FACULTY OF MEDICINE AND HEALTH SCIENCES

# IN VITRO GENERATION OF STEM CELL DERIVED GAMETES


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Mentor: Swati Mishra
Faculty of Medicine and Health Sciences, Department of Reproductive Medicine



A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Science in Biomedical Sciences.

Academic year: 2017-2018


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# SUMMARY

**Background:** Successful *in vitro* derivation of gametes would be a major breakthrough in reproductive medicine. Several reports about *in vitro* derivation of human primordial germ cell-like cells (hPGCLCs) have been made. However, the efficiency of these protocols remains questionable. In this study, we compare the effect of pluripotency state and human leukaemia inhibitory factor (hLIF) concentration on hPGCLC derivation efficiency.

**Methods:** HPGCLCs were derived from (1) human embryonic stem cells (hESCs) containing three pluripotent states ('4i', 'RSeT' and primed) and from (2) hESCs that were additionally pre-induced for two days with basic fibroblast growth factor (bFGF) and Activin A. The importance of hLIF concentration was tested by comparing high and low concentrations during hPGCLC differentiation. By immunostaining for hPGCLC-specific markers OCT4, SOX17 and PDPN, the efficiency of each culture condition was determined.

**Results:** A significantly higher capability for hPGCLC derivation was observed from '4i' hESCs. No significant difference was observed between the 'RSeT' and primed condition. When comparing pre-induced to conventional conditions, a significant lower efficiency was observed. Additionally, no hPGCLCs were observed when cultured in the presence of a lower hLIF concentration.

**Conclusion:** We demonstrated that the competence of hESCs for PGCLC fate is determined by their pluripotent state and that high hLIF concentrations are obligatory. Further, our results showed that gamete development is not completely conserved between mice and human, as pre-induction of hESCs resulted in a low efficiency. However, more in-depth research is required to clarify the underlying processes and to provide more insights into human gametogenesis.

# SAMENVATTING

**Achtergrond:** Succesvolle *in vitro* generatie van gameten kan een grote doorbraak betekenen in reproductieve geneeskunde. Verschillende studies hebben *in vitro* generatie van humane *primordial germ cell-like cells* (hPGCLCs) gerapporteerd. Desalniettemin wordt de efficientie van deze protocollen nog in vraag gesteld. Hier onderzoeken we het effect van pluripotentie staat en *human leukaemia inhibitory factor* (hLIF) concentratie op hPGCLC generatie efficiëntie.

**Methoden:** HPGCLCs werden gegenereerd van (1) humane embryonale stamcellen (hESCs) in drie verschillende pluripotente staten (‘4i’, ‘RSeT’ en *primed*) en van (2) hESCs die bijkomend gedurende twee dagen werden gepreïnduceerd met *basic fibroblast growth factor* (bFGF) and Activine A. Het belang van *human leukaemia inhibitory factor* (hLIF) werd onderzocht door het vergelijken van hoge en lage concentraties tijdens hPGCLC differentiatie. De efficiëntie van elke cultuurconditie werd bepaald via immunokleuring voor hPGCLC-specifieke merkers OCT4, SOX17 en PDPN.

**Resultaten:** Een significant betere differentiatie naar hPGCLCs werd gezien bij ‘4i’ hESCs. Tussen 'RSeT' en' primed' condities werd geen significant verschil waargenomen. Gepreïnduceerde hESCs in vergelijking met conventionele hESCs vertoonden een significant lager rendement. Bovendien werd geen hPGCLC differentiatie waargenomen bij het cultiveren in een lagere hLIF-concentratie.

**Conclusie:** We hebben aangetoond dat de aanleg van hESCs voor hPGCLCs bepaald wordt door hun pluripotente toestand en dat een hoge hLIF concentratie noodzakelijk is. Verder toonden onze resultaten aan dat gameetontwikkeling niet volledig geconserveerd is tussen soorten aangezien gepreïnduceerde hESCs leidde tot een lagere efficiëntie. Echter, diepgaander onderzoek is nodig om de onderliggende processen te verklaren en meer inzichten te verschaffen in de menselijke gametogenese.

# 1 INTRODUCTION

For a long time, *in vitro* generation of gametes has been an important goal in reproductive medicine. An impediment to this, however, is a lack of knowledge regarding the mechanisms that regulate human gametogenesis. This is mainly due to ethical restrictions that limit access of human embryonic material at crucial stages of gamete development. Nonetheless, extensive research in mice has aided the understanding of mammalian germ cell development. Although these studies have provided us with essential information, one should be cautious with its direct extrapolation, especially for human-centric studies. As research advances, more and more significant differences between the mouse and the human model are coming to light. Therefore, more research is required to elucidate these differences and further map the complex network of processes that govern human gametogenesis.

Research has demonstrated that stem cells can serve as a starting point for the *in vitro* generation of gametes [1,2]. This is because of their capability to give rise to any cell type of the mammalian body, a feature better known as pluripotency. Multiple types of stem cells exist, based on their source, mode of derivation and culture systems. Research groups have managed to successfully reconstruct *in vitro* gametogenesis from pluripotent stem cells in mice [3,4]. However, in humans, success has been limited, but some important molecular processes have already been identified [2,5].

The development of stem cell-derived (SCD) gametes would present a promising breakthrough, which can further elucidate the underlying regulatory mechanisms of gametogenesis and aid in countering infertility. However, it is important to bear in mind that, despite the great interest in SCD gametes, guaranteeing their efficacy and safety before any clinical applications is important since gametes transmit crucial genetic information from one generation to the other [6]. Therefore, the development of a robust method to assess the impact of *in vitro* induction on the properties of derived gametes will be necessary before any further application can be planned in the future.

## 1.1 STEM CELLS AS PROGENITORS FOR GAMETES

Stem cells are defined by their ability for long term self-renewal and their potential to differentiate towards specialized cell types. Because of these properties, stem cells are a frequently used tool in regenerative medicine. As discussed earlier, one of the possible applications of stem cells is the *in vitro* generation of gametes. For this field of study, pluripotent stem cells (PSCs) are used to investigate and elucidate the processes of gametogenesis. Two types of pluripotent stem cells are available: *embryonic stem cells (ESCs)*, harvested from the inner cell mass (ICM) of blastocysts, and *induced pluripotent stem cells (iPSCs)*, reprogrammed from somatic cells. For further clinical applications, the derivation of patient-specific SCD gametes will be important. These patient-specific gametes can be obtained from *reprogrammed patient somatic cells (iPSCs)* or through *somatic cell nuclear transfer (SCNT)*.

### 1.1.1 Embryonic stem cells and their characteristics

#### 1.1.1.1 A brief history of ESCs

Embryonic stem cells (ESCs) are pluripotent stem cells derived from the inner cell mass (ICM) of pre-implantation blastocysts. This was first achieved in 1981 when the ICM of mouse pre-implantation blastocysts was isolated and cultured *in vitro*, thus creating the first mouse ESCs line [7]. Later, in 1998, the first human ESC line was successfully cultivated [8]. Although mESCs (mouse ESCs) and hESCs (human ESCs) were both derived from pre-implantation blastocysts, their characteristics and culture requirements were found to be markedly different. In 2007, a novel type of pluripotent stem cells was derived from mice post-implantation epiblasts, labelled mouse epiblast stem cells (mEpiSCs) [9]. For ethical reasons, no equivalent derivations have been attempted from human embryos. In comparison to mESCs, mEpiSCs show significant differences in their characteristics and function. Hence, mESCs and mEpiSCs were defined as having different pluripotent states, respectively *naive* and *primed* pluripotency. Since hESCs share more pluripotency features with mEpiSCs than with mESCs, they are considered to be primed stem cells.

#### 1.1.1.2 The different states of pluripotency: Primed vs. naïve

Different states of pluripotency in ESCs are defined based on differences in their origin, culture requirements, morphology, differentiation behaviour and molecular profile as indicated in Table 1. Naïve ESCs have a reduced expression of lineage commitment factors, have (in female cell lines) two activated X chromosomes, can contribute to chimera when injected into mice blastocyst and represent a more homogenous state of pluripotency [10,11]. When cultured, naïve stem cells form domed, small colonies and demonstrate the ability to be passaged as

single cells. On a molecular level, naïve stem cells will have a high expression of pluripotency factors, such as OCT4, SOX2, NANOG, KLF2 and KLF4. In comparison, primed stem cells will have an upregulation of lineage commitment factors, an inactivated X chromosome (in female cell lines) and higher DNA methylation levels without undergoing differentiation. They maintain expression of some pluripotency factors, such as OCT4 and SOX2, but have downregulated naïve pluripotency factors. Also, at the gene regulatory level, naïve and primed pluripotent cells differ and as a result, different culture conditions are needed. Naïve stem cells are most stabile when cultivated in LIF/Stat3 conditions, while primed stem cells need conditions containing bFGF and Activin A [10].

**Table 1** Characteristics of naïve and primed ES cells.

| | **Naïve stem cells** | **Primed stem cells** |
|---|---|---|
| **Predisposition** | None | Some predisposition towards certain cell types |
| **Expression of early differentiation markers** | Low | Elevated |
| **Can form chimaera** | Yes | No |
| **X Chromosome status** | XaXa (both active) | XaXi (X inactivation) |
| **Morphology** | Domed colonies | Flat colonies |
| **Survival rate as single cells** | High | Low |
| **Stabilization** | LIF/Stat3 | bFGF, Activin A |

As mentioned before, hESCs are considered to be primed pluripotent stem cells due to their shared traits with mEpiSCs [9]. However, not all characteristics of hESCs are in line with the pluripotency state that mEpiSCs attain. MEpiSCs only display primed features, whereas hESCs exhibit features that gives them certain naïve properties. Therefore, it is important to bear in mind that human ESCs are not identical to primed mEpiSCs, but that they are more likely to be *primed-like* cells with some naïve features [12].

The existence of these different types of pluripotent states was initially attributed to the difference in species and different sources of origin [12]. However, further research demonstrated that naïve pluripotent stem cells can be converted *in vitro* into the primed state by adjusting culture conditions [13]. This indicates that the pluripotency state of stem cells also depends on the used culture conditions, next to their derivation source. To date, multiple studies have provided us with evidence that it is indeed possible to generate alternative pluripotency states in hESCs [11,14]. Nevertheless, it is important to keep in mind that

pluripotency has a dynamic range rather than just the primed and the naïve state of pluripotency. Subsequently, this means that it is important to properly analyse and compare these different culture conditions in order to determine which is the best fit for the research question being answered. Also in this research project, the aim is to find the most ideal state of pluripotency suited for derivation of stem cell derived gametes.

### 1.1.2 Patient-specific PSCs

As genetic parenthood is valued by most patients, the use of patient-specific stem cells will take a central role in clinical applications of SCD gametes. Two techniques can be used to obtain patient-specific PSCs: reprograming somatic cells to obtain induced pluripotent stem cells (iPSCs) or conducting Somatic Cell Nuclear Transfer (SCNT).

#### 1.1.2.1 Induced pluripotent stem cells (iPSCs)

The first successful reprogramming of human somatic cells into pluripotent stem cells was demonstrated by Yamanaka et al. [15]. By using retroviral vectors, human fibroblasts were reprogrammed to iPSC lines by overexpressing four transcription factors: OCT4, SOX2, KLF4 and c-MYC. The obtained human iPSCs were similar to hESCs in various ways, such as morphology, proliferation and gene expression. However, the safety of these iPSCs differs from that of hESCs. Retroviral vectors as a reprogramming method poses the risk of insertional mutagenesis and both KLF4 and c-MYC are known oncogenes. Therefore, alternative methods, both viral and non-viral, were developed and are being used today.

#### 1.1.2.2 Somatic Cell Nuclear Transfer (SCNT)

Somatic Cell Nuclear Transfer (SCNT) has proven to be an alternative route for the derivation of patient-specific PSCs [16]. In this technique, the nucleus of a donor oocyte is first removed. Next, the nucleus of a somatic cell is implanted into the cytoplasm of the enucleated oocyte (Figure 1). Cytoplasmic factors reprogram the nucleus and, eventually, the cell begins to divide like an embryo. After formation of the blastocyst, embryonic stem cells can be isolated from the ICM. These hESCs contain the same genetic material as the somatic cell donor and as a result, patient-specific SCD gametes can be derived. Since no reprogramming vectors are necessary, this technique could offer a safer alternative over iPSCs. However, additional research is still required in order to determine which route is most safe for the generation of patient-specific gametes.

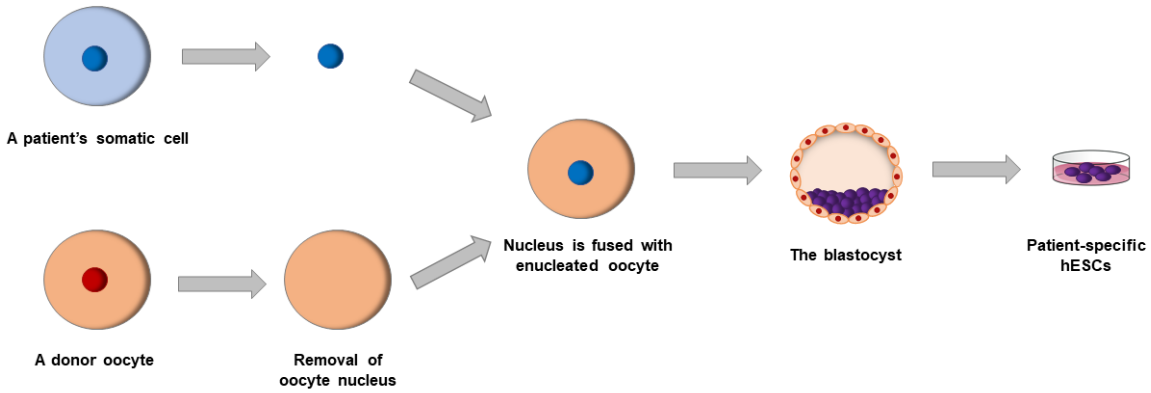


**Figure 1** A schematic representation of the technique of Stem Cell Nuclear Transfer (SCNT).

Despite the great interest in SCD gametes in the field of reproductive medicine, there is still a long way to go until these techniques can be applied safely in clinical practice. Research is still ongoing to clarify the biological processes of gametogenesis and, ultimately, to obtain human SCD gametes. Once this goal is achieved, it will be essential to assess and guarantee the safety of these SCD gametes through robust analytical methods.

