypical blastocyst were found per concentration. In the 10ng/mL mGM-CSF group 27% of the blastocysts showed an atypical expression pattern, whereas no ones were found in the lower concentration of mGM-CSF. All groups showed an equal proportion of absolute and proportional cell numbers (Figure 24 & Figure S8).

Table 7 Mean values of all cell lineages, standard deviation and n-values of all experimental groups.

Group		CDX2 positive		SOX17 positive		NANOG positive		CDX2+NANOG positive		n
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	
CD1 normal expression	0 ng/mL GM-CSF	76.5	17.9	9.7	3.6	6.0	2.9	/	/	13
	2 ng/mL hGM-CSF	73.4	13.6	11.4	4.5	6.0	2.3	/	/	48
	10 ng/mL hGM-CSF	59.0	14.8	7.0	3.8	5.4	2.1	/	/	42
	2 ng/mL mGM-CSF	76.1	15.2	10.5	3.8	6.0	2.3	/	/	47
	10 ng/mL mGM-CSF	77.3	11.8	10.5	3.3	5.7	2.3	/	/	38
CD1 atypical expression	2 ng/mL hGM-CSF	73	/	12	/	9	/	2	/	1
	10 ng/mL hGM-CSF	62	/	7	/	4	/	3	/	1
	10 ng/mL mGM-CSF	69.6	13.3	7.7	3.4	5.2	2.7	2.0	1.5	14

3.7 Embryo transfer experiment

To investigate the effect of GM-CSF on fetus and placenta parameters, E4.5 blastocysts were transferred back into foster mothers. After 11 days the foster mothers were sacrificed to collect the placentas and fetuses.

3.7.1 Development rates in the embryo transfer experiment

For the embryo transfer experiment a total of 724 eggs and zygotes were retrieved on day 0.5. A total of 474 4-cell stage embryos were cultured further until E4.5, 144 were transferred back into foster mothers. Two different concentrations of mGM-CSF (2 and 10ng/mL) and the *in vitro* control medium KSOM(aa) were used for this experiment. The mean development rate for the in vitro control medium was 73%, for the 2ng/mL mGM-CSF 77% and for the 10ng/mL mGM-CSF group 85% (Figure 27 & Table S4).

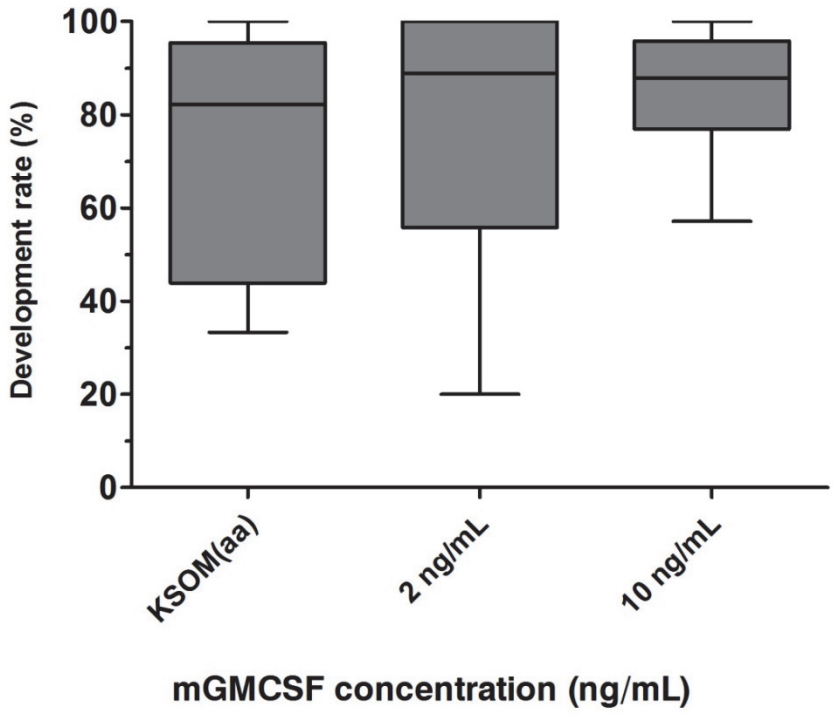

Figure 27 Development rates for the embryo transfer experiment. Shown is the percentage of 4-cell stage embryos, which developed into blastocysts (E2.5-E4.5). Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance.

3.7.2 Transferred blastocysts, implantation and fetal rate

In total, 144 blastocysts were transferred back into foster mothers (Table 8). The range of implantation among the concentration was 52-74%, but includes the number of implanted embryos which did not develop further into fetuses. The fetus rate ranges between 26 and 48% in the 2 and 10ng/mL mGM-CSF, respectively.

Table 8 Media groups and numbers of transferred embryos into foster mothers, number of implantation and number of implantations which lead to a fetus.

Medium	**Transferred Embryos**	**Implantations** (n/%)	**Fetuses** (n/%)
KSOM(aa)	38	25 (66)	11 (34)
2 ng/mL mGM-CSF	46	34 (74)	19 (26)
10 ng/mL hGM-CSF	60	31 (52)	15 (48)

3.7.3 Fetus length

A total of 45 fetuses were collected, sampled and measured to assess the effect of GM-CSF on fetus development. No significant change in the fetus length were found among the two different concentrations and the *in vitro* control medium (Figure 28; mean values: 0 ng/mL mGM-CSF: 0.156±0.1; 2 ng/mL: 1.535±.017; 10 ng/mL: 1.538±0.11).

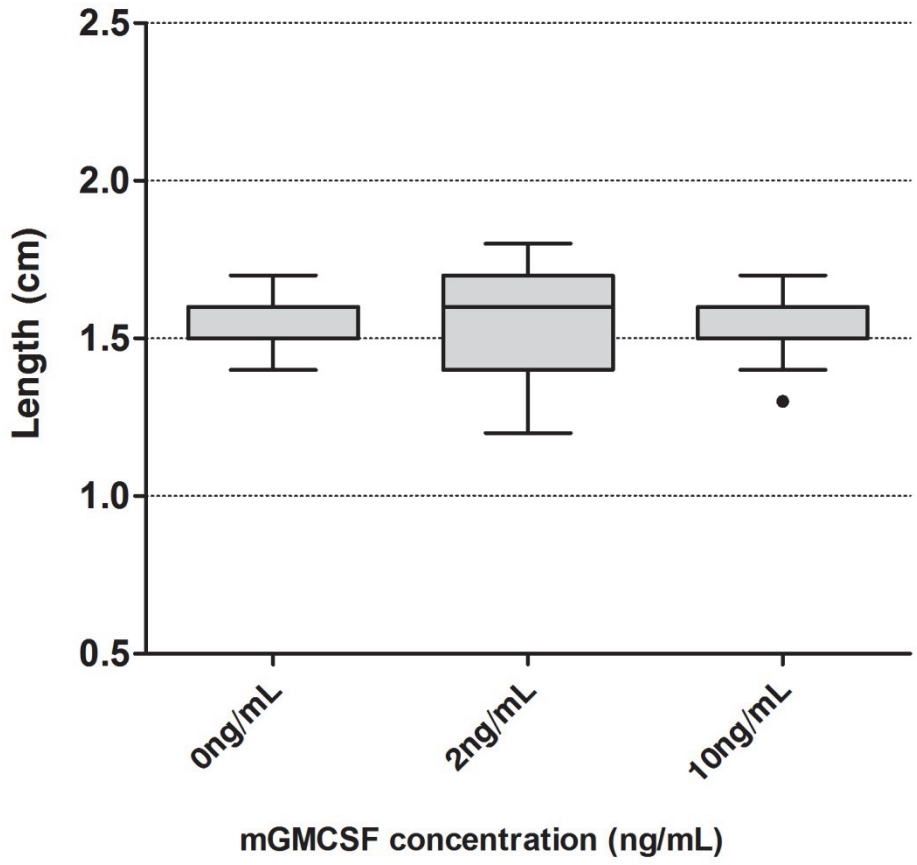

Figure 28 Effect of mGM-CSF on fetus length. Shown is the length (cm) of fetuses after 11 days of development in foster mothers. Embryos where cultured in two different concentration of mGM-CSF (2 and 10 ng/mL) and the *in vitro* control (KSOM(aa); 0 ng/mL). Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance; dots not included between the whiskers indicate outliers.

3.7.4 Fetus weight

A difference in fetus weight was found between the *in vitro* control medium and the 2ng/mL mGM-CSF group (Figure 29; mean values: 0 ng/mL mGM-CSF: 0.169±0.02; 2 ng/mL: 0.213±0.05; p<0.001 One-Way ANOVA followed by Bonferroni post-hoc test).