## 1.2 GAMETOGENESIS: THE *IN VIVO* AND *IN VITRO* PROCESS

### 1.2.1 *In vivo* gametogenesis

Gametogenesis is a complex process, crucial for assuring the reproductive health of the individual and the correct transmission of genetic and epigenetic information across generations. Primordial germ cell development starts early on in mammalian embryogenesis. After fertilization, the zygote starts a series of cell divisions and after reaching a 16-cell stage, the blastocyst is formed. This blastocyst is composed of two cell types: the pluripotent inner cell mass (ICM) and an outer monolayer of trophectoderm (TE). After implantation into the uterine wall, the ICM separates into pluripotent epiblast and primitive endoderm. At the stage of pre-gastrulation, the development of germ cells begins when the primordial germ cells (PGCs) originate in the base of the embryo's allantois [17,18] (Figure 2). These PGCs are the earliest embryonic progenitor cells that will ultimately differentiate into germ cells. PGC development initiates around 6,5-7,25 days post coitum (dpc) in mice and around 28-36 dpc in humans [17]. After their initial formation, the PGCs migrate towards the genital ridges and start to colonize the primordial gonads. During this journey and upon arrival in the gonadal ridges, the PGCs are characterized by two essential processes: extensive epigenetic remodelling and reacquisition of pluripotency. They start to express OCT4 [18], a known marker of pluripotency, and they begin epigenetic remodelling [19], including global DNA

demethylation and chromatin modifications, to reset parental imprinting. Upon formation of the ovaries and testes, the PGCs finally start a sex-specific differentiation process into oogonia or pre-spermatogonia, respectively called oogenesis and spermatogenesis. The germ cell development trajectory continues into adulthood in both male and female.

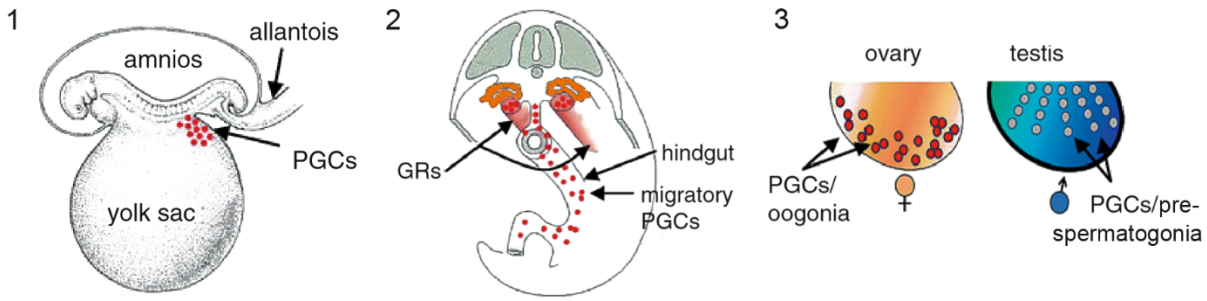


**Figure 2 Formation of primordial germ cells (PGCs) during the early embryonic development.** 1) Development of PGCs at the base of the allantois 2) Migration of PGCs towards the genital ridges (GRs) and colonization of the primordial gonads 3) Sex-specific differentiation into oogonia and pre-spermatogonia in respectively the ovary and testis. *Adapted from De Felici et al.* [17]

Due to both ethical constraints and the practical inaccessibility of the human embryo at the PGC specification stages, we are far from understanding the complex series of molecular events underlying human PGC (hPGC) development. To counter this, initial research towards SCD gametes has mostly been performed on model organisms. However, this is hampered by developmental differences between animals and human. Nonetheless, development of SCD gametes can be of great importance to obtain valuable knowledge that can clarify crucial aspects of molecular pathways and provide an insight into the *in vivo* gametogenesis process.

### 1.2.2 Artificial gametes: The *in vitro* process

#### 1.2.2.1 Mouse-based studies

As mentioned before, most of our current knowledge about mammalian gametogenesis originates from mouse models. Extensive research towards mice germ cell development has identified key signalling and transcription factors [1] and has led to the generation of mouse primordial germ cell-like cells (mPGCLCs) [1,20]. Eventually, this research resulted in the successful *in vitro* reconstitution of the processes of oogenesis and spermatogenesis [3,4,21].

##### 1.2.2.1.1 Induction of mouse primordial germ cell-like cells (mPGCLCs)

The first important steps towards *in vitro* generation of mouse gametes were reported in 2011 by Hayashi et al. [1]. Their research revealed that bone morphogenetic factor 4 (BMP4) is a

critical signalling factor for PGCLC induction. Subsequently, BLIMP1 (PRDM1), PRDM14 and TFAP2C were identified as key transcription factors for mouse PGC specification. BLIMP1 inhibits certain target genes, such as somatic cell genes and, in contrast, PRDM14 activates pluripotency-related and germ cell-specific genes. By regulating the expression of these key transcription factors, BMP4 ensures the suppression of somatic genes and the upregulation of germ cell lineage.

This study also demonstrated that direct conversion of mESCs to mPGCLCs is inefficient. However, when culturing mESCs in the presence of basic fibroblast growth factor (bFGF) and Activin A, mouse epiblast-like cells (mEpiLCs) were formed [1] (Figure 3). Compared to mESCs, these mEpiLCs showed a high competence for mPGCLCs that reached a peak after 48 hours of culture, but was largely lost after 72 hours. Subsequently, by exposure to BMP4, leukaemia inhibitory factor (LIF), stem cell factor (SCF) and epidermal growth factor (EGF), the obtained mEpiLCs differentiated to mPGCLCs. Notably, the induction of mPGCLCs starting from primed mEpiSCs was not successful. This suggested that *in vivo* PGC specification possibly occurs during transition from ESCs to EpiSCs [1].

Another study confirmed the importance of the three key transcription factors BLIMP1 (PRDM1), PRDM14 and TFAP2C. In this study, mEpiLCs were differentiated into mPGCLCs with high efficiency by overexpressing these three transcription factors without the addition of BMP4 in the medium [20]. They also reported that the overexpression of PRDM14 alone should suffice for the induction of mPGCLCs.

#### 1.2.2.1.2 Differentiation of mPGCLCs into mature gametes

Initiation and completion of meiosis, which is a process unique to oocytes and sperm cells, is one of the major challenges of generating mature germ cells. Therefore, the main characteristics of meiosis have been defined in a set of golden standards to which *in vitro* derived gametes must fulfil. These golden standards include the following: appropriate nuclear and chromosomal localization and organisation, holding a normal number of chromosomes, possessing correct nuclear DNA content at various stages of meiosis and, importantly, owning the capability to produce a viable euploid offspring [22].

The potential of mPGCLCs for spermatogenesis and oogenesis has been explored using various methods for each process (Figure 3). To reproduce the process of oogenesis, mPGCLCs were aggregated with female gonadal somatic cells to form reconstituted ovaries (rOvaries) [21]. Further, an *in vivo* step was necessary to obtain functional oocytes. For this, the rOvaries were transplanted into the ovaries of mice. The oocytes were fertilized by *in vitro*

fertilization (IVF) and, although the efficiency of this protocol was very low, healthy and fertile progeny was obtained.

More recently, the entire process of oogenesis has been successfully reconstituted *in vitro*. The previously described protocol was improved and mPGCLCs were differentiated into mature oocytes by following three steps: *in vitro* differentiation (IVD), *in vitro* growth (IVG) and *in vitro* maturation (IVM). During IVD, rOvaries were formed by aggregating mPGCLCs with female gonadal somatic cells. The rOvaries were then cultured with a combination of media to form primary oocytes. To further grow these primary oocytes, IVG medium containing follicle-stimulating hormones was used. The obtained fully-grown oocytes were further matured using IVM conditions. The generated mature oocytes were fertilized with IVF and, after transplantation into pseudo-pregnant mice, fertile offspring was obtained [3].

In one of the studies to *in vitro* reconstruct the entire male germline pathway, mPGCLCs were co-cultured with neonatal testicular somatic tissue in the presence of retinoic acid (RA), BMPs and Activin A. After 6 days, the cultures were exposed to a combination of three sex hormones: follicle stimulating factor (FSH), testosterone and bovine pituitary extract. Eventually, the mPGCLCs developed into haploid spermatid-like cells (SLCs) that displayed elements of successful meiosis: correct chromosome content and nuclear DNA, erasure of genetic imprinting and, importantly, the ability to generate viable and fertile progeny after intracytoplasmic injection into donor oocytes [4].

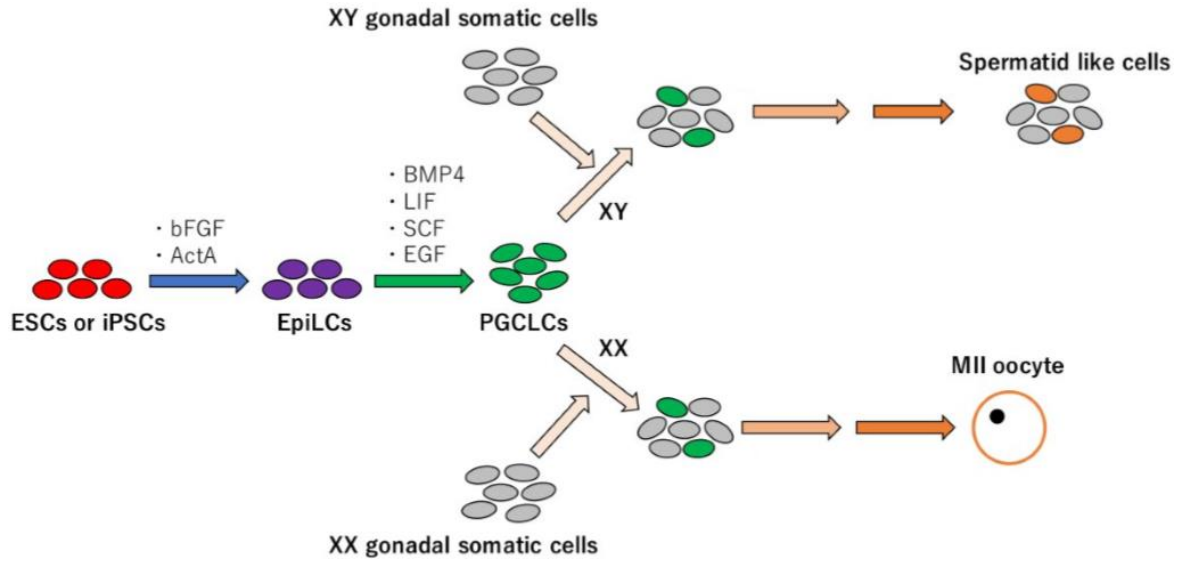


**Figure 3** The methods for *in vitro* induction of gametes in the mouse. *Adapted from Hayashi et al.* [23]

#### 1.2.2.2 Human-based studies

Most of our knowledge on human germline development is based on extrapolation of research in mice. However, more recent studies on gametogenesis in other animal models have revealed marked developmental differences with mouse models [2,24,25]. These results suggest that PGC specification is not entirely conserved between mammals and that direct extrapolation of the findings of mouse-based studies onto human development may not be entirely accurate. Remarkably, these differences are situated at different levels of development. For example, during early post-implantation, mouse epiblasts develop as a cup-shaped egg cylinder with extraembryonic ectoderm (ExE) positioned on top. In contrast, pig and human epiblasts develop as a bilaminar flat disk. This demonstrates that, in terms of morphology, mice and men do not have an equivalent early developmental structure (Figure 4).

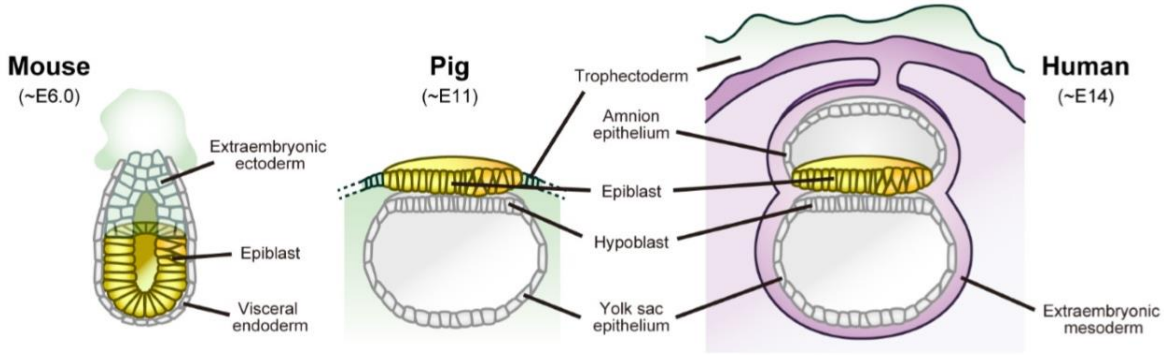


**Figure 4** Early post-implantation development in mice (1), porcine (2) and humans (3) [25].

The previously discussed conclusion was supported by various studies. In these studies, *in vitro* generation of early human primordial germ cells (hPGCs) was investigated and, importantly, key regulators of human PGC specification were identified. Moreover, it was observed that certain key regulators for mouse PGC induction do not have a similar significant role in human development.

Initial attempts for the induction of germ cell from human PSCs were performed using various methods. For one, haploid germ cell-like cells were derived from hESCs by inducing over-expression of DAZL - a late germ cell marker - and closely related genes DAZ and BOULE [26]. It was demonstrated that these haploid germ cell-like cells attained a pre-meiotic stage. In another study, haploid spermatid-like cells were induced from iPSCs via spontaneous differentiation onto monolayers of human fibroblasts [27]. However, due to a lack of

understanding of the key regulators of human PGCs, the efficiency of these studies was limited.