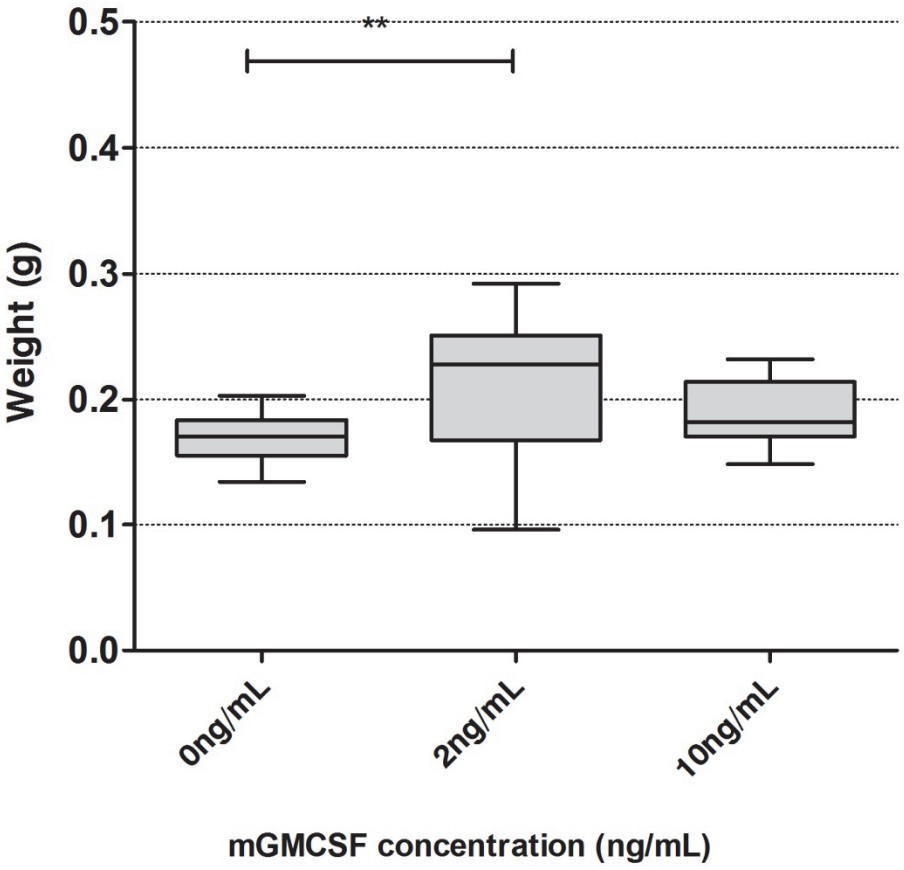

Figure 29 Effect of mGM-CSF on fetus weight. Shown is the weight (g) of fetuses after 11 days of development in foster mothers. Embryos where cultured in two different concentration of mGM-CSF (2 and 10 ng/mL) and the *in vitro* control (KSOM(aa); 0 ng/mL). Significance values: P<0.001 (**). Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance;

3.7.5 Placenta weight

No significant changes in the placenta weight were found among the two different concentrations and the *in vitro* control medium (Figure 30; mean values: 0 ng/mL mGM-CSF: 0.112±0.03; 2 ng/mL: 0.123±0.05; 10 ng/mL: 0.121±0.02).

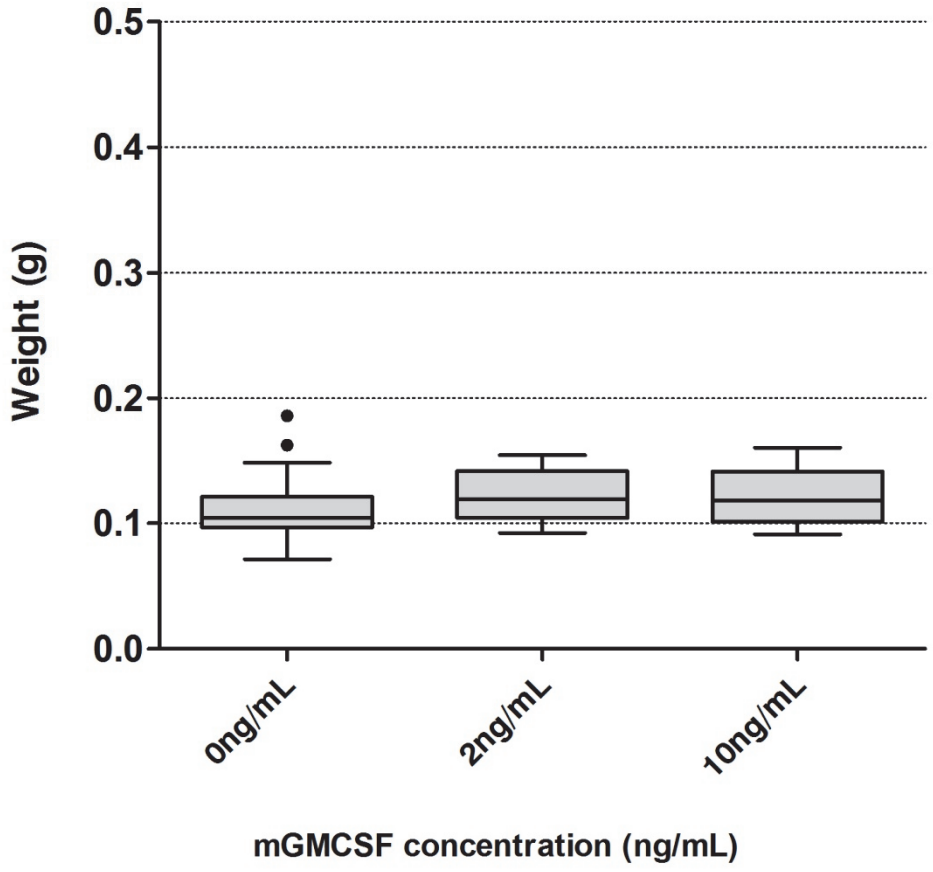

Figure 30 Effect of mGM-CSF on placenta weight. Shown is the weight (g) of fetuses after 11 days of development in foster mothers. Embryos where cultured in two different concentration of mGM-CSF (2 and 10 ng/mL) and the *in vitro* control (KSOM(aa); 0 ng/mL). Boxes illustrate median and quartiles and the whiskers extend for 1.5x the inter quartile distance; dots not included between the whiskers indicate outliers.

4 Discussion

4.1 Influence of GM-CSF on early embryo development

The success of ART is a healthy live birth. To reach this goal researchers and clinicians try to increase pregnancy rates by supporting the pre-implantation embryo as good as possible. However, it is not only important to support the embryo at its best, but it is also equally important to do no harm to the embryo. This holds true also for a culture medium that should have no influence on the normal developmental program of an embryo. Keeping this in mind it seems contrary, regarding normal embryo development that ART media and new compositions of media do neither need to fulfill official requirements nor have to pass clinical trials before they are released to the market. Therefore, this study was designed to investigate the effect of GM-CSF in ART media which aim at supporting embryo development, without knowing how it affects the cells of the embryo, especially the ICM and its cell identity.

In our study, we found that embryo culture media supplemented with GM-CSF do influence pre-implantation development of mouse embryos by enhancing cell numbers and inducing an atypical expression of the pluripotency marker NANOG in trophectodermal cells. We used an intraspecies approach and saw that mouse GM-CSF clearly had an influence on total cell numbers; they increased in the culture medium supplemented with increasing concentrations of mGM-CSF in comparison to our control medium. We did not see this increase of cell numbers in the embryos cultured in ART media with or without GM-CSF or in culture medium supplemented with different concentrations of hGM-CSF. The changes we found were caused by a marked difference in TE and primitive endoderm cell numbers (CDX2 and SOX17 positive) but not due to a change in epiblast cell numbers (NANOG positive).

Different animal models were used in the past to investigate the question of how GM-CSF might be able to influence cell proliferation. In pigs, mRNA expression of CSF2 (GM-CSF) was measured in the endometrium during the estrogen cycle and pregnancy (Jeong *et al.*, 2014). It was shown that expression was significantly increased during the implantation period. The proliferation rate of primary trophectoderm cells was increased in higher concentrations of 20 and 100 ng/mL CSF2, compared to lower concentrations (≤1 ng/mL). A mouse study compared the blastulation rate of embryos cultured in different concentrations of GM-CSF (0-10 ng/mL) and found that the rate is decreased in higher concentrations (5 and 10 ng/mL) and that cell numbers decrease in these groups. However, they did not

subdivide the total cell number of embryos in single lineages (Elaimi *et al.*, 2012). This fits partially to our findings, where we also found an increase of cells in the lowest concentration of 0 and 5 ng/mL GM-CSF, but also a decrease of total cell numbers in the highest concentration of 10 and 20 ng/mL GM-CSF. On the other hand, we did not find a decrease in blastulation rate. Our data show no differences between different concentrations of mGM-CSF. Furthermore, Elaimi *et al.* investigated if embryos show different rates of chromosomal abnormalities but found no difference. At variance with these findings, others did not find any changes in different concentrations of mouse GM-CSF in human ART culture media at all (Karagenc *et al.*, 2005). These finding may be due to increased survivability of cells in the early embryo. It was found that GM-CSF affects pre-implantation development by reducing apoptosis rate and thereby changing cell numbers in the mouse (Behr *et al.*, 2005). In human it was shown, that pre-implantation embryos express the GM-Rα subunit in each developmental stage until the blastocyst stage (Sjoblom, 2002). Together with the subunit βc, GM-Rα is part of the heterodimeric receptor complex, which binds GM-CSF. Development rates increased with medium supplemented with GM-CSF (2 ng/mL), in contrast to medium without GM-CSF or medium with GM-CSF and receptor neutralizing antibodies against GM-Rα and βc. The study also found that embryos cultured without GM-CSF have an increased rate of cell death in the ICM, which leads, together with a higher amount of TE cells, to a higher total cell number in embryos cultured with GM-CSF. The study did not subdivide the ICM in its two single-cell lineages, the primitive endoderm and epiblast. Comparing these data to our findings remains difficult due to the use of human embryos and hGM-CSF, but we found the same results of increased numbers of TE and PrE cells in our homologous system of mouse embryos and mGM-CSF

We also investigated the question if GM-CSF may influence implantation rate and fetal and placental development. Transferring E4.5 blastocysts back into the uterus of foster mothers was not an established method and was never performed before in our animal facility. Technical circumstances led to a delay of experiments, and at the end, prevented us from performing more embryo transfer experiments. Thus, we were not able to reach our aim of collecting at least 25 samples of fetuses and placentas per experimental group (determined by a power analysis). Therefore, we cannot answer, if the increase of TE and ICM cell numbers by incubation of pre-implantation embryos with GM-CSF is the sole reason for better survival or implantation of the embryo. In total, we collected 11 to 19 individual samples of fetuses and placentas per group. Implantation rate among the three different groups in this project ranges between 48 and 56% (Table S5). It is known that different culture media lead to different outcomes, regarding embryo development. Schwarzer et al.

were able to show that culture media support embryos differently before they were transferred back into the uterus and led to very poor values of implantation and fetal rates, even though the blastocysts rate showed high values at the beginning (Schwarzer *et al.*, 2012). Also, the cell lineage composition was affected by different culture media. A mouse study by Sjöblom *et al.* found no influence on embryos which were cultured in media with or without GM-CSF (2 ng/mL), compared to *in vivo* developed embryos, on the likelihood of foster mothers to become pregnant. But, GM-CSF led to an increase in viable offspring (Sjöblom *et al.*, 2005). They also show an increase of weight of fetuses cultured in medium containing GM-CSF. Additionally, offspring from the *in vitro* group shows an increase of weight later in life compared to the *in vivo* group, which was partially compensated by offspring from the GM-CSF treated embryos. Keeping this in mind, our embryo transfer data are in line with the literature, but should be interpreted carefully, because the size of experimental groups is too small. Therefore, embryo transfer experiment should be repeated in future investigations to increase the individual group size of different concentrations. Additionally, fetal organs and placentas were collected to analyze the possible effect of GM-CSF on these tissues. It was already shown that fetal and placental morphology did not differ after embryos were cultured in different media (Hemkemeyer *et al.*, 2014). Placentas and fetal organs of our project will be processed and analyzed in future projects. Further animal studies investigating the implantation and post-implantation processes and fetal development would be necessary. However, a glance at human clinical data shows a significant increase in survival and live birth rates, when pre-implantation embryos were cultured in media with GM-CSF, especially in women with previous miscarriages (Ziebe *et al.*, 2013). Additionally, chromosomal analysis showed that there is no effect on the genome, as human embryos cultured in ART media with hGM-CSF have the same ploidy rates as embryos cultured without hGM-CSF (Agerholm et al., 2010), which was also shown in mice (Elaimi *et al.*, 2012). In mice, qPCR and immunochemistry analysis revealed that mGM-CSF is able to suppress stress response and apoptosis *in vitro* (Chin *et al.*, 2009). Interestingly, the presence and concentration of human serum albumin (HSA) in a culture medium may play an important role in the occurrence of a GM-CSF effect. While Karagenc et al. found no effect if HSA was present (Karagenc *et al.*, 2005), Ziebe et al. saw that there was an effect in the presence of HSA which was even enhanced if HSA concentrations were increased from 2 ng/mL to 5 ng/mL (Ziebe *et al.*, 2013). In all our experiments we used culture media containing HSA (2 ng/mL) and found a clear effect of GM-CSF in our mouse blastocysts.

4.2 GM-CSF alters cell identity of trophectodermal cells

Unexpectedly, we found that among all embryos cultured either in mouse or in human GM-CSF supplemented media embryos showing an atypical expression pattern of the different cell lineages. This unexpected finding was an ectopic expression of NANOG among CDX2-positive TE cells in both, human ART media (with and without GM-CSF) and at increasing concentrations in the mouse and the human GM-CSF supplemented media. NANOG is a well-known transcription factor, which fulfills a key role in ICM and epiblast lineage establishment and maintenance (Boroviak et al., 2015). It also represses the trophoblast gene Cdx2 (Chen et al., 2009) which of course should be silenced in the ICM.

25% of the all analysed embryos were positive for a NANOG/CDX2 co-expression in human ART media and there already in the medium without supplementation of GM-CSF. It seems that this specific human ART medium has already the feasibility to change cell fate. Among all mGM-CSF groups, we identified a total amount of 9% embryos with an ectopic NANOG expression, and actually among the two highest concentrations an amount of 40 %. Furthermore, we identified a total of 18% embryos with ectopic NANOG expression in media supplemented with hGM-CSF. While embryos cultured in the mouse medium supplemented with mouse GM-CSF showed atypical expression only in the highest and possible supraphysiological concentrations, embryos cultured in human GM-CSF showed this co-expression already from the lowest concentration onwards. Although the frequency of embryos with atypical expression pattern was different, the absolute number of double positive cells in each embryo was equal and therefore comparable. It is remarkable that although human GM-CSF showed no influence on embryo development regarding cell numbers at all, it had a dose depended effect on the occurrence of NANOG positive trophectoderm cells in mouse embryos.

These findings are divergent from a previous study, where genes regulating the NANOG pathway were downregulated in bovine trophectoderm cells after these embryos were cultured in a medium containing 10 ng/mL GM-CSF (Ozawa *et al.*, 2016). In total, 242 upregulated and 703 downregulated genes were found in the ICM and 401 upregulated and 485 downregulated genes in the TE. Whereby, 74 genes were regulated in both ICM and TE. After a more stringent adjustment, 25 genes in the ICM and 23 genes in the TE were still up- or downregulated. These genes are partially involved in pluripotency pathways, which are regulating and maintaining pluripotency of ICM cells and in the suppression of apoptosis pathways. In contrast to our co-expression of NANOG/CDX2 in TE cells under the influence of GM-CSF, they found that genes connected to the Nanog pathway in bovine TE cells are

downregulated. The suppression of stress and apoptosis pathways were also shown in mice in an earlier study, as well as the increase of cells in the ICM and TE after embryos were cultured in GM-CSF (Chin *et al.*, 2009). Therefore, it may be speculated that GM-CSF affects the cell fate specification processes. CDX2 and NANOG are part of a transcriptional network regulating the development of trophectoderm and inner cell mass, and GM-CSF may influence this molecular machinery (Sasaki, 2010). Two scenarios explaining this ectopic expression are conceivable: 1) an error of programming of cell identity, or 2) a false positioning of correctly programmed cells. Either way, these errors could impact the pre- and post-implantation processes, possibly also in the human. A caveat of our study is that the interspecies combination with mouse embryos in a medium with the human growth factor is rather non-physiological. And we have to be cautious to translate our findings one-to-one to the human situation. Although mouse and human pre-implantation development appear to be very equal, still, a lot of differences exist, e.g. it is believed that the first and second cell lineage decision in the human is not separated as it is in the mouse, it occurs at the same time. Cells in the human embryo seem to retain plasticity longer than the cells in the mouse (Wamaitha and Niakan, 2018). Still, we believe that all these considerations do not question the message that GM-CSF has an effect on cell identity, which might probably also occur in the human. We would like to propose that the addition of e.g. growth factors to human culture media should not be done without enough clinical evidence or preceding basic research. Importantly, further work is required to identify exact effects on implantation and fetal development in animal models like mice.

4.3 Excluding mouse strain and laboratory-specific effects

To exclude mouse strain-specific and laboratory-specific effects we repeated our experiments with a different strain of mice (CD1) and in a different laboratory without changing experimental parameters. First, we used CD1 male mice, instead of C57Bl6 males, to fertilize B6C3F1 females. It was shown that development and quality of blastocysts are influenced by the choice of a specific mouse strain and that strain specificities have the potential to alter cell allocation at the blastocyst stage (Pfeiffer *et al.*, 2014). In our study we tested two different concentrations of mouse and hGM-CSF (2 and 10 ng/mL) and our *in vitro* control. Again, we were able to show the effect on cell numbers in trophectoderm and primitive endoderm in the mGM-CSF group, as described above. No effects were detectable in the NANOG cell lineage in the mGM-CSF group or in any hGM-CSF group. Surprisingly, we found only one positive embryo per concentration with coexpression of NANOG/CDX2 in the mGM-CSF group. As it was shown for the hGM-CSF group in the previous experiments we found more than 20% embryos positive for ectopic expression of NANOG at 10 ng/m.