In the study of Irie et al. [2], human primordial germ cell-like cells (hPGCLCs) were induced in accordance with the protocol in mice as defined by Hayashi et al. [1]. Human ESCs were cultured for 4-5 days in the presence of bone morphogenetic protein 2/4 (BMP2/4), epidermal growth factor (EGF), stem cell factor (SCF) and human leukaemia inhibitory factor (hLIF) in order to derive hPGCLCs (Figure 5). A first prominent result of this study was their conclusion that 'pre-induction' of human naïve ESCs to a more *primed-like* state is not necessary, in contrast to mice. Since pre-induction resulted in a very low efficiency, this differentiation protocol was further examined using primed hESCs. However, also primed hESCs responded with a low efficiency (0-5%). As a final alternative, naïve hESCs maintained in four-inhibitor-containing (4i) medium were investigated because it was observed that gene expression of these hESCs resembles that of pre-induced hESCs after two days [2]. The 4i medium contains four inhibitors: GSK3β, MEK, p38, and JNK. More recently, the 4i culture condition was defined as a *naïve-like* culture condition which has a weak expression of naïve markers such as KLF4, similar to the primed condition [28].

After culturing the '4i' hESCs, hPGCLCs were derived with a high yield. However, some inconsistencies were observed when differentiating hPGCLCs. These results demonstrates the inefficiency of 'pre-induction' and the high competence of human naïve ESCs for hPGCLC fate, whereas this is not the case for mouse ESCs [2].

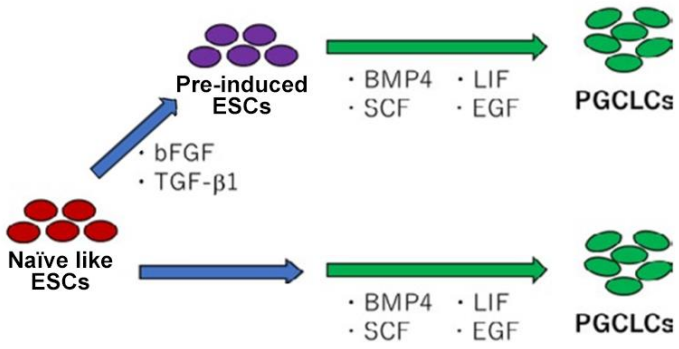


**Figure 5 Protocol as described by Irie et al. for the generation of hPGCLCs.** Human ESCs are 'pre-induced' to a more *primed-like* state by culture in bFGF and tumour growth factor (TGFβ). *Adapted from Hayashi et al.* [23]

Another striking result of this study was the identification of SOX17 - a marker for endodermal lineage - as the key regulator for human PGC specification. Obtained hPGCLCs expressed

TNAP, NANOS3 and BLIMP1, similar to that of mice PGCLCs (mPGCLCs). However, in contrast to mPGCLCs, downregulation of SOX2 and PRDM14 was observed. Furthermore, this study demonstrated that SOX17 appeared to regulate downstream BLIMP1 expression, which is a critical factor for mPGCLCs [2].

An alternative two-step differentiation system was developed to differentiate human pluripotent stem cells into hPGCLCs (Figure 6). Firstly, human ESCs and iPSCs were converted to incipient mesoderm-like cells (iMeLCs) by exposure to Activin A and WNT agonist CHIR99021 – a GSK-3β inhibitor - for 2 days. The iMeLCs exhibited a flat epithelial-like morphology and a similar expression level of naïve markers as hiPSCs. Secondly, further culture in the presence of BMP4, hLIF, EGF and SCF differentiated iMeLCs to hPGCLCs expressing BLIMP1 and TFAP2C with an efficiency of ~30%. However, no expression of late germ cell markers DDX4 and DAZL was observed [29].

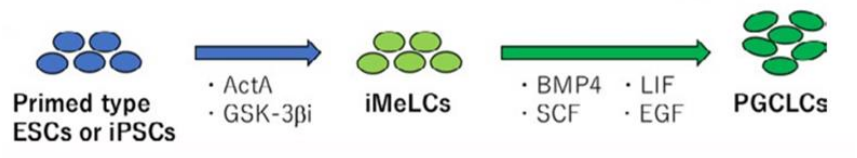


**Figure 6** An alternative differentiation protocol described by Sasaki et al. [29]. *Adapted from Hayashi et al.* [23]

Yet another step-wise approach was developed for generating pre-migratory hPGCLCs starting from human ESCs and iPSCs [5]. Primed hPSCs were converted to a mesoderm-like state by 2 days of culture in the presence of Activin A, BMP4 and bFGF. Further culture in medium containing BMP4, hLIF and ROCK inhibitor for 4 days, induced hPGCLCs. By performing molecular analysis, it was demonstrated that PRDM14, which is crucial in mice PGCLCs, has no significant role in the survival of human PGCLCs.

Research published by Duggal et al. [30] demonstrated an increased propensity of stem cells derived in Activin A towards the germ cell lineage. Additional supplementation of Activin A during germ cell differentiation upregulated early germ cell markers such as DPPA3 (otherwise known as Stella). Supplementation of BMP4 along with Activin A provided an additional boost to the culture allowing the expression of late germ cell markers such as DPPA3 and DAZL. When culturing the obtained hPGCLCs cultured further in *in vitro* maturation (IVM) medium, formation of germ cell-like clusters and induction of meiotic gene expression was observed [30].

From all previously discussed studies can generally be concluded that PGC specification in the mouse and human differ on certain levels. Some key regulators of human PGC fate have no detectable role in mouse germ cell development and vice-versa. In mice, BLIMP1 is the key regulator for mPGC development, however in humans SOX17 controls BLIMP1 expression and is essential for hPGC fate. Also, SOX2 plays no detectable role in human PGC induction, whereas it is a crucial gene for mouse PGCs. This confirms that mammalian development is conserved to a certain level, but that molecular differences between species occurs. Therefore, direct extrapolation of our knowledge is not entirely accurate, but extensive research into the development of each species is necessary. These studies have unravelled parts of the complex network of processes that govern human gametogenesis, even though many processes still remain unclear. It must be noted that the repeatability and efficiency of these methods remains debatable.

## 1.3 RELEVANCE OF STEM CELL-DERIVED GAMETES

### 1.3.1 Clinical use of SCD gametes

Assisted Reproduction Technology (ART) has been an important breakthrough in reproductive medicine. In the general population, 15% of couples are dealing with infertility [31] and although ART has provided a solution for many of these patients, some of them cannot be helped. Among those patients are men and women dealing with the absence of viable gametes, but also same sex couples, single parents and post-menopausal women [31,32]. For these patients, gamete donation by unrelated donors is the only available treatment and this, unfortunately, does not lead to genetic parenthood. Correspondingly, another difficulty is the lack of donor gametes, mainly oocytes due to the risk of complications during and after the procedure of donation [33,6]. Our lack of knowledge regarding human germ cell development has proven a major difficulty in providing these patients with a solution to have genetically related children. However, the development of SCD gametes could provide an answer for many of these patients, even for same sex couples or for post-menopausal women. It might be possible to differentiate patient-specific induced pluripotent stem cells (iPSCs) into gametes of any gender and, ultimately, this would lead to genetically related offspring. Importantly, the development of SCD oocytes or sperm cells could also provide a solution for the lack of donor gametes.

### 1.3.2 Scientific use of SCD gametes

Apart from clinical applications, stem cell-derived gametes can also be used in a scientific context where they could provide essential knowledge in the field of germ cell biology. First of

all, SCD gametes can aid the understanding of human gametogenesis [33]. Germ cell development occurs early on in embryogenesis and due to ethical restrictions, there is a major lack of human material at these early stages. As a result, research towards human gametogenesis is limited and therefore, mostly animal models are used. This situation is far from ideal, as gametogenesis is a very species-specific process. SCD gametes, on the other hand, could provide an unlimited source of more relevant research material. In this way, the largely unknown processes of human germ cell development can be clarified. Further, *in vitro* generated gametes could aid in finding new therapeutic targets to counter infertility, as well as aid in testing the effect of drug toxicity on fertility [31,33]. Also, it could help in testing the impact of environmental factors on infertility or it could provide an insight into diseases which particularly affect the germ cells [33]. Furthermore, apart from all the application mentioned above, many other possible applications for SCD gametes exists [33].

### 1.3.3 Ethics and safety concerning SCD gametes

Research on human materials is a delicate field where ethical and safety concerns constantly arise. Especially in the field of SCD gametes these concerns are current due to the significant risks and ethical implications. Even though the prospect of SCD gametes proposes promising applications, stringent guidelines and procedures will be important to guarantee the safety and functionality of these methods [34]. However, determining the safety of SCD gametes will be challenging due to various reasons. First of all, the development of robust functional and safety assays will be necessary to assess the features of the SCD gametes. Therefore, defining a number of golden standards that gametes must meet could be imperative in the future. In this way, assessing the properties and profiles of normal *in vivo* developed gametes can be of aid. Additionally, current legislations and ethical constraints in many countries prohibit the creation of embryos for research purpose. This poses a difficulty for the functional assessment of SCD gametes, which can only be done by creating *in vitro* embryos [34].

For further clinical applications, the derivation of patient-specific gametes will be essential. As discussed earlier, two methods can be used in order to obtain such gametes, iPSCs or SCNT, however, both methods have their own safety and ethical concerns [6,34]. By example, several studies have reported the occurrence of chromosomal abnormalities in continuously cultured hiPSC lines [35]. This indicates that if hiPSCs will be used as a method for the induction of patient-specific gametes, the assessment of their chromosomal properties will be essential. Further, the SCNT method uses human oocytes as a source in order to derive patient-specific oocytes. One of the concerns here is to what extent it is ethically justified to use donor oocytes in this manner, since there is a major lack of donor gametes. As both of these methods have

their own ethical and safety concerns, the comparison of gametes obtained via either route will be necessary in order to determine which route is most reliable.

Nonetheless, even though there is still a long way to go until SCD gametes can be used for any clinical or scientific applications, it is important to bear in mind that the development of these gametes holds great promise for the future.

## 1.4 PROBLEM DESCRIPTION

### 1.4.1 The importance of research on various states of pluripotency

Although there have been several claims for differentiating human PSCs towards PGCLCs, the repeatability and efficiency of these methods remain debatable. Irie et al. [2] described a robust protocol for differentiating hESCs towards hPGCLCs, however, this differentiation protocol was tested using only the '4i' condition and a single cell line. As described above, pluripotency is a highly dynamic state and the comparison between various culture conditions is essential in order to understand the impact it has on hPGCLC derivation. Therefore, in this project, the efficiency of this differentiation protocol will be examined using multiple hESC culture conditions.

### 1.4.2 Pre-induction: Necessary or not?

Research on mouse ESCs demonstrated that pre-induction towards mEpiLCs is required to gain mPGCLCs. Irie et al. [2] examined the effect of pre-induction on human ESCs and came to the conclusion that, in contrast to mESCs, hESCs do not have a need for pre-induction. However, this theory was confirmed using only the 4i condition, which has a *naïve-like* morphology but is transcriptomically more similar to the primed condition. Therefore, it is necessary to re-evaluate the need of pre-induction by examining its effect on purely naïve conditions in deriving hPGCLC. Because of its importance during mouse PGCLC induction, it will also help elucidate differences or similarities between the mouse and the human models.

### 1.4.3 The need for a high LIF concentration

Human leukaemia inhibitory factor (hLIF) is an important component of stem cell culture and mouse PGCLC differentiation [2]. However, hLIF is an expensive component and the used concentration in the differentiation medium is 10x more than during stem cell culture. Also, the reason for a high hLIF concentration during hPGCLC differentiation is not completely known apart from the fact that it improves the maintenance and induction of mouse germline cells. Therefore, the effect of a lower hLIF concentration, equivalent to stem cell cultures, during hPGCLC differentiation will be investigated.

### 1.4.4 Study design

This thesis project investigates three main research questions as described above. An important part of this research is the use of different hESC lines and various states of pluripotency. Below, in table 2, the cell lines and culture conditions used to conduct the experiments have been listed. All the hESC lines and conditions used were characterized to

ascertain their pluripotency profile using immunofluorescence. Further, for the differentiation of hPGCLCs, the protocol defined by Irie et al. [2] was used. The experimental setup of this project is represented in Figure 7.