Therefore, we believe that our findings on early embryo development are mouse strain independent. Additionally, we repeated parts of our experiments at the animal facility of Max Planck Institute of Biomedicine in Münster, without changing any experimental parameters. We repeated the experiment two times and investigated the cell numbers and occurrence of co-expression of NANOG/CDX2. For this limited amount of repeats, we decided to perform the experiment with two different concentrations of hGM-CSF only (2 and 10 ng/mL). In our experiments, we detected no differences regarding cell numbers but found ectopic expression of NANOG at both concentrations. In the repeated experiments we found a difference of SOX17 and NANOG positive cells among 2 and 10 ng/mL of hGM-CSF, which we did not find in our experiment. Regarding the occurrence of ectopic NANOG expression, both experiments showed higher rates, compared to our experiments. Especially, the second experiment at MPI shows more than 80% blastocysts with ectopic expression of NANOG at 2 ng /mL of hGM-CSF. The only difference between the second and first repeat at the MPI was the first day of hormone injection being on another day of the week, which then was followed by a cage cleaning by the staff of the animal facility, this directly before the mice were sacrificed. The day of injection was changed against the normal schedule to conduct the experiment in the same week. This would suggest that this change may be probably due to the stress of a cage cleaning, which would be a striking result. Another set of experiments should be planned to repeat the culture at the Max Planck Institute confirming these results. Despite this, we were able to show the same results in a different laboratory and believe that our findings on early embryo development are laboratory independent.

5 Conclusion & Outlook

It is still not fully understood if and how the GM-CSF acts on embryo development, implantation, and survival. In our study, we observed an increase of trophectoderm cells (CDX2 positive) in media with increasing concentrations of mGM-CSF and assume this may improve the embryo's capacity to implant and develop (Figure 10). Although, we found an effect of GM-CSF on fetus weight, this needs to be investigated with further embryo transfer experiments in the future, testing different concentrations, adding more control groups, mouse strains and maybe targeting morphological parameters of offspring. However, transcriptome and proteome analysis should be the next step, regarding our findings of altered cell identity and the cell lineage composition. There are two possible settings for experiments, regarding ‘omic’ approaches. First, pooling blastocysts and comparing the data between experimental groups, with the disadvantage of combining cell lineages and embryos. Secondly, dissecting the ICM and the TE cells to analyze them separately. This could also be conducted as a single-cell approach. Afterwards, it would be possible to cluster these cells, on the basis of their genetic profile and to compare them to different conditions. These attempts are already feasible and would be expanded and adjusted to our setting (Durruthy-Durruthy *et al.*, 2016; Petropoulos *et al.*, 2016). All retrieved placentas and fetal organs of collected fetuses must be processed further to possibly find differences among the different groups.

We also were able to detect an ectopic expression of NANOG in TE cells, while the embryo development or the number of cells did not differ among most of the experimental settings. To identify the embryos with ectopic expression and use them for transcriptome, proteome or embryo transfer experiments it would be necessary to use a marker to detect affected embryos as long as they are still alive. In our study, embryos had to be fixed and stained to investigate cell numbers. It would be possible to use a mouse strain, which expresses GFP along with or coupled with NANOG. Then, we would be able to identify embryos with ectopic expression of NANOG in trophectoderm cells and separate them from the unaffected embryos. Of course, control experiments must proof if this strain and its embryos develop, like the embryos in our experiments did.

We included the cross-species combination of mouse embryos cultured in human ART media with or without human growth factor and our *in vitro* control medium supplemented with hGM-CSF to mimic the clinical and mouse embryo assay (MEA) situation. The MEA is routinely used to assess human culture media before they are launched for the human ART market. Every human culture medium passes the test if it supports mouse embryo

development by reaching an 80 % blastocyst formation rate. Concerns exist whether this method is sufficient or sensitive enough to detect all the effects, which an artificial environment may have on the pre-implantation embryo. Investigating the cell lineage composition of embryos cultured in new media and may find abnormalities could be a future approach for such media.

Our findings may serve as a basis for further studies investigating the effect of GM-CSF on early embryo development, focusing on the cell fate decision and cell identity.

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6 Appendix

6.1 Supplementary data & statistics

6.1.1 Supplement Tables

Table S1 Development rate (%) for the ART experiment; 4-cell stage to blastocyst

ART experiments	KSOM(aa)	BlastGen™/ EmbryoGen	CleaveTM/Blast™
Experiment 1	n.a.	n.a.	n.a.
Experiment 2	81.8	93.3	78.6
Experiment 3	71.4	74.2	62.5
Experiment 4	87.5	79.3	79.2
Experiment 5	71.4	57.4	84.6

Table S2 Development rate (%) for the m- and hGM-CSF experiment; 4-cell stage to blastocyst; rates below 50% are highlighted.

Experiment	Concentration of GM-CSF					
mGM-CSF	**0 ng/mL**	**1 ng/mL**	**2 ng/mL**	**5 ng/mL**	**10 ng/mL**	**20 ng/mL**
Experiment 1	27.6	3.8	43.3	46.2	25.0	14.3
Experiment 2	73.3	53.7	53.8	53.7	71.4	55.0
Experiment 3	33.3	26.7	37.5	30.0	72.7	54.5
Experiment 4	52.0	76.5	69.6	76.1	46.7	60.0
Experiment 5	/	/	/	/	45.5	58.8
hGMCSF	**0 ng/mL**	**1 ng/mL**	**2 ng/mL**	**5 ng/mL**	**10 ng/mL**	**20 ng/mL**
Experiment 1	57.1	48.1	47.8	53.8	58.3	38.9
Experiment 2	85.7	90.0	54.5	66.7	85.7	85.7
Experiment 3	45.5	59.3	15.2	36.4	42.9	30.0
Experiment 4	50.0	60.0	86.4	57.1	77.8	85.7
Experiment 5	78.9	47.4	50.0	73.7	57.9	27.8
Experiment 6	60.0	/	/	28.0	16.7	43.5
Experiment 7	55.6	/	/	61.5	57.1	68.8

Table S3 development rate (%) for the CD1 experiment; 4-cell stage to blastocyst; rates below 50% are highlighted.

GM-CSF		hGM-CSF		mGM-CSF	
Concentration	**0 ng/mL**	**2 ng/mL**	**10 ng/mL**	**2 ng/mL**	**10 ng/mL**
Experiment 1	83.3	100.0	78.9	94.7	84.0
Experiment 2	44.4	78.9	77.8	92.9	75.0
Experiment 3	66.7	60.7	44.7	70.6	66.7

Table S4 development rate (%) for the embryo transfer experiment; 4-cell stage to blastocyst; rates below 50% are highlighted.

GM-CSF		mGM-CSF	
Concentration	**0 ng/mL**	**2 ng/mL**	**10 ng/mL**
Experiment 1	100.0	75.0	87.9
Experiment 2	78.6	100.0	81.8
Experiment 3		57.1	100.0
Experiment 4	95.7	88.9	90.9
Experiment 5	85.7	100.0	87.5
Experiment 6	94.7	100.0	100.0
Experiment 7	40.0	95.2	91.7
Experiment 8	33.3	20.0	57.1
Experiment 9	55.6	54.5	72.0

Table S5 Implantation data

KSOM(aa)	ET3	ET4	ET5 M1	ET5 M2	ET6 M1	ET6 M2	ET7	ET8 M1	ET8 M2	Total
Fetus rate (%)	x	25	37.5	12.5	0	0	x	60	60	
Implantation site only (%)	x	0	37.5	25	0	75	x	20	40	
Total		25	75	37.5	0	75	0	80	100	49.06

KSOM(aa)+2ng/mL mGMCSF	ET3	ET4	ET5 M1	ET5 M2	ET6 M1	ET6 M2	ET7	ET8		Total
Fetus rate (%)	x	75	25	12.5	12.5	62.5	x	66.6		
Implantation site only (%)	x	25	62.5	50	25	0	x	33.3		
Total		100	87.5	62.5	37.5	62.5	0	100	0	56.25

KSOM(aa)+10ng/mL mGMCSF	ET3	ET4	ET5 M1	ET5 M2	ET6 M1	ET6 M2	ET7	ET8 M1	ET8 M2	Total
Fetus rate (%)	0	x	75	25	0	0	37.5	37.5	12.5	
Implantation site only (%)	0	x	25	75	0	12.5	62.5	25	0	
Total		0	100	100	0	12.5	100	62.5	12.5	48.44