**Table 2 The used hESC lines and culture conditions according to the performed experiment.**
Research question 1: Comparing the efficiency of various culture condition for hPGCLC derivation,
Research question 2: Investigating the effect of pre-induction on hPGCLC differentiation,
Research question 3: HPGCLC induction in the presence of a lower hLIF concentration.

| Research Question | Used hESC line | Used culture conditions |
|---|---|---|
| **1** | UGent11-4A | Primed, 4i and RSeT |
| **2** | UGent11-4A | 4i and RSeT |
| **3** | UGent11-4A and UGent11-2 | Primed, 4i, RSeT and GDN |

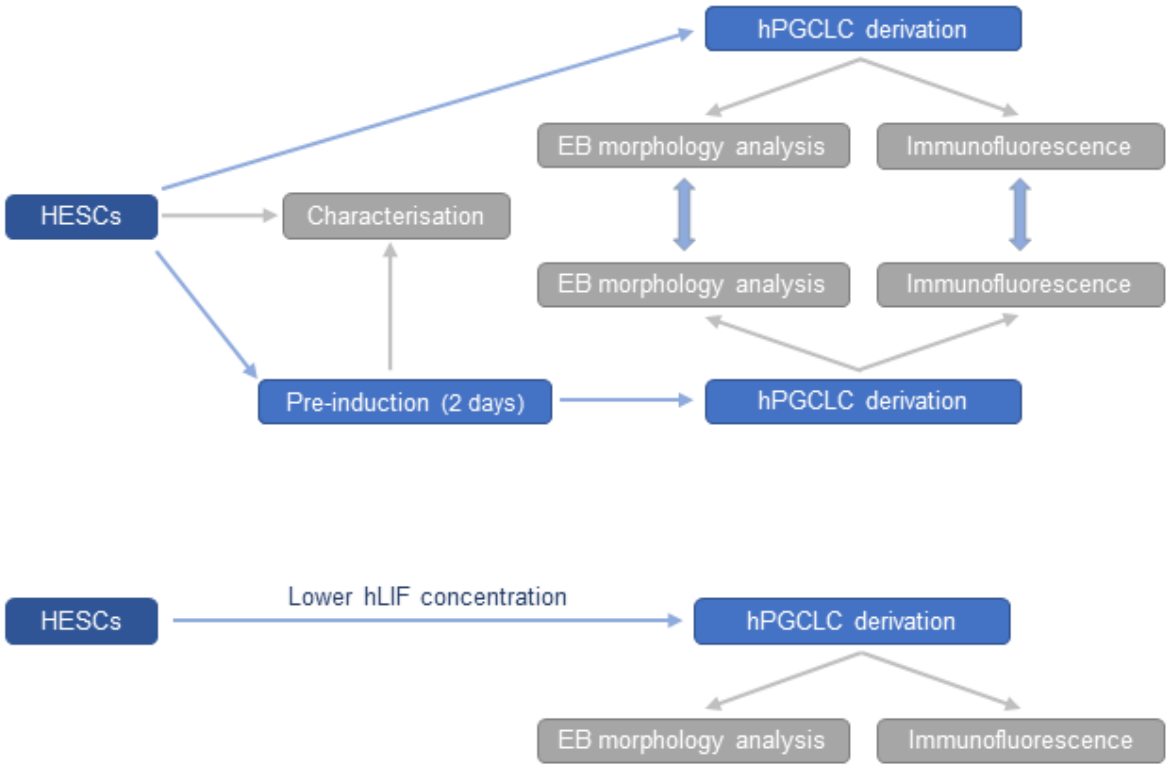


**Figure 7 Study design of this research project.**
Top schema: A representation of the workflow for research question 1 and 2.
Bottom schema: A representation of the workflow for research question 3.

# 2 MATERIALS AND METHODS

## 2.1 CULTURE OF HESCS

### 2.1.1 Culture of primed hESCs

The hESC line UGent11-4A (46, XX) and UGent11-2 (46, XX) used for these experiments were derived and cultured in house as previously described by respectively Duggal et al. [36] and Van der Jeught et al. [37]. The hESCs were maintained on a feeder layer of mitomycin C inactivated mouse embryonic fibroblasts (MEFs) and cultured in hESC medium supplemented with 4 ng/ml basic fibroblast growth factor (bFGF). The hESC medium is composed of 80% Knock-Out Dulbecco's Modified Eagle's Medium (KO-DMEM), 20% Knock-Out Serum (KOSR), 2 mM L-Glutamine, 100 U-µg/ml Penicillin/Streptomycin, 200 mM Non-Essential Amino Acids (NEAA) and 0.1 mM β-mercapthoethanol. The primed hESC cultures were refreshed daily and enzymatically passaged every 4-5 days using CTK (Collagenase Trypsin Knockout serum replacement) to ensure the undifferentiated state of the hESCs. The primed hESCs were cultured to 80-90% confluency in low oxygen conditions of 5% $O_2$ and 6% $CO_2$ at 37°C.

### 2.1.2 Culture of naïve hESCs

Both primed UGent11-4A and UGent11-2 cell lines were converted towards naïve pluripotency by performing routine passaging and culture in various conversion media, namely 4i, GDN and RSeT medium. For GDN and 4i medium, hESC medium is supplemented with small molecules as described in Table 3. Since RSeT, a naïve medium, is patented and commercially available from STEMCELL TECHNOLOGIES, no formulation data is clearly accessible. The naïve hESCs were maintained on freshly inactivated MEFs and enzymatically passaged every 3-4 days, using TrypLE Express. The naïve hESCs were cultured to 80-90% confluency at 37°C.

**Table 3** Formulation of naïve conversion media 4i and GDN

| ***Small molecules*** | **4i** | **GDN** |
|---|---|---|
| **hLIF** | 20 ng/ml | 10 ng/ml |
| **bFGF** | 8 ng/ml | 12 ng/ml |
| **TGFβ** | 1 ng/ml | - |
| **PD0325901** | 1 μM | 1 μM |
| **CHIR99021** | 3 μM | 3 μM |
| **SP600125** | 5 μM | - |
| **SB203580** | 5 μM | - |
| **ROCKi** | 10 μM | - |
| **Ascorbic Acid** | - | 50 ng/ml |
| **Forskolin** | - | 10 μM |

**Table 4** Formulation of N2B27 medium for pre-induction and hPGCLC medium

| | **N2B27 medium** | **hPGCLC medium** |
|---|---|---|
| **DMEM/F12** | 0.48 μl/ml | - |
| **Neurobasal medium** | 0.48 μl/ml | - |
| **N2** | 20 μl/ml | - |
| **B27** | 10 μl/ml | - |
| **BSA** | 5 μg/ml | - |
| **GMEM** | - | 95.8% |
| **KOSR** | - | 15% |
| **L-glutamine** | 2 mM | 2 mM |
| **Penicillin / Streptomycin** | 100 U-μg/ml | 100 U-μg/ml |
| **NEAA** | 0.1 mM | 0.1 mM |
| **β-mercapthoethanol** | 0.1 mM | 0.1 mM |
| **Sodium Pyruvate** | - | 1 mM |
| **BMP4** | - | 500 ng/ml |
| **SCF** | - | 100 ng/ml |
| **EGF** | - | 50 ng/ml |
| **ROCKi** | - | 10 μM |

## 2.2 DIFFERENTIATION OF hESCs TOWARDS hPGCLCs

### 2.2.1 Induction of hPGCLCs starting from hESCs

#### 2.2.1.1 Preplating

After culture to 80-90% confluency, the hESCs were washed using 1x Dulbecco's phosphate-buffered saline (DPBS), dissociated from the feeder layer using TrypLE Express and neutralized with MEF medium, composed of 88% 1x DMEM, 10% Fetal Bovine Serum (FBS), 2mM L-Glutamine and 100 kU-µg/ml penicillin/streptomycin. The cells were spun in a centrifuge at 750 rpm for primed cells and 800 rpm for naïve cells for 5 mins. With the supernatant discarded, the pellet was resuspended in fresh naïve/primed medium supplemented with 10uM ROCKi. These cells were preplated for 1 hour at 37°C onto gelatine coated T75 flasks. The preplating allows the remaining MEFs to attach to the coated flask in an hour while unattached hESC can be used for further experiments

#### 2.2.1.2 Embryoid Body formation

After preplating, the hESCs were plated onto ultra-low attachment U-bottom 96-well plates at a cell density of 6,500 cells/well in 100 µl PGCLC medium supplemented with either 1 µg/ml hLIF or 0.1 µg/ml hLIF according to the experiment and spun in a balanced plate centrifuge at 400g for 2 mins to force the formation of embryoid bodies. The composition of PGCLC medium is described in Table 4. Subsequently, the hESCs were allowed to differentiate as embryoid bodies (EBs) for 4.25 days at 37°C.

### 2.2.2 Induction of hPGCLCs starting from pre-induced hESCs

#### 2.2.2.1 Pre-induction of hESCs

Naïve hESCs are pre-induced according to the protocol as described by Irie et al. [2]. The hESCs were washed using 1x DPBS, dissociated from the feeder layer using TrypLE Express and neutralized with MEF medium. The cells were spun in a centrifuge at 800 rpm for 5 mins. The obtained pellet was resuspended in fresh naïve medium supplemented with 10uM ROCKi. Next, the cells were preplated for 1 hour at 37°C onto gelatine coated T75 to remove the MEFs from the cell suspension. After the preplating step, the hESCs were plated on Matrigel-coated 12-well plates at a cell density of 400,000 cells/well in 1 ml of N2B27 medium/well supplemented with 1% KOSR, 10 ng/ml bFGF, 20 ng/ml Activin A and 10 µm ROCKi. The composition of N2B27 medium is described in Table 4. The cells were cultured for 2 days at 37°C and medium was refreshed after 1 day.

#### 2.2.2.2 Differentiation to hPGCLCs by embryoid formation

After 2 days of pre-induction, the hESCs were washed using 1x DPBS, dissociated from the Matrigel using TrypLE Express and neutralized with MEF medium. Next, the hESCs were plated onto ultra-low attachment U-bottom 96-well plates at a cell density of again 6,500 cells/well in 100 µl PGCLC medium supplemented with either 1 µg/ml hLIF or 0.1 µg/ml hLIF according to the experiment. To form embryoid bodies, the cells were spun in a balanced plate centrifuge at 400g for 2 mins. Subsequently, the hESCs were allowed to differentiate as embryoid bodies for 4.25 days at 37°C.

## 2.3 IMMUNOFLUORESCENCE

### 2.3.1 Immunofluorescence of hESCs and pre-induced cells

Both hESCs and pre-induced cells, cultured on gelatine coated-glass coverslips, were washed with 1x PBS (phosphatase buffered saline) and fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Following three washing steps with 1x PBS, the cells were permeabilized for 8 minutes with 1x PBS/0.1% Triton X. Next, after a washing step with 1x PBS, the cells were blocked for 1 hour at room temperature using 1x PBS/0.5% BSA/10% Fetal calf serum (FCS). After blocking, primary antibodies (Table 5) were applied and the cells were incubated overnight at 4°C. After incubation overnight, the coverslips were washed three times with 1x PBS and incubated with secondary antibodies (Table 6) for 1 hour at room temperature. Lastly, the cells were washed in 1x PBS, incubated with DAPI for 20 minutes and then mounted in DABCO.

**Table 5** Primary antibodies used for immunostaining of coverslips

| Primary antibody | Species | Dilution factor |
|---|---|---|
| **OCT3/4** | Mouse | 1:50 |
| **NANOG** | Goat | 1:50 |
| **SOX17** | Goat | 1:50 |
| **PDPN** | Rat | 1:50 |
| **KLF2** | Rabbit | 1:50 |

**Table 6** Secondary antibodies used for immunostaining of coverslips

| Secondary antibody | Species | Dilution factor |
|---|---|---|
| **Alexa 488** | Donkey anti goat | 1:250 |
| **Alexa 594** | Donkey anti rat | 1:250 |
| **Alexa 647** | Donkey anti mouse | 1:250 |
| **Alexa 488** | Donkey anti rabbit | 1:250 |
| **Alexa 594** | Goat anti Rabbit | 1:250 |

### 2.3.2 Immunofluorescence of embryoid bodies

After 4.25 days of culture, the EBs were washed in 1x PBS and fixed in 4% PFA for 1 hour at room temperature. Next, the EBs were washed three times with a washing solution of 1x PBS/0.05% BSA for 5 minutes and then permeabilized in 1x PBS/0.5% Triton X for 1 hour. After a wash step with washing solution, the EBs were blocked for 2 hours at room temperature in a blocking solution containing 1x PBS/0.5% BSA/10% Fetal calf serum (FCS). Further, the EBs were incubated with primary antibody, diluted in the blocking solution, at 4°C overnight. The used primary antibodies are described in Table 7.

**Table 7** Primary antibodies used for immunostaining of EBs.

| Primary antibody | Species | Dilution factor |
|---|---|---|
| **OCT3/4** | Mouse | 1:175 |
| **SOX17** | Goat | 1:175 |
| **VASA** | Goat | 1:175 |
| **NANOG** | Goat | 1:175 |
| **PRDM14** | Rabbit | 1:175 |
| **cKIT** | Rabbit | 1:175 |
| **PRDM1** | Rat | 1:175 |
| **PDPN** | Rat/ Mouse | 1:175 |

After incubation overnight, three washing steps with washing solution were performed and the secondary antibodies were applied for 1 hour at room temperature. The secondary antibodies are listed in Table 8. Following three washing steps with washing solution, the EBS were placed in DAPI for 30 minutes and then mounted in DABCO.

**Table 8** Secondary antibodies used for immunostaining of EBs.

| Secondary antibody | Species | Dilution factor |
|---|---|---|
| **Alexa 488** | Donkey Anti Goat | 1:250 |
| **Alexa 488** | Donkey Anti Rabbit | 1:250 |
| **Alexa 594** | Donkey Anti Goat | 1:250 |
| **Alexa 594** | Donkey Anti Rat | 1:250 |
| **Alexa 647** | Donkey Anti Mouse | 1:250 |
| **Alexa 488** | Goat Anti Rat | 1:500 |
| **Alexa 594** | Goat Anti Rabbit | 1:250 |

### 2.3.3 Cryosection and positive controls

Fresh cryosections of mouse testis were positively stained according to the protocol described in Appendix III.

## 2.4 WORKFLOW

### 2.4.1 Positive controls

Antibodies used for the analysis of the experiments were tested positively on 10um cryosections of mouse testis. Positive controls for all the used antibodies as described in Tables 5-8 were performed according to 2.3.3. This was done to determine the specificity of the primary antibody.

### 2.4.2 Characterisation of the hESCs

Primed and naïve hESC were cultured according to 2.1.1 and 2.1.2, respectively. Depending on the experiment, different cell lines and culture conditions were used as indicated in Table 2. Before each differentiation experiment was performed, the morphology of the hESC cultures was evaluated and the characteristics of each hESC culture was determined by immunofluorescence analysis.

#### 2.4.2.1 Morphology of the cultured hESCs

Before starting each differentiation experiment, the morphology of the cultured hESCs was examined to assure confluent and clear colonies.

#### 2.4.2.2 Analysis of the characteristics of the hESCs

To further analyse the characteristics of the cultured hESCs, immunostaining was performed according to 2.3.1. The stem cells were stained for pluripotency markers OCT4 and NANOG and naïve marker KLF2 to determine their pluripotency and naïve profile. It is important that each hESC culture exhibits OCT4 and NANOG as a conformation of their pluripotency features. In contrast, KLF2 is only expected to appear in naïve hESC cultures. Further, primed and naïve hESCs were stained for SOX17 and PDPN to determine if these markers occurred before differentiation. Correspondingly, negative controls were performed for each staining to exclude non-specific binding of the primary antibodies. After staining, the hESCs were analysed using EVOS FL Cell Imaging System.

### 2.4.3 Comparing the efficiency of various culture condition

In the first part of this research, we determined the efficiency of various culture conditions for induction of hPGCLCs. The compared culture conditions are indicated in Table 2. The hESCs were cultured until 80-90% confluency and were next differentiated towards hPGCLCs according to the protocol as described in 2.2.1. The morphology of the obtained embryoid bodies was examined and immunofluorescence analysis was performed to determine the characteristics of the embryoid bodies.

#### 2.4.3.1 Morphology of the cultured embryoid bodies

Embryoid bodies of various conditions were cultured for 4.25 days and daily pictures were taken with EVOS XL Core Cell Imaging System. The size of each embryoid bodies was examined.

#### 2.4.3.2 Immunofluorescence analysis to determine hPGCLC features

For further analysis, we stained the obtained embryoid bodies (EBs) according to the protocol as described in 2.3.2. Immunostaining was performed for various markers as indicated in Table 9. After staining, the EBs were imaged using a Leica SP8 Confocal microscope and before analysis, all the images were processes according to 2.4.6. We also performed negative controls for each staining to exclude nonspecific binding of the primary antibodies.

**Table 9** Immunostaining of the embryoid bodies was performed for the markers described below.

| Markers | Characteristics |
|---|---|
| **OCT4** (POU5F1) | Pluripotency marker<br>Indicates hPGCLCs when it occurs together with SOX17 and PDPN |
| **SOX17** | Endodermal maker<br>Indicates hPGCLCs when it occurs together with OCT4 and PDPN |
| **PDPN** | Germ cell marker<br>Indicates hPGCLCs when it occurs together with OCT4 and SOX17 |
| **cKIT** | Late germ cell marker |
| **VASA** (DDX4) | Late germ cell marker |
| **NANOG** | Pluripotency marker |
| **PRDM14** | Mouse-specific germ cell marker |
| **BLIMP1** (PRDM1) | Mouse-specific germ cell marker |

##### 2.4.3.2.1 Comparing the efficiency of various culture conditions on the derivation of hPGCLCs

According to Irie et al. [2], cells expressing both OCT4 and SOX17 can be regarded as hPGCLCs. However, a more recent study has identified Podoplanin (PDPN) as another hPGCLC-specific surface marker [24]. Therefore, in this thesis, embryoid bodies were stained for the combination of OCT4, SOX17 and PDPN. When a cell shows to be positive for all these three markers, we regarded it as an hPGCLC. This triple staining was performed on four embryoid bodies of each condition. To determine the effect of each culture condition on hPGCLC induction, the number of triple positive cells were counted and the numbers of each culture condition were compared. Statistical analysis of the obtained data was performed using the PRISM 7 software from Graphpad in order to assess range, distribution and standard deviation. Column statistics analyses was performed to determine if the data of the replicates

followed a normal distribution. Individual data sets were compared using a multiparametric one way-ANOVA test to determine significant differences, if any, between hPGCLC differentiation from the various hESC conditions. Further, the percentage of hPGCLCs was calculated to the total number of DAPI positive cells in each embryoid body as indicated in Equation 1.

$$\%\ hPGCLCs = \frac{Number\ of\ triple\ positive\ cells}{Total\ count\ of\ DAPI\ positive\ cells}$$

**Equation 1** Calculation of the percentage of hPGCLCs of each culture condition

### 2.4.4 The effect of pre-induction on hPGCLC differentiation

The second part of this research aimed at evaluating the effect of pre-induction on PGCLC differentiation. In order to achieve this, hESCs in RSeT and 4i conditions were cultured until 80-90% confluency and were then pre-induced according to 2.2.2.1. Subsequently, the characteristics of the pre-induced cells were examined using immunofluorescence analysis to determine what effect pre-induction has on the features of the cells. After two days of culture, the pre-induced cells were differentiated to hPGCLCs by following the protocol as described in 2.2.2.2. The morphology of the obtained EBs was assessed and immunofluorescence analysis was performed to determine the number of triple positive cells within each EB.

#### 2.4.4.1 Assessment of the properties of the cells during pre-induction

During the two days of pre-induction, the morphology of the cells was examined. Again, pictures were taken each day with EVOS XL Core Cell Imaging System.

Since we do not know what effect pre-induction has on the properties of the stem cells, immunofluorescence analysis was performed for further characterization. The pre-induced cells were stained according to the protocol as discussed at 2.3.1. By staining the cells for pluripotency markers OCT4 and NANOG and naïve marker KLF2, their stem cell-specific properties were studied. Further, to check the status of hPGCLC markers in the pre-induced cells, we performed a staining for SOX17 and PDPN. Negative controls were performed for each staining to eliminate non-specific binding of the primary antibodies. The stained hESCs were analysed using EVOS FL Cell Imaging System.

#### 2.4.4.2 Differentiation of the pre-induced cells to hPGCLCs

As mentioned before, the pre-induced cells were differentiated as embryoid bodies in medium to induce hPGCLCs. During culture of EBs, their morphology was studied and pictures were taken every day. To further analyse the effect of pre-induction on hPGCLC differentiation, we performed immunostaining for the markers as described in Table 9. The stained embryoid

bodies were imaged using a Leica SP8 Confocal microscope and the images were processed according to 2.4.6. By staining the embryoid bodies for OCT4, SOX17 and PDPN, the effect of pre-induction was determined. Negative controls for each staining were also performed to dismiss nonspecific binding of the primary antibodies.

#### 2.4.4.2.1 Determining the effect of pre-induction on hPGCLC derivation

Four embryoid bodies of each pre-induced condition were triple stained for markers OCT4, SOX17 and PDPN. The number of hPGCLCs was counted for each pre-induced culture conditions and compared to each other. Also, the percentage of hPGCLCs of each condition was calculated using Equation 1. Subsequently, in order to assess the effect of pre-induction, the obtained numbers of hPGCLCs of each condition were compared with the earlier counted numbers of their 'non-pre-induced' counterpart. Statistical analysis was performed according to 2.4.3.2.1 in order to determine if the observed differences were significant.

### 2.4.5 hPGCLC formation in the present of low hLIF concentration

In order to investigate the effect of a high hLIF concentration during hPGCLC induction, pre-characterized hESCs were differentiated towards hPGCLCs according to the protocol as described at 2.2.1, however with a slight adjustment: instead of 1 µg/ml hLIF, the concentration of 0.1 µg/ml hLIF was used. The used hESC cultures were pre-characterized, and their pluripotency profile was ascertained.

For further analysis, the obtained embryoid bodies were stained for hPGCLC-specific markers OCT4, SOX17 and PDPN. Next, they were examined by confocal microscopy. In order to assess the efficiency of this adjusted protocol, the calculations as previously described in 2.4.3.2.1 were followed. Negative controls were again performed to exclude nonspecific-binding of the primary antibodies.

### 2.4.6 Image analysis using FIJI

Following imaging with a Leica SP8 Confocal microscope, the obtained images were further processed using FIJI (ImageJ), an open source image processing package based on ImageJ. In order to count the number of DAPI positive cells, the RGB images were first converted to 8-bit grayscale. Subsequently, the DAPI positive cells were manually counted using the FIJI plugin 'Cell Counter'. Further, an overlay of the OCT4, SOX17 and PDPN images was made using 'Merge Channels' in order to manually count the number of triple positive cells (hPGCLCs).

# 3 RESULTS

## 3.1 CHARACTERISATION OF THE HESCS

### 3.1.1 Morphology of the hESCs

Primed hESCs were cultured and converted into three different naïve conditions as described in 2.1.2. After several passages, stable cultures were obtained with major morphological differences. The hESCs cultured in primed condition formed large colonies with a flat morphology (Figure 8). HESCs cultured in 4i and GDN naïve conversion medium formed small dome-shaped colonies, indicating that the hESCs were converted towards naïve pluripotency. HESCs cultured in RSeT naïve conversion medium also formed dome-shaped colonies. However, these were clearly smaller than the colonies in the '4i' or 'GDN' condition. When comparing primed and naïve hESCs, the primed flat colonies were visibly larger than the naïve domed colonies. Importantly, an undifferentiated morphology was exhibited by all conditions since round colonies with clear edges could be observed.

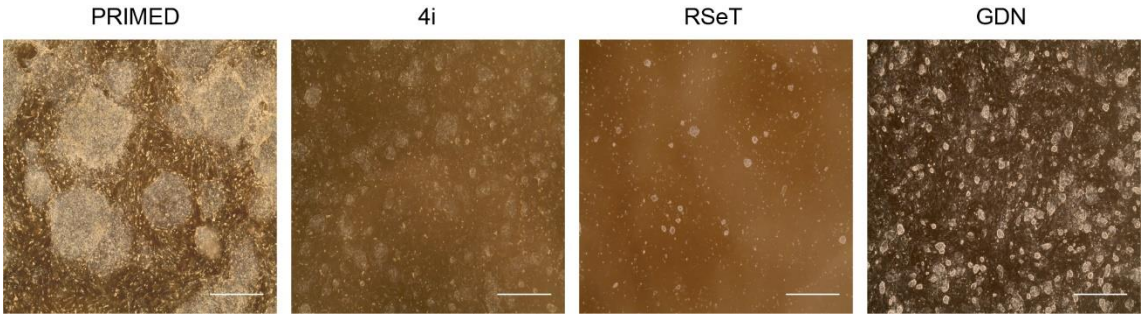


**Figure 8** Colonies of hESC line UGent11-4A cultured in the primed, 4i, RSeT and 'GDN' condition. Scale bar = 400 µm

### 3.1.2 Analysis of the characteristics of the hESCs

To determine whether the cultured primed and naïve hESCs exhibited pluripotent features, the hESCs were stained for pluripotent markers OCT4 and NANOG and naïve marker KLF2. Both primed and naïve hESCs showed nuclear expression of the markers OCT4 and NANOG (Figure 9). As expected, nuclear KLF2 expression was observed in the naïve hESCs, while the primed hESCs showed no expression (Figure 10). Primed, naïve '4i' and naïve 'RSeT' hESCs were also stained for OCT4, SOX17 and PDPN, however, no triple positive cells were observed. This demonstrates that no preliminary differentiation towards hPGCLCs occurred. The performed negative controls for each staining eliminated the occurrence of non-specific binding (Figure S1-S3).

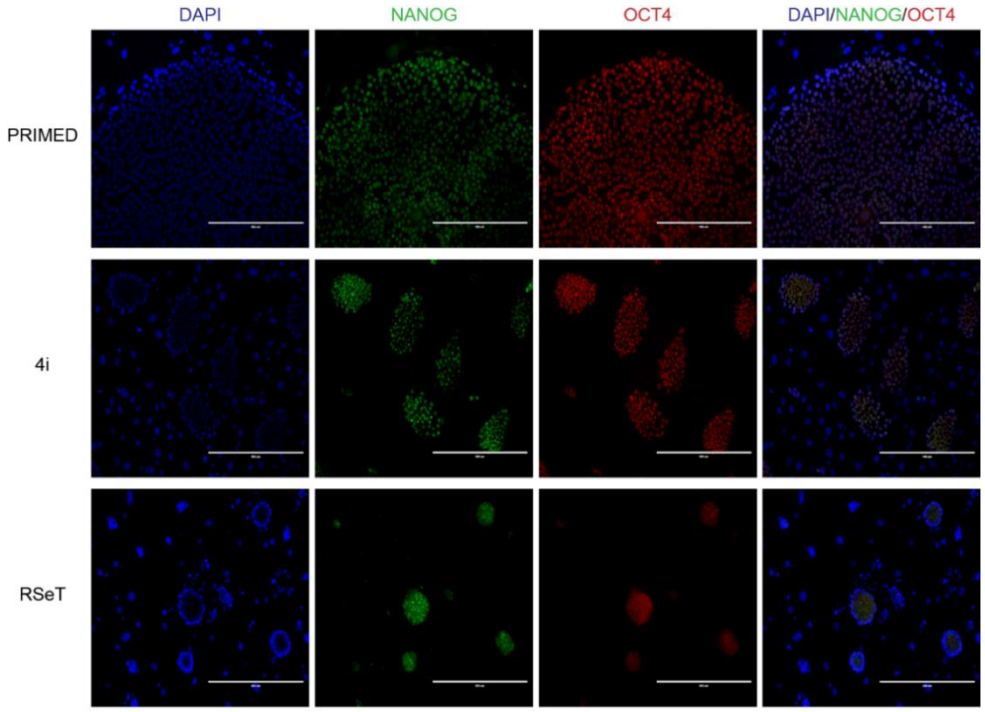


**Figure 9 Immunofluorescence analysis of UGent11-4A in primed, 4i and RSeT conditions.** Nuclear staining was performed using DAPI (4',6-diamidino-2-phenylindole, blue) and the cells were stained for NANOG (green) and OCT4 (red) expression. An overlay of all stainings is shown in the last column. Scale bar = 400µm.