6.1.2 Supplement figures

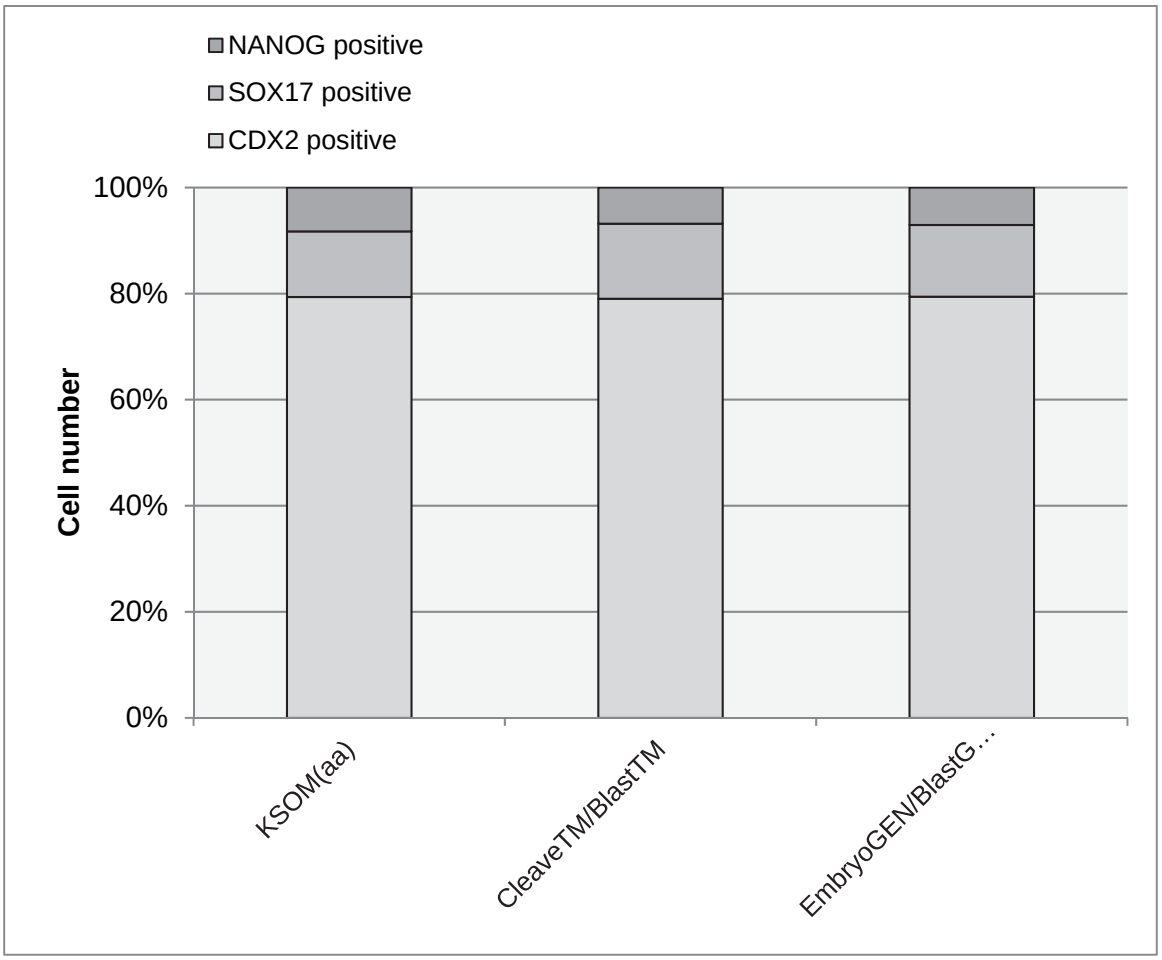

Figure S1 Proportional cell number of different lineages of the ART experiment.

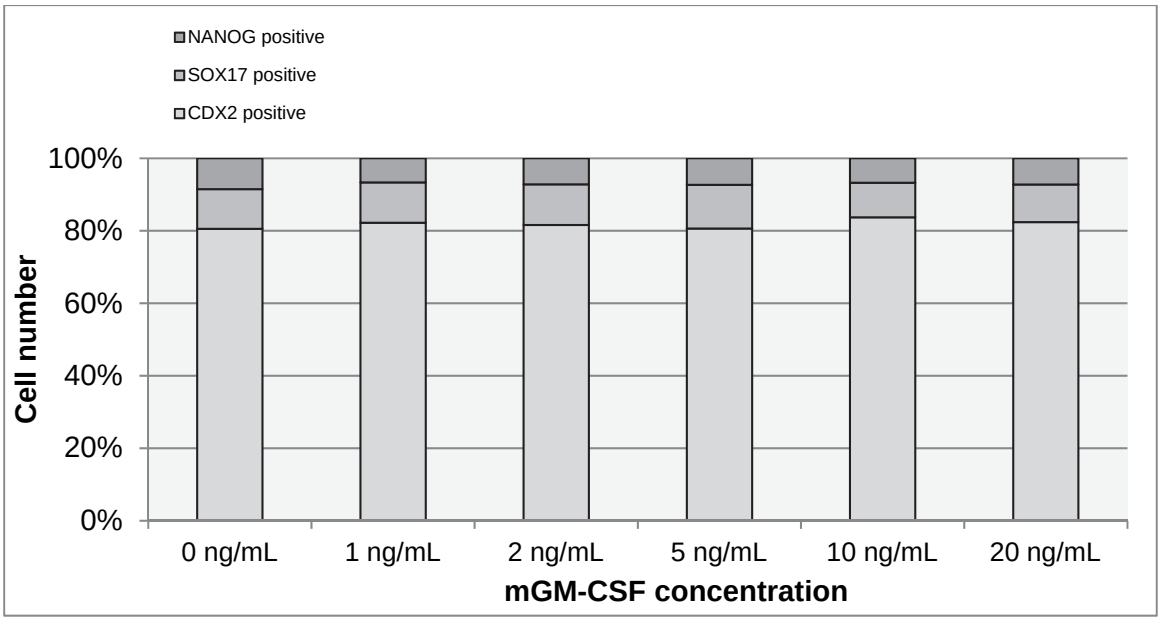

Figure S2 Proportional cell number of different lineages of the mGM-CSF experiment.

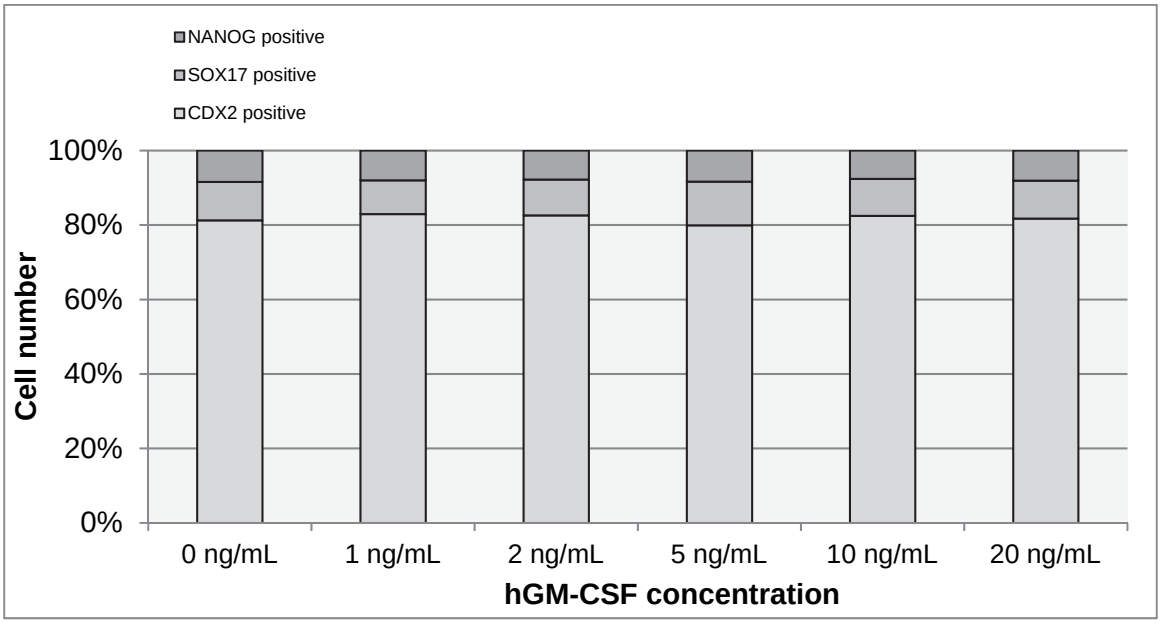

Figure S3 Proportional cell number of different lineages of the hGM-CSF experiment.