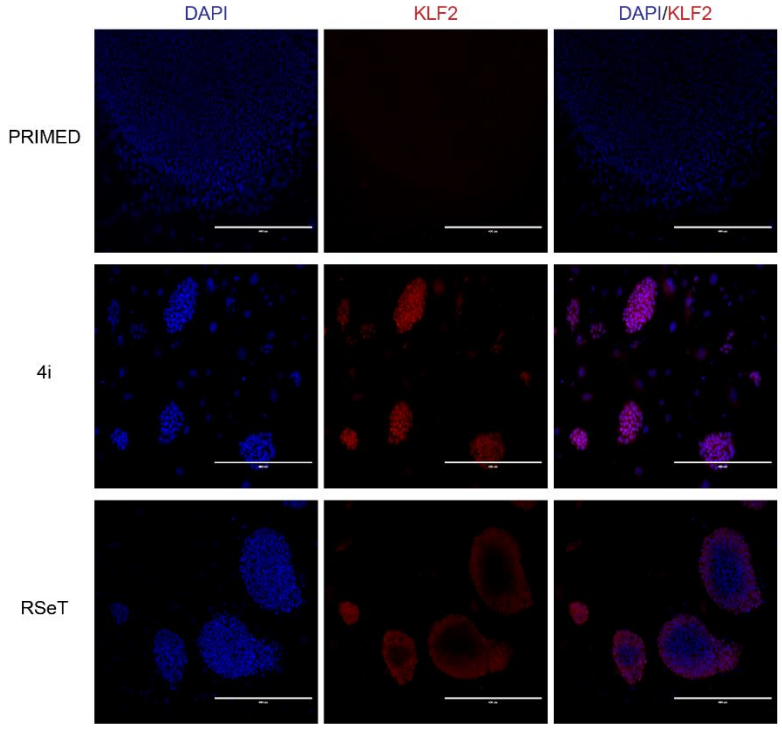


**Figure 10 Immunofluorescence analysis of UGent11-4A in primed, 4i and RSeT conditions.** Nuclear staining was performed using DAPI (4',6-diamidino-2-phenylindole, blue) and the cells were stained for KLF2 (red) expression. An overlay of all stainings is shown in the last column. Scale bar = 400µm

## 3.2 HPGCLC FORMATION: A COMPARISON BETWEEN CULTURE CONDITIONS

### 3.2.1 Morphology of the cultured embryoid bodies

Embryoid bodies were cultured for 4.25 days and pictures were taken daily (Figure 11). During culture, the EBs became more round and compact and in some EBs, growth could be observed. Each condition resulted in EBs from different sizes where the primed condition formed the smallest EBs and the 4i condition the largest. However, slight variation in size between EBs of the same condition could also be observed.

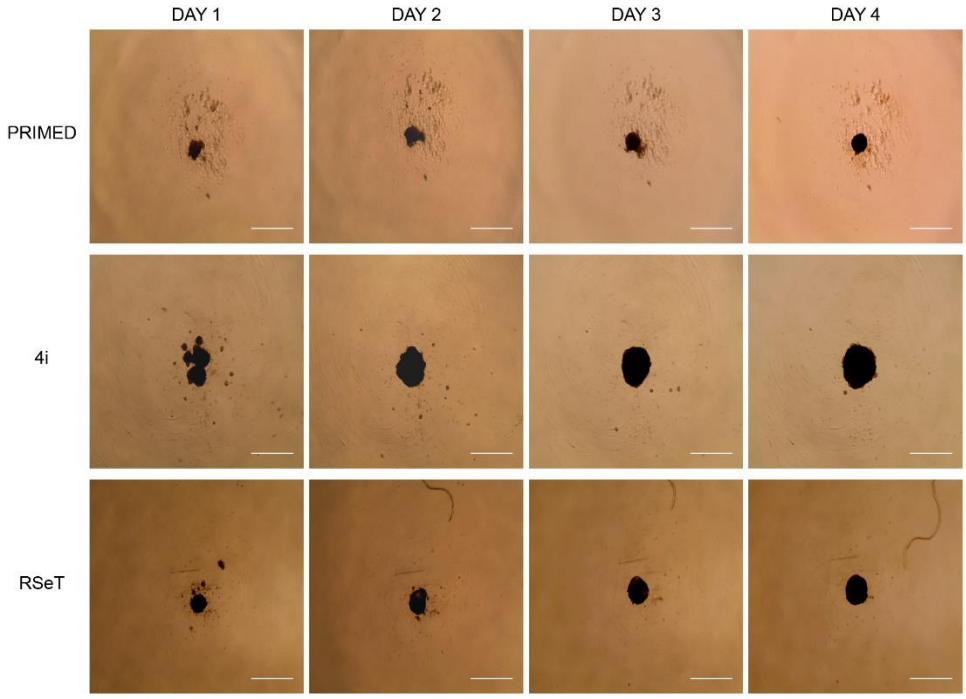


**Figure 11** Embryoid bodies generated from primed, 4i and RSeT hESCs during 4.25 days of culture. Scale bar = 400 µm

### 3.2.2 Immunofluorescence analysis to determine hPGCLC features

Immunofluorescence analysis was performed on the obtained embryoid bodies to further determine their characteristics. Therefore, embryoid bodies of each condition were stained for various markers as indicated in Table 8.

#### 3.2.2.1 Staining for OCT4, SOX17 and PDPN to determine the yield of hPGCLCs

EBs were stained for the markers OCT4, SOX17 and PDPN. When a cell shows to be positive for these three markers, we regard it to be an hPGCLC. Four EBs of the primed, 4i and RSeT condition were stained, however, since one EB of each culture condition was lost during staining, the results of only three stainings per condition are shown. In general, all EBs stained positive for OCT4, SOX17 and PDPN (Figure 14). Correspondingly, triple positive cells were

observed in each EB, indicating that hPGCLCs were present (Figure 14). Further, nonspecific binding of the primary antibodies could be excluded due to the performed negative controls (Figure S3).

The number of DAPI, OCT4, SOX17 and triple positive (OCT4/SOX17/PDPN) cells of all EBs were counted (**Table 10**). Subsequently, the number of triple positive cells of each EB was compared (Figure 12). Further, the percentage of hPGCLCs in each EB was calculated (**Table 10**) and compared between each culture condition (**Figure 13**).

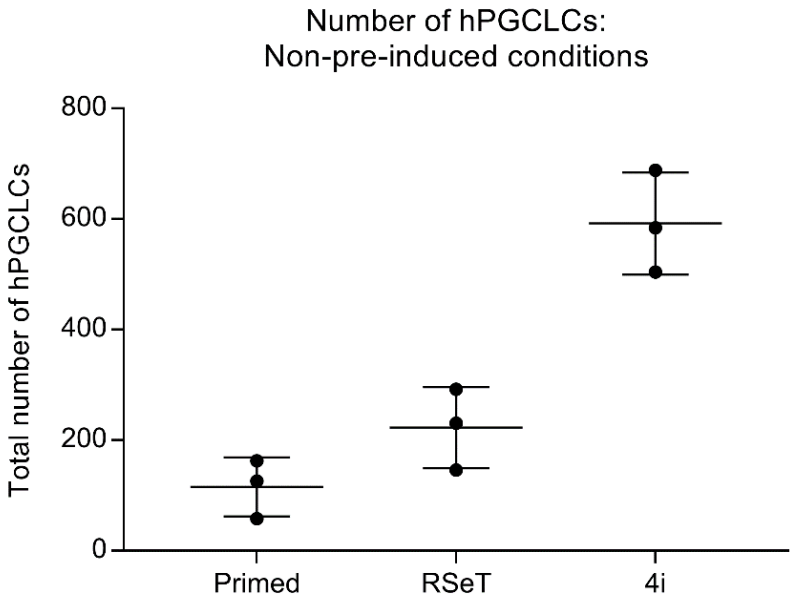


**Figure 12 Number of hPGCLCs of each culture condition.** Four embryoid bodies of the primed, RSeT and 4i culture condition were stained for OCT4, SOX17 and PDPN. The number of triple positive cells (hPGCLCs) were counted for three EBs of each condition and presented in this graph. The error bars denote the mean and standard deviation.

The number of triple positive cells of three EBs from the primed, RSeT and 4i condition were compared and differences were observed between the various culture conditions (Figure 12). The number of hPGCLCs counted in the primed condition were 58, 126 and 163. Further, 146, 231 and 292 hPGCLCs were counted in 'RSeT' EBs. In EBs derived from the 4i condition resulted in 504, 584 and 688 hPGCLCs per EB. Within each culture condition, slight variation in the number of hPGCLCs could be observed.

To determine if the observed differences between all culture conditions are significant, a multiparametric one way-ANOVA test was performed and the p-values were calculated (Table 11). Notably, the three replicates of all conditions followed a normal distribution.

These results indicate that a significantly higher number hPGCLCs was obtained from 4i EBs. When comparing RSeT and primed, a slightly elevated number of hPGCLCs can be observed in the RSeT condition, however, this elevation is not statistically significant.

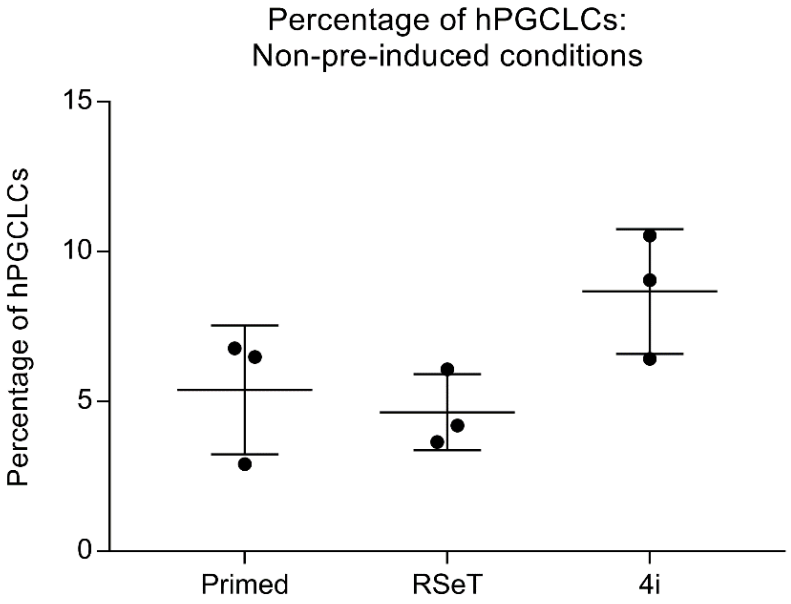


**Figure 13 Percentage of hPGCLCs of each culture condition.** For three EBs of each condition, the triple positive cells were counted and percentages were calculated to the total number of DAPI positive cells in the EB. In this graph, the percentage of hPGCLCs for each culture condition is shown with error bars that represent the mean and standard deviation.

From primed, RSeT and 4i EBs, the percentage of triple positive cells of three EBs was calculated and compared to one another (**Figure 13**). The 4i condition resulted in an hPGCLC formation efficiency of 6.43%, 9.06% and 10.54%. The RSeT condition had a hPGCLC differentiation efficiency of 3.66%, 4.20% and 6.08%, while the primed condition resulted in a differentiation efficiency of 2.92%, 6.49% and 6.78%. An elevated percentage of hPGCLCs was observed in the 4i condition. However, this elevation is less pronounced as it is in the number of hPGCLCs. Here, the primed condition has a marginally higher percentage of hPGCLCs compared to the RSeT condition. Slight variation was again observed in the percentages within the individual samples of each condition.

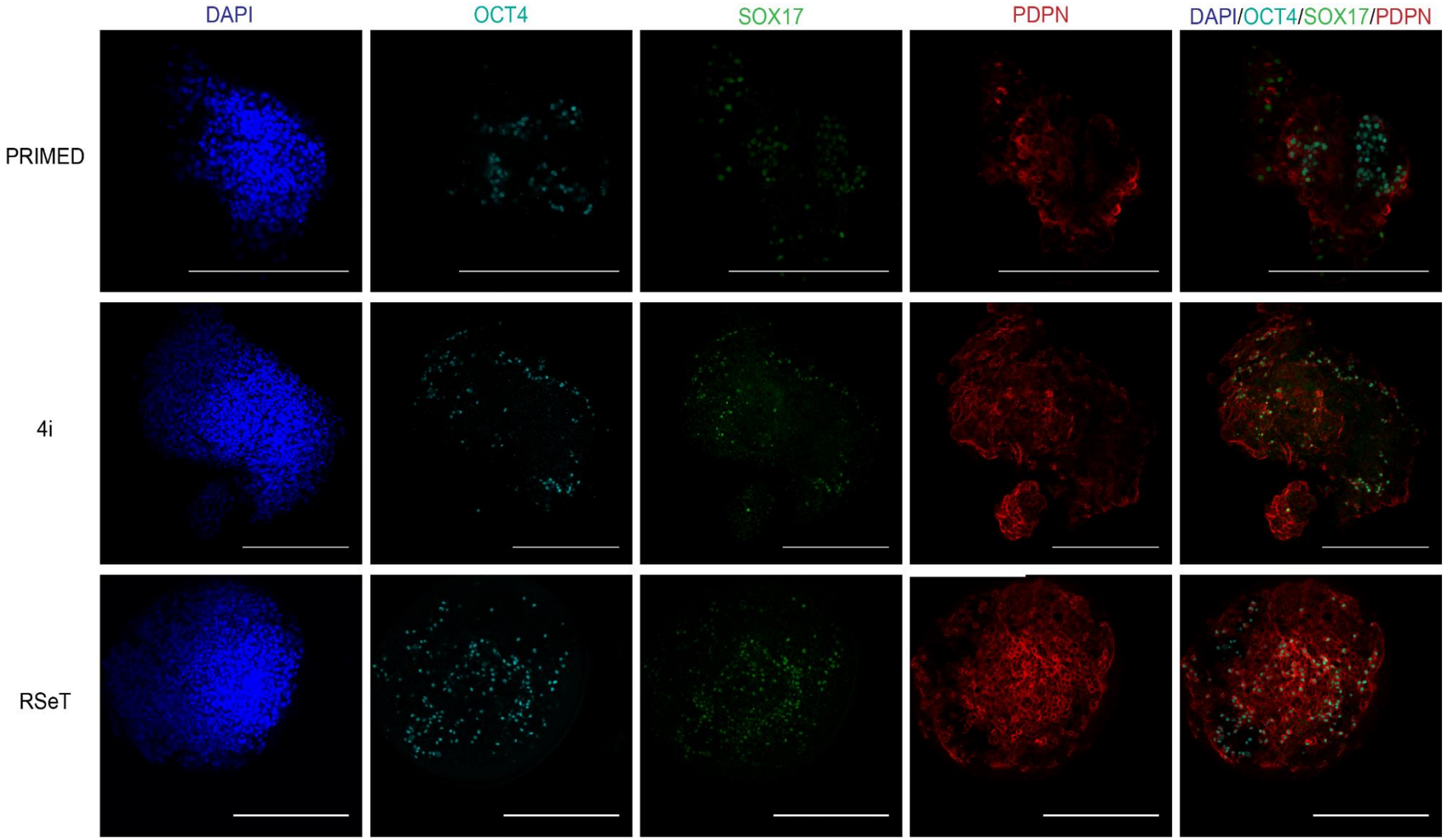


**Figure 14 Immunocytochemistry analysis of embryoid bodies from the primed, 4i and RSeT culture conditions.** The cells were stained for OCT4 (cyan), SOX17 (green) and PDPN (red) expression. Nuclear staining was performed using DAPI (4',6-diamidino-2-phenylindole, blue). In the last column, an overlay of the different stainings is shown. Scale bar = 100 µm

**Table 10** Raw data of all the counts of non-pre-induced embryoid bodies from the '4i', RSeT and primed conditions.

| ***Total number of cells*** | **4i** Staining 1 | **4i** Staining 2 | **4i** Staining 3 | **RSeT** Staining 1 | **RSeT** Staining 2 | **RSeT** Staining 3 | **Primed** Staining 1 | **Primed** Staining 2 | **Primed** Staining 3 |
|---|---|---|---|---|---|---|---|---|---|
| **DAPI positive cells** | 7839 | 6444 | 6528 | 6945 | 3984 | 3798 | 1988 | 1941 | 2403 |
| **SOX17 positive cells** | 282 | 750 | 938 | 1149 | 1135 | 722 | 118 | 288 | 932 |
| **OCT4 positive cells** | 1373 | 966 | 1185 | 1201 | 1173 | 1190 | 639 | 366 | 384 |
| **OCT4/SOX17/PDPN Triple positive cells** | 504 | 584 | 688 | 292 | 146 | 231 | 58 | 126 | 163 |
| **Percentage of hPGCLCs** | 6.43% | 9.06% | 10.54% | 4.20% | 3.66% | 6.08% | 2.92% | 6.49% | 6.78% |

**Table 11** To determine the significance of the obtained number of hPGCLCs, multiparametric one way-ANOVA test was performed. **α = 0.05**

| **Compared conditions** | **p-value** | **Significant?** |
|---|---|---|
| 4i vs. RSeT | <0.0001 | Yes |
| 4i vs. primed | <0.0001 | Yes |
| RSeT vs. primed | 0.2326 | No |

#### 3.2.2.2 Immunostaining of embryoid bodies for multiple other markers

Apart from the stainings for hPGCLC markers OCT4, SOX17 and PDPN, the embryoid bodies were also stained for the presence of pluripotency marker NANOG (Figure S8), late germ cell markers VASA and cKIT (Figure S9) and mouse-specific PGCLC markers BLIMP1 and PRDM14 (Figure S7-S8). Each EB was additionally stained for marker OCT4 as a positive control. All conditions stained positive for pluripotency marker NANOG. Further, staining for markers cKIT, VASA, BLIMP1 and PRDM14 were negative in all conditions. Correspondingly, all EBs stained positive for marker OCT4, as expected. The performed negative controls showed that non-specific binding did not occurred (Figure S4-S6).

## 3.3 THE EFFECT OF PRE-INDUCTION ON hPGCLC DIFFERENTIATION

To determine the effect of pre-induction on hPGCLC differentiation, naïve 4i and RSeT hESCs were pre-induced for two days according to 2.2.2.1.

### 3.3.1 Assessment of the cells during pre-induction

#### 3.3.1.1 Morphology of the pre-induced cells

During two days of pre-induction, the morphology of the hESCs was assessed. It could be observed that the hESCs started to exhibit a spindle-shaped morphology and that no clear colonies were formed (Figure 15).

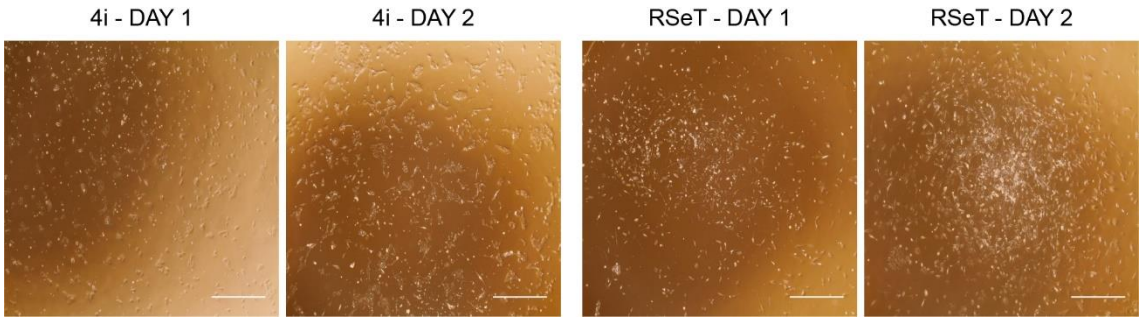


**Figure 15** Morphology of 4i and RSeT hESCs during two days of pre-induction. Scale bar = 400 µm

#### 3.3.1.2 The effect of pre-induction on the stem cell characteristics

To further determine the features after two days of pre-induction, the hESCs were stained for naïve marker KLF2 and pluripotency markers OCT4 and NANOG. Correspondingly, both 4i and RSeT pre-induced hESCs showed expression of KLF2, OCT4 and NANOG (Figure 16). This indicates that the pre-induced cells still exhibit stem-cell specific features. Further, the pre-induced hESCs were stained against OCT4, SOX17 and PDPN to determine if preliminary differentiation towards hPGCLCs occurred. However, no triple positive cells were observed.

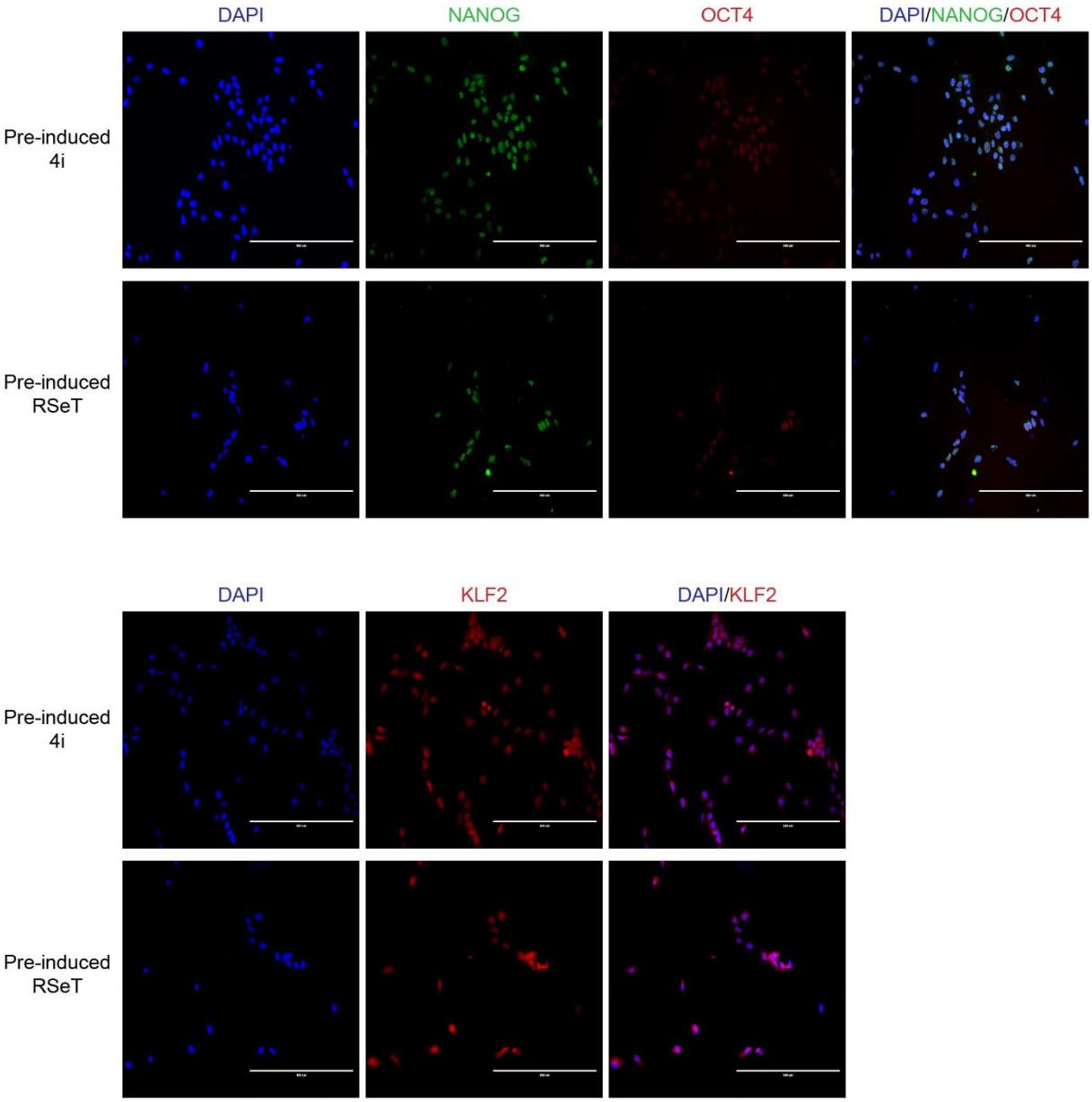


**Figure 16 Immunofluorescence analysis of 4i and RSeT hESCs after two days of pre-induction.** Top picture: The pre-induced hESCs were stained for NANOG (green) and OCT4 (red) expression. Bottom picture: The pre-induced hESCs were stained for KLF2 (red) expression. For both stainings, nuclear staining was performed using DAPI (4',6-diamidino-2-phenylindole, blue) and an overlay of the stainings is shown in the last column. Scale bar = 400 µm

### 3.3.2 Differentiation of pre-induced cells to hPGCLCs

#### 3.3.2.1 Morphology of the embryoid bodies in culture

After two days of pre-induction, the naïve 4i and RSeT hESCs were differentiated towards hPGCLCs. During culture, the morphology of the EBs was examined and compared with EBs obtained from the previous experiment. The pre-induced EBs became more compact and rounder. Further, growth could be observed in some EBs (Figure 17). When comparing pre-induced (Figure 17) and non-pre-induced EBs (Figure 11), a difference in size could be observed, where the pre-induced EBs are smaller. However, no difference in size could be observed between 4i and RSeT pre-induced EBs.

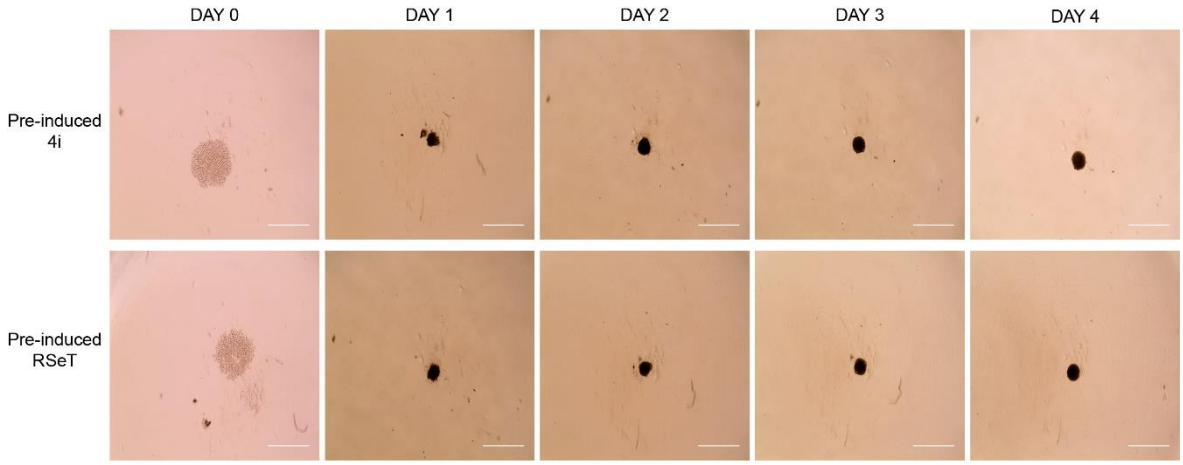


**Figure 17** Embryoid bodies from pre-induced 4i and RSeT hESCs during 4 days of culture. Scale bar = 400 µm

#### 3.3.2.2 Immunofluorescence analysis of pre-induced embryoid bodies

To determine the characteristics of the pre-induced EBs, immunofluorescence analysis was performed for various markers as indicated in Table 8.

##### 3.3.2.2.1 Determining the effect of pre-induction in hPGCLC derivation

Four EBs of both the pre-induced 4i and RSeT condition were stained for the markers OCT4, SOX17 and PDPN to determine the effect of pre-induction on hPGCLC formation (Figure S10). However, the results of only three stainings per conditions are shown since one EB of each culture condition was lost during staining. The number of DAPI, OCT4, SOX17 and triple positive (OCT4/SOX17/PDPN) cells was counted of each EB and, correspondingly, the percentages of hPGCLCs were calculated (Table 12).