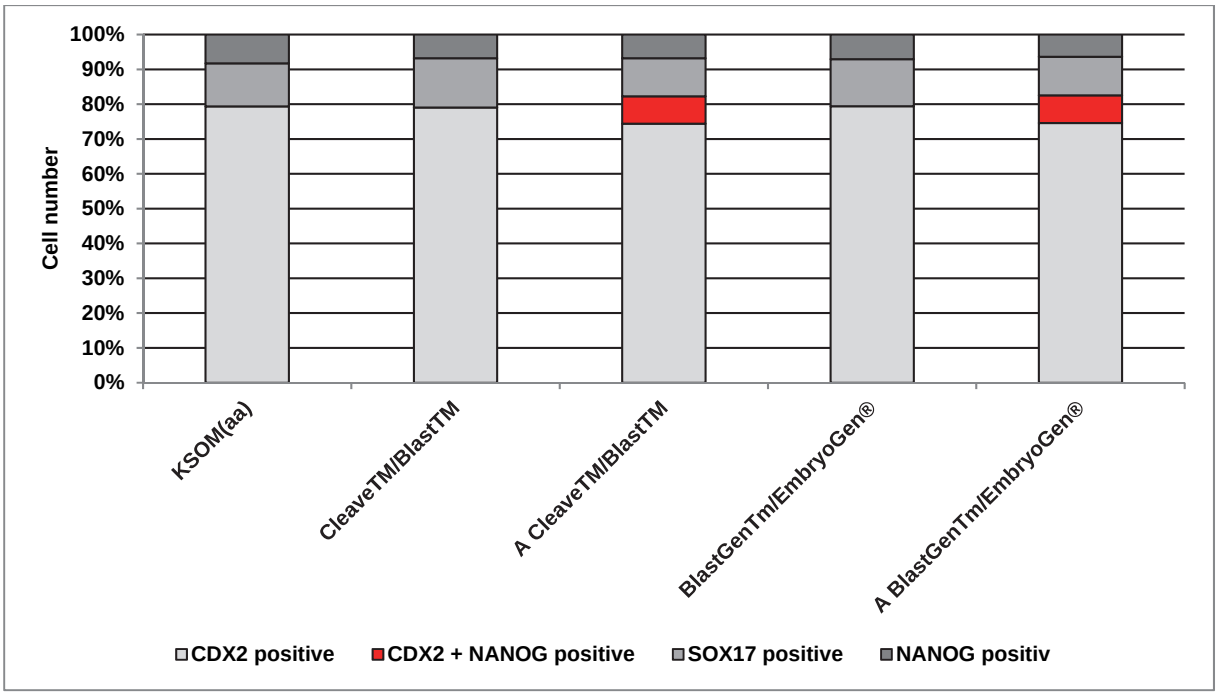

Figure S4 Proportional cell number of different lineages in ART media and with groups of embryos with ectopic NANOG expression in TE cells (red).

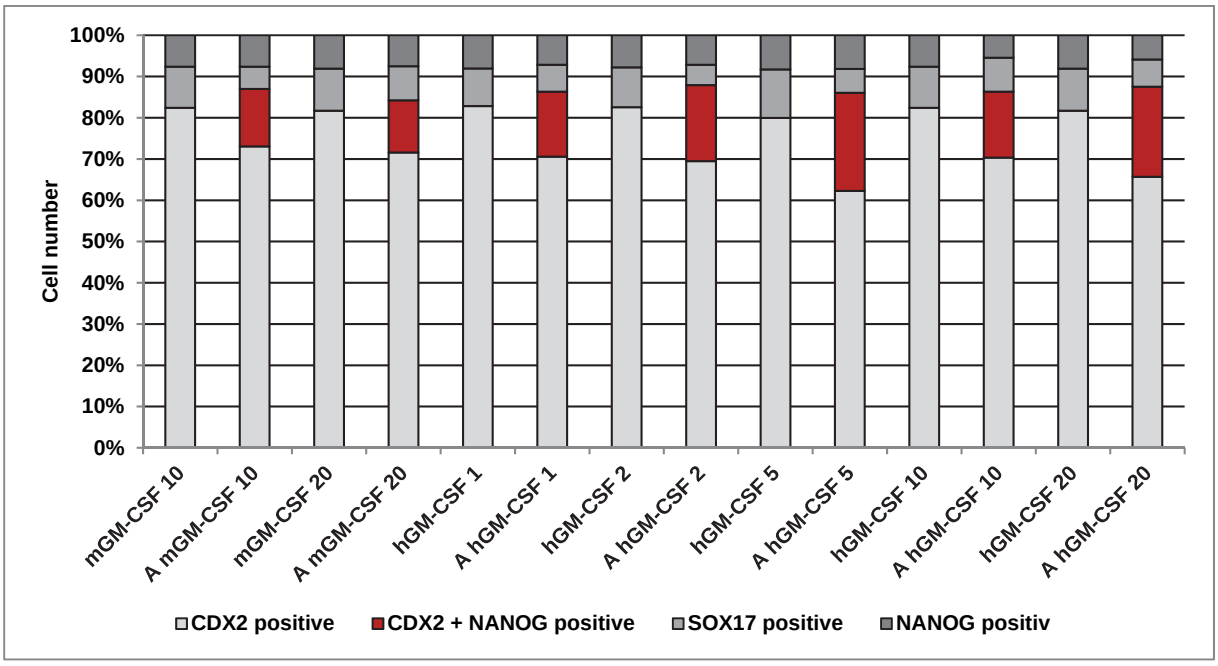

Figure S5 Proportional cell number of different lineages in ART media and with groups of embryos with ectopic NANOG expression in TE cells (red).

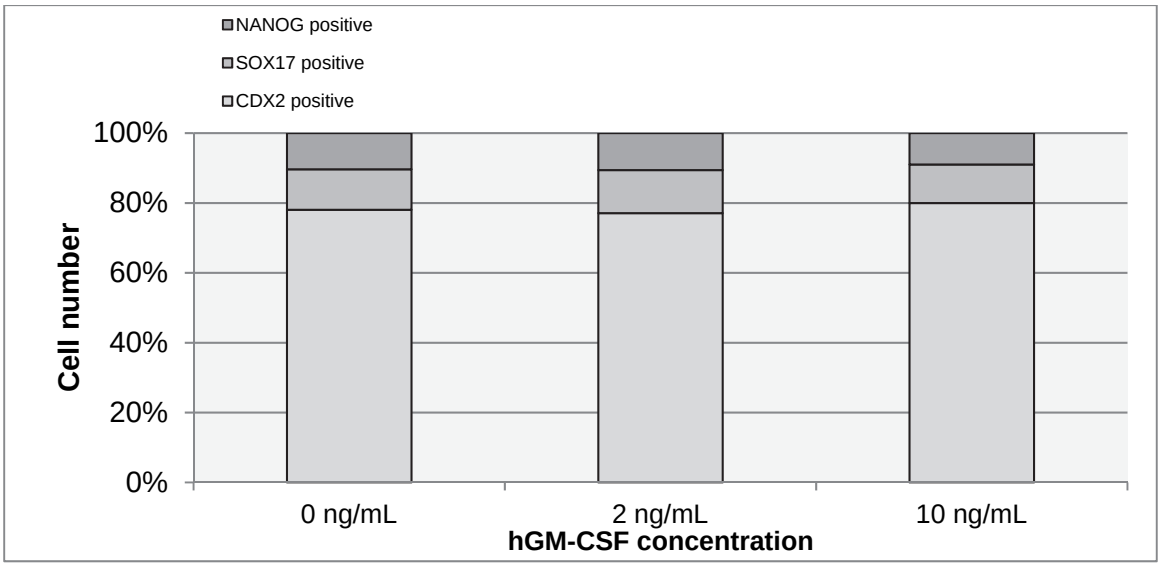

Figure S6 Comparison of mean proportional numbers of different cell lineages in the MPI experiment.

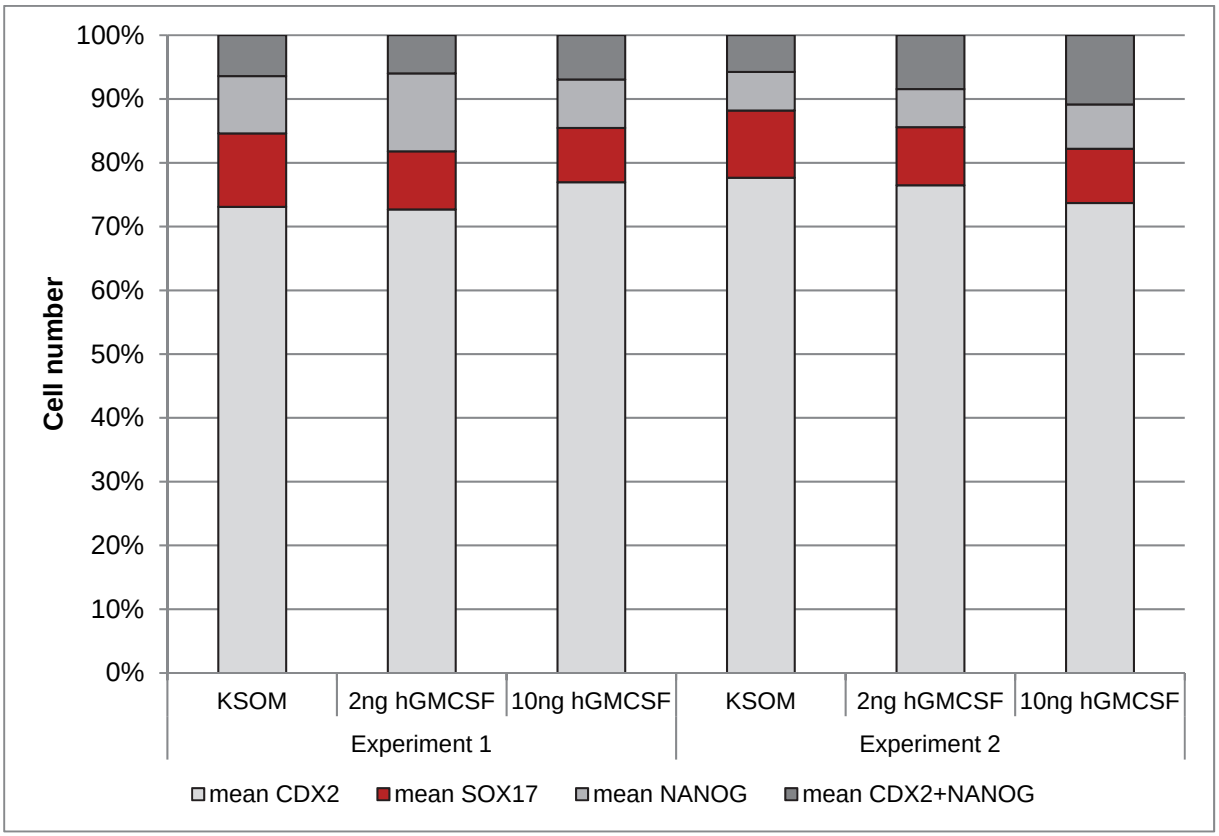

Figure S7 Mean proportional numbers of different cell lineages in blastocysts with ectopic expression of NANOG in the MPI experiment.

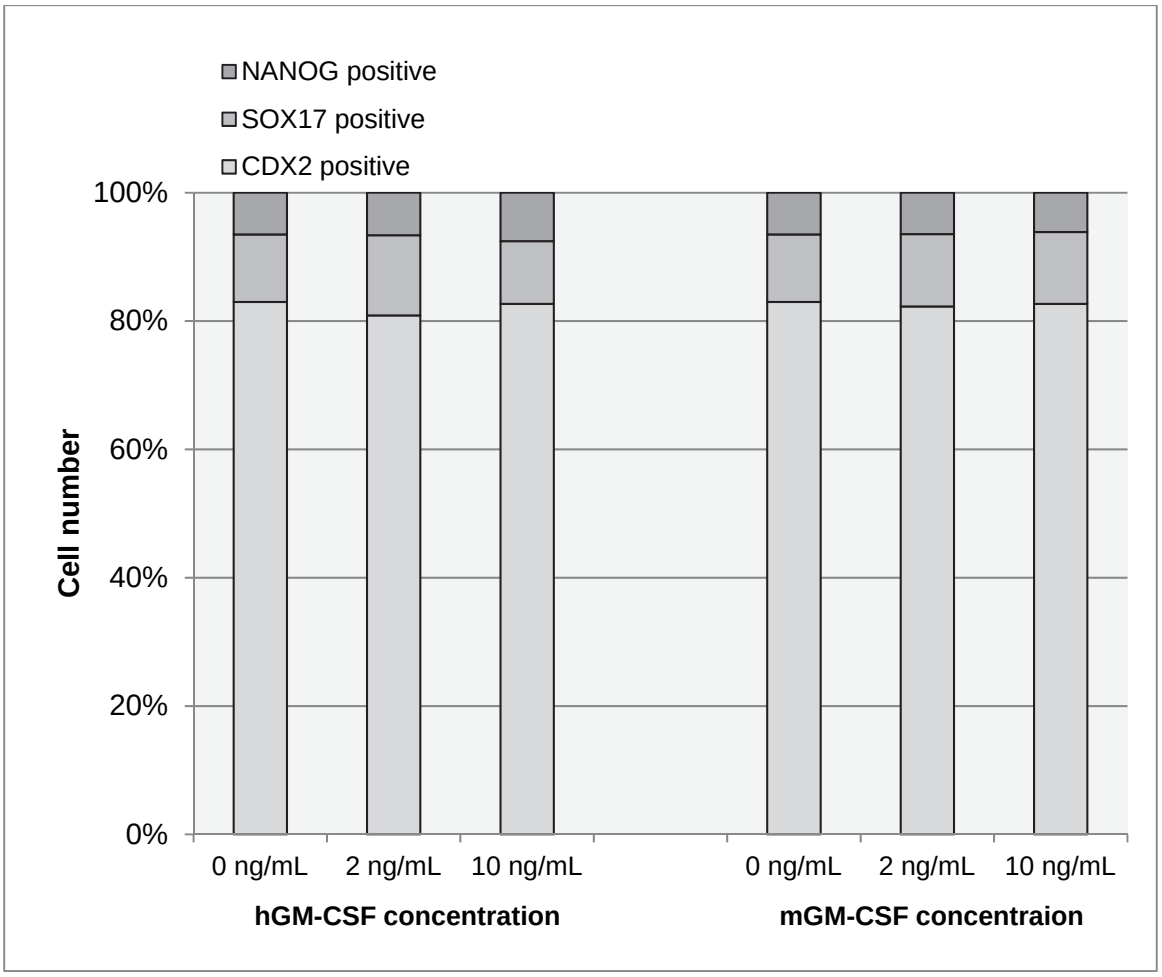

Figure S8 Comparison of mean proportional numbers of different cell lineages in the CD1 experiment.

6.2 Materials

6.2.1 Antibodies

Table S6 Primary antibodies

Antigen	Species/Isotype	Manufacturer	Catalogue #
Cdx2	Mouse	abcam	Ab157524
Sox17	Goat	R&D Systems	AF1924
Nanog	Rabbit	Cosmo Bio Co., LTD.	RCAB002P-F

Table S7 Secondary antibodies

Secondary antibody	Host	Wavelength	Manufacturer	Catalogue #
Anti-mouse	Donkey	488 nm	ImmunoResearch	711-166-152
Anti-Goat	Donkey	568 nm	ThermoFisher	A-11057
Anti-Rabbit	Donkey	647 nm	abcam	Ab150075

6.2.2 Growth factors

Table S8 Growth factors

Growth factor	Manufacturer	Catalogue #
Recombinant mouse GM-CSF	R&D Systems	415-ML-010
Recombinant human GM-CSF	R&D Systems	7954-GM-010

6.2.3 Chemicals and reagents

Table S9 Chemicals and reagents

Chemical and reagents	Catalogue #	Manufacturer
Bovine Serum Albumin (BSA)	A9647	Sigma Aldrich
Donkey serum	S30-100ML	Merck Millipore
Ethanol	/	Pharmacy UKM
Formaldehyde	1.04002.2500	Merck
Glacial acetic acid	1.00062.2500	Merck
Glycine	G7126	Sigma-Aldrich Chemie GmbH
Ovogest® (hCG)	/	MSD Tiergesundheit

Human Serum Albumin (HSA)	9988	Irvine Scientific
Hyaluronidase	H2126	Sigma-Aldrich Chemie GmbH
Mineral oil	ART-4008-5P	SAGE
Penicillin	13752	Sigma-Aldrich Chemie GmbH
Paraformaldehyde (PFA)	1.04005.100	Merck KGaA
Phosphate-buffered saline	59331C-1000	Sigma Aldrich
Picric acid	33600	Riedel-de Haen
PMSG	/	/
Streptomycin	S1277	Sigma-Aldrich Chemie GmbH
Triton X-100	X100	Sigma-Aldrich Chemie GmbH
Tween-20	P1379	Sigma-Aldrich Chemie GmbH
Tyrode's solution	T1788	Sigma-Aldrich Chemie GmbH

6.2.4 Culture media

Table S10 Culture media

Medium	Catalogue #	Manufacturer
BlastGEN	12050003	Origio®
Blast™	83060010	Origio®
Cleave™	83040010	Origio®
EmbryoGEN	12040003	Origio®
KSOM(aa)	/	In house (Michele Boiani)
M2	M7167	Sigma-Aldrich Chemie GmbH

6.2.5 Consumables

Table S11 Consumables

Consumables	Catalogue #	Manufacturer
BD Microlance	362200	Becton Dickinson
Biosphere Filter Tips 10	70.115.210	Sarstedt
Biosphere Filter Tips 100	70.760.211	Sarstedt
Biosphere Filter Tips 1000	70.762.211	Sarstedt
Cryotubes 1.6 mL red	73.380.002	Sarstedt
Eppendorf tube	0030 120.086	Eppendorf

Falcon Cellstar tube 15 mL	188271	Greiner bio-one
Falcon Cellstar tube 50 mL	227261	Greiner bio-one
Glas tube with snap-on lid	/	/
Multidish 4 wells	176740	ThermoScientific
Omnifix®-F	9161406V	Braun
Tissue culture dish 3 mm	353001	Falcon
Tissue culture dish 6 mm	353004	Falcon
WillCo Dish 3 mm	GW57-5040	/
96-well plate, round	82.1582	Sarstedt
96-well plate lid	82.1584	Sarstedt

6.2.6 Equipment

Table S12 Microscope & cameras

Microscope & cameras	Manufacturer
Microscope BX61	Olympus
Retiga 4000R camera	Qimaging
Microscope Optiphot	Nikon
AxioScan	ZEISS
Stereo microscope	Kern Optics

Table S13 Laboratory devices

Laboratory devices	Manufacturer
pH-Meter 766	Knick

6.2.7 Software

Table S14 Software

Software	Manufacturer
Olympus software	ZEISS Zen Blue
GraphPad Prism 5.0	GraphPad Software, Inc.
Miscrosoft Office software	Microsoft Corporation
ImageJ/Fiji	/

6.3 Power analysis for the ART experiment

PASS 14.0.7 22.12.2016 14:47:42 1

One-Way Analysis of Variance F-Tests

Numeric Results
Means: 30 35 40

Power	Average n	G	Total N	K	Std Dev of Means σm	Standard Deviation σ	Effect Size	Alpha
0,8148	21,00	3	63	1,00	4,08	10,00	0,4082	0,0500
0,8060	45,00	3	135	1,00	4,08	15,00	0,2722	0,0500

References

Desu, M. M. and Raghavarao, D. 1990. Sample Size Methodology. Academic Press. New York.
Fleiss, Joseph L. 1986. The Design and Analysis of Clinical Experiments. John Wiley & Sons. New York.
Kirk, Roger E. 1982. Experimental Design: Procedures for the Behavioral Sciences. Brooks/Cole. Pacific Grove, California.

Report Definitions
Power is the probability of rejecting a false null hypothesis. It should be close to one.
n is the average group sample size.
G is the number of groups.
Total N is the total sample size of all groups combined.
K is the group means multiplier.
σm is the standard deviation of the group means under the alternative hypothesis.
σ is the within group standard deviation.
The Effect Size is the ratio of σm and σ.
Alpha is the probability of rejecting a true null hypothesis. It should be small.

Summary Statements
In a one-way ANOVA study, sample sizes of 21, 21, and 21 are obtained from the 3 groups whose means are to be compared. The total sample of 63 subjects achieves 81% power to detect differences among the means versus the alternative of equal means using an F test with a 0,0500 significance level. The size of the variation in the means is represented by their standard deviation which is 4,08. The common standard deviation within a group is assumed to be 10,00.

In a one-way ANOVA study, sample sizes of 45, 45, and 45 are obtained from the 3 groups whose means are to be compared. The total sample of 63 subjects achieves 81% power to detect differences among the means versus the alternative of equal means using an F test with a 0,0500 significance level. The size of the variation in the means is represented by their standard deviation which is 4,08. The common standard deviation within a group is assumed to be 15,00.

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Fallzahlschätzung

Forschungsvorhaben: The effect of growth factor supplemented ART culture media on mouse embryo development

Allgemeine Bemerkungen
Die Fallzahlschätzung bezieht sich ausschließlich auf das oben genannte Projekt. Die Verwendung der Ergebnisse der Fallzahlschätzung im Rahmen eines anderen als des oben genannten Projektes bedarf der Zustimmung des Instituts für Biometrie und Klinische Forschung (IBKF).

Fallzahlschätzung
Die notwendige Fallzahl des Tierversuchs beträgt insgesamt **4x45=180 auswertbare Mausembryos**. Die Fallzahl ergibt sich wie folgt:

Es gibt 4 Gruppen, die sich bezüglich des Mediums unterscheiden:

1) Origio-Medium
2) Origio-Medium mit Wachstumsfaktor GM-CSF
3) KSOM (*in vitro*-Kontrolle)
4) *In vivo*-Kontrolle.

Die primäre Zielgröße ist die mittlere Gesamtzellzahl (GZZ) an Tag 4,5 (bzw. 3,5 in Gruppe 4), diese Größe ist normalverteilt. Ziel ist es zu zeigen, dass sich die mittlere Gesamtzellzahl in den Gruppen 1 bis 3 unterscheidet, also dass sie in mindestens zwei der Gruppen unterschiedlich ist.

Die Hypothesen lauten:

$$H_{123}: \mu_1 = \mu_2 = \mu_3 \text{ gegen } A_{123}: \mu_i \neq \mu_j \text{ für ein Paar } i \neq j \in \{1, 2, 3\},$$

wobei μ_i jeweils die mittlere GZZ in Gruppe i bezeichnet.

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Aufgrund von Voruntersuchungen werden folgende Annahmen für die mittlere Zellzahl in den Gruppen gemacht:

- $\mu_3 = 35$ (bekannt)
- $\mu_1 = 30$ (15% niedriger als in Gruppe 3)
- $\mu_2 = 40$ (15% höher als in Gruppe 3)

Die Standardabweichung in Gruppe 3 beträgt 12. Die Standardabweichung in den Gruppen wird sich vermutlich unterscheiden, sollte im Mittel jedoch bei ca. 30% der mittleren GZZ liegen, das entspricht einer maximalen Standardabweichung von 10-15.

Um die Hypothese H_{123} konfirmatorisch zu testen wird eine einfaktorielle ANOVA (Analysis of Variance) durchgeführt. Um mit diesem Test klinisch relevante Unterschiede (siehe Annahmen) mit einer Power von 80% zu einem Signifikanzniveau von 5% aufzudecken, wird eine Fallzahl von 3x45 auswertbaren Mausembryos benötigt (siehe Anhang). Da Gruppe 4 als Vergleichsgruppe mitgeführt wird und die Anzahl in den Gruppen ausbalanciert sein soll, werden auch in Gruppe 4 mindestens 45 Embryos benötigt. Das resultiert in einer Gesamtzahl von 180 auswertbaren Mausembryos.

Anmerkungen
Nach der Durchführung der ANOVA sollen die paarweisen Vergleiche durchgeführt werden und insbesondere die beiden folgenden Hypothesen konfirmatorisch getestet werden:

- H_{12}: $\mu_1 = \mu_2$
- H_{13}: $\mu_1 = \mu_3$

Nach dem Abschlusstestprinzip ist dafür (3-Gruppen-Vergleich) eine Anpassung des Signifikanzniveaus nicht notwendig [1]. Daher können die paarweisen Vergleiche zum vollen Signifikanzniveau von 5% konfirmatorisch getestet werden, z.B. mit dem Zweistichproben-t-Test. Die Unterschiede sind dann signifikant, wenn sowohl die globale Nullhypothese H_{123} als auch die paarweise Hypothese abgelehnt wurde.

Schlussbemerkungen
Um das Erreichen der Anzahl auswertbarer Mausembryos zu garantieren, muss die Anzahl eventuell geeignet erhöht werden.

Alle Berechnungen wurden mit PASS 14 durchgeführt.

Referenzen:
[1] Marcus, R., Eric, P., & Gabriel, K. R. (1976). On closed testing procedures with special reference to ordered analysis of variance. *Biometrika*, *63*(3), 655-660.

8 Danksagung

An dieser Stelle danke ich all denen, die mir stets mit Rat und Tat zur Seite standen und mir die Möglichkeit eröffnet haben, meine Doktorarbeit anzufertigen.

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9 Versicherungen & Eidesstattliche Erklärungen

9.1 Versicherung nach § 6 Abs. 3 (2)

Hiermit versichere ich, dass ich nicht wegen einer Straftat zu einer Strafe von mehr als einem Jahr Freiheitsentzug verurteilt worden bin, zu deren Begehung ich meine wissenschaftliche Qualifikation missbraucht habe.

I hereby declare that I have not been sentenced to more than one year imprisonment as a punishment for a crime for which I have abused my scientific qualifications.

Münster, ...

(signature)

9.2 Versicherungen nach § 6 Abs. 3, Nr. 5, 6, 9

Hiermit versichere ich, dass ich bisher noch keinen Promotionsversuch unternommen habe.

I hereby declare to not have tried to obtain a doctorate earlier.

Münster, ...

(signature)

Hiermit versichere ich, dass ich die vorgelegte Dissertation selbst und ohne unerlaubte Hilfe angefertigt, alle in Anspruch genommenen Quellen und Hilfsmittel in der Dissertation angegeben habe und die Dissertation nicht bereits anderweitig als Prüfungsarbeit vorgelegen hat.

I hereby declare that I have produced the presented thesis by myself and without unpermitted help, that all sources and aids used are indicated, and that this dissertation has not been presented elsewhere as an examination paper.

Münster, ...

(signature)

Hiermit erkläre ich mich mit der Zulassung von Zuhörerinnen/Zuhörern beim öffentlichen Teil der Disputation einverstanden. *

I hereby agree to admit an audience to the public part of my disputation.*

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