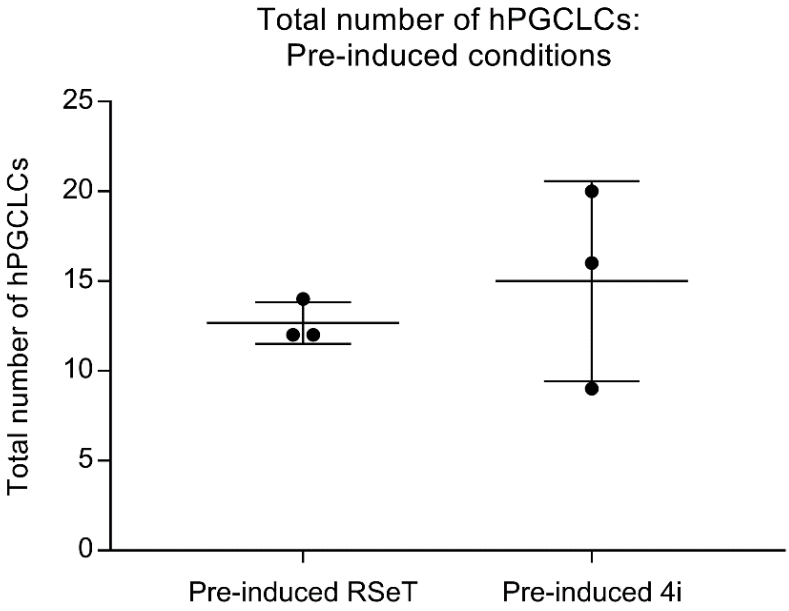


**Figure 18 Total number of hPGCLCs of pre-induced conditions.** For both pre-induced 4i and RSeT culture conditions, four EBs were stained for OCT4, SOX17 and PDPN. The number of triple positive cells (hPGCLCs) of three EBs per condition was counted. In this graph, the number of hPGCLCs is presented with error bars that denote the mean and standard deviation.

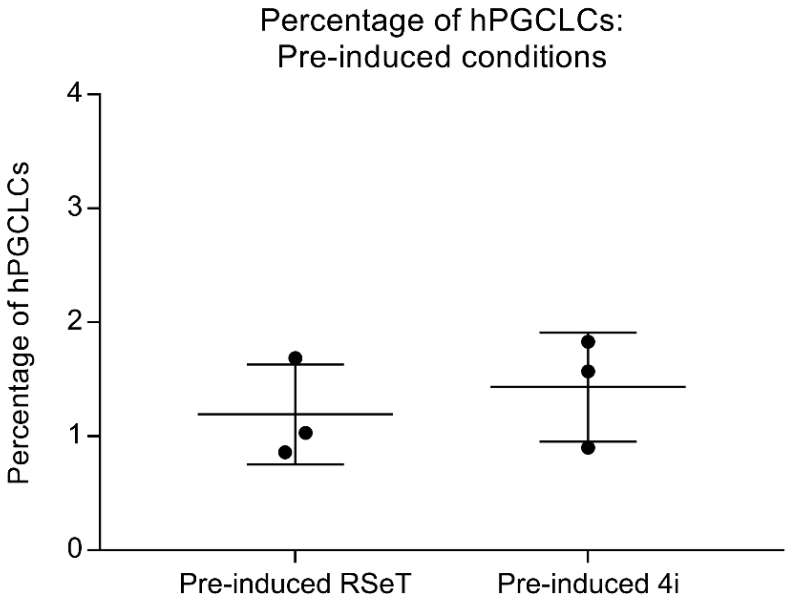


**Figure 19 Percentage of hPGCLCs in the pre-induced conditions.** For both pre-induced 4i and RSeT culture conditions, the number of hPGCLCs were counted and percentages were calculated against the total number of DAPI positive cells. The calculated percentages are presented in this graph. The error bars denote the mean and standard deviation.

**Table 12** Raw data of all the counts of pre-induced (PI) embryoid bodies from the '4i' and RSeT conditions.

| ***Total number of cells*** | **PI - 4i** Staining 1 | **PI - 4i** Staining 2 | **PI - 4i** Staining 3 | **PI - RSeT** Staining 1 | **PI - RSeT** Staining 2 | **PI - RSeT** Staining 3 |
|---|---|---|---|---|---|---|
| **DAPI positive cells** | 996 | 1276 | 873 | 1401 | 1354 | 709 |
| **SOX17 positive cells** | 62 | 44 | 392 | 198 | 711 | 211 |
| **OCT4 positive cells** | 12 | 40 | 82 | 23 | 34 | 20 |
| **Triple positive cells** | 9 | 20 | 16 | 12 | 14 | 12 |
| **Percentage of hPGCLCs** | 0.90% | 1.57% | 1.83% | 0.86% | 1.03% | 1.69% |

**Table 13** To determine if the differences between pre-induced and non-pre-induced are significant, a multiparametric one way-ANOVA test was performed. **$\alpha = 0.05$**

| **Compared conditions** | **p-value** | **Significant?** |
|---|---|---|
| 4i vs. pre-induced 4i | <0.0001 | Yes |
| RSeT vs. pre-induced RSeT | 0.0062 | Yes |

When comparing the number of triple positive cells (Figure 18) and the percentages of hPGCLCs (**Figure 19**) of the pre-induced 4i and RSeT condition, no major differences can be observed. The pre-induced 4i condition resulted in 9,16 and 20 hPGCLCs in each EB. Correspondingly, this lead to an efficiency of 0.90%, 1.83% and 1.57%, respectively. The pre-induced RSeT condition had a total number of 12, 12 and 14 hPGCLCs which corresponded with an efficiency of 0.86%, 1.69% and 1.03%, respectively. Notably, the three replicates of each condition followed a normal distribution, even though some variation in the number of hPGCLCs was observed.

To further determine the effect of pre-induction, the number of hPGCLCs of the pre-induced conditions were compared to the number of hPGCLCs in the corresponding 'non-pre-induced' condition (Figure 20). To determine if the observed differences were significant, the p-values were calculated by a multiparametric one way-ANOVA test (Table 13).

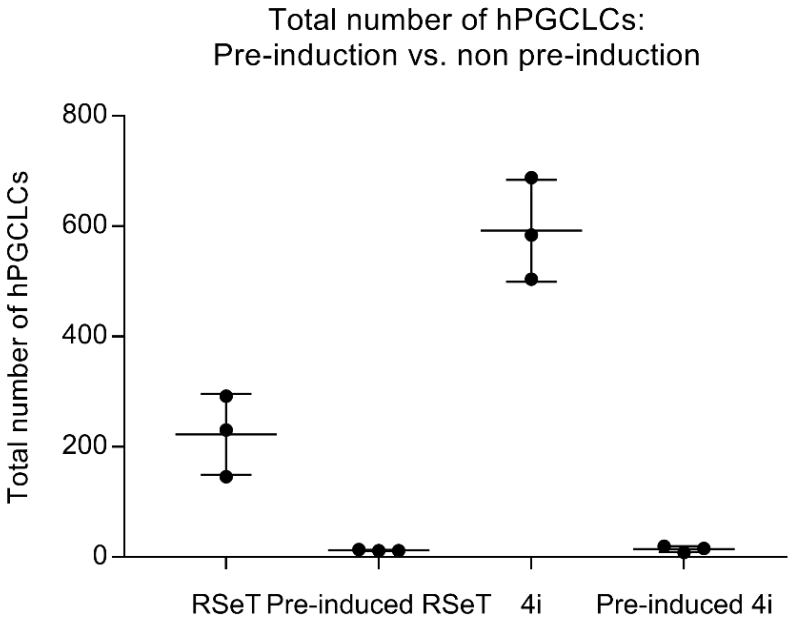


**Figure 20 Total number of hPGCLCs for pre-induced and non-pre-induced conditions.** In this graph, the total number of triple positive cells of pre-induced and non-pre-induced 4i and RSeT EBs are compared. The error bars denote the mean and standard deviation.

These results demonstrate that the number of hPGCLCs decreased significantly when starting from pre-induced hESCs compared to non-pre-induced hESCs (Figure 20). The decrease in the number of hPGCLCs was statistically significant for both 4i and RSeT pre-induced hESCs (Table 13).

Further, we observed that the number of OCT4 positive cells was lower in pre-induced EBs compared to non-pre-induced EBs. Also, more nuclear fragmentation is observed in pre-induced EBs compared to the non-pre-induced EBs, as can be seen on the DAPI channel (Figure 14 & Figure S10). The performed negative control indicated that no non-specific binding occurred (Figure S3).

#### 3.3.2.2.2 Immunostaining of embryoid bodies for other markers

Besides OCT4, SOX17 and PDPN staining, the embryoid bodies obtained from pre-induced 4i and RSeT hESCs were also stained for pluripotency marker NANOG, late germ cell markers VASA and cKIT and mouse-specific PGCLC marker PRDM14 (Figure S11-S12). Additionally, OCT4 staining was performed on each EB as a positive control. The EBs stained positive for pluripotency markers NANOG. However, staining for the markers VASA, cKIT and PRDM14 was negative. Corresponding negative controls for each staining showed that nonspecific binding did not occur (Figure S4-S6).

## 3.4 HPGCLC FORMATION IN THE PRESENCE OF LOW hLIF CONCENTRATION

Human LIF is used at very high concentrations in human and mouse PGCLC derivation experiments without a clear explanation. Therefore, the effect of a lower hLIF concentration on hPGCLC induction was investigated. Pre-characterized hESCs were differentiated towards hPGCLCs, however, instead of 1 µg/ml hLIF, the concentration of 0.1 µg/ml hLIF was used. Further, hESCs were pre-induced for two days (according to 2.2.2.1) and then differentiated towards hPGCLCs in a lower hLIF concentration.

### 3.4.1 Morphology of the pre-induced cells

After two days of pre-induction, the morphology of the hESCs was examined and a spindle-shaped morphology could be observed (**Figure S13**).

### 3.4.2 Morphology of the embryoid bodies

After 4.25 days in culture, the morphology of non-pre-induced and pre-induced EBs was assessed and compared to EBs cultured in a higher hLIF concentration. In general, the size of EBs cultured in a lower hLIF concentration had increased (Figure 21).

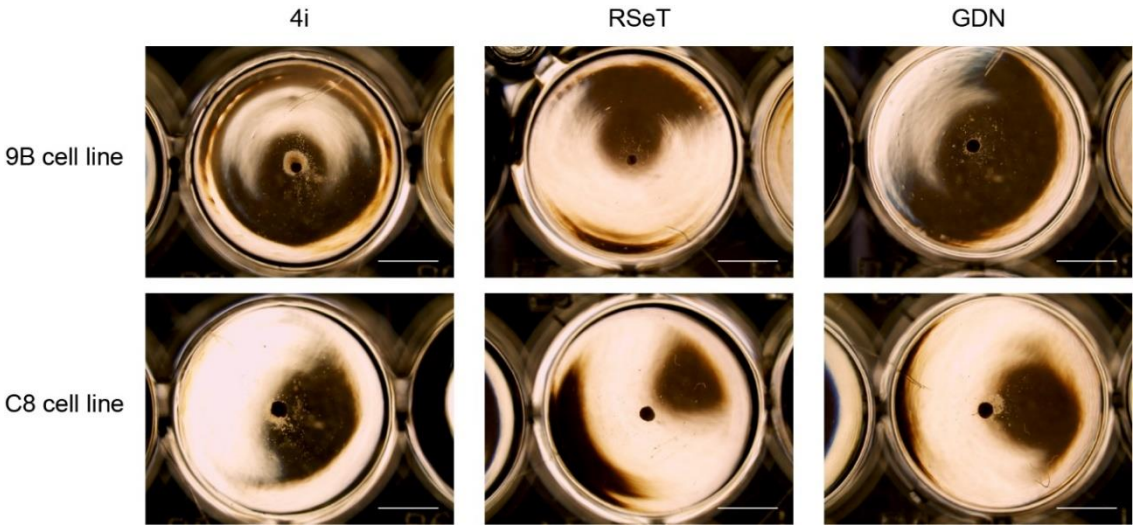


**Figure 21** Morphology of 'pre-induced' embryoid bodies cultured in the presence of a lower hLIF concentration. Scale bar = 400 µm

### 3.4.3 The efficiency of hPGCLC derivation in lower hLIF concentration

The embryoid bodies obtained from culture in lower hLIF concentration were stained for OCT4, SOX17 and PDPN to determine the number of hPGCLCs. Although the EBs stained positive for PDPN and OCT4, no triple positive cells were detected when examining the embryoid bodies of each culture condition (Figure S14). Non-specific binding could be excluded due to the performed negative control (Figure S3).

# 4 DISCUSSION

Even though several studies have reported the differentiation of human pluripotent stem cells into germ cell-like cells [2,5,26–29], a very efficient method for the derivation of hPGCLCs has not been developed yet. Irie et al. [2] described a differentiation protocol that showed to be robust for hESCs culture in the 4i medium. However, since they examined '4i' hESCs only, we investigated this protocol using various pluripotent states of hESCs, induced by different culture conditions.

The pluripotency profile of stem cells can be assessed by examining the expression of OCT4, KLF2, KLF4, SSEA3, NANOG and TRA-1 [12]. All the stem cell conditions used in our experiments showed expression of OCT4 and NANOG and were therefore considered to be pluripotent. Naïve conditions, such as 4i and RSeT, also expressed the naïve marker KLF2. The confirmed pluripotency profile of these stem cells assured that they could now be used to derive cells of any lineage. More specifically for this project, the stem cells could now be used to derive the precursor cells of the germ line. In order to aid differentiation, many protocols dictate that pluripotent stem cells need to be cultured as embryoid bodies (EBs). This, because it represents an *embryo-like* culture environment and increases cell-cell contact. In our study, EB culture was used to differentiate the hESCs towards hPGCLCs. By evaluating the morphology of the cultured EBs, minor differences in size could be observed between culture conditions, however no major conclusions can be based upon these observations. In further investigations, performing a live-death assay on EBs in culture would prove to be interesting. In this way, a better estimation of the most efficient hESC culture can be made.

According to Irie et al. [2], cells expressing both OCT4 and SOX17 can be assessed as hPGCLCs. Correspondingly, Podoplanin (PDPN) was identified as an additional hPGCLC-specific surface marker by another study [31]. Therefore, in our research, a cell expressing all three markers was regarded to be an hPGCLC. Markedly, the 4i condition resulted in a significantly higher number of hPGCLCs compared to the 'primed' and RSeT condition. Further, between the 'primed' and RSeT conditions, no significant differences were observed. In this way, our results are comparable with the results of Irie et al. [2], where it was indicated that the 4i condition shows the highest competence for hPGCLC fate. Correspondingly, we can conclude that derivation from a more naïve condition (RSeT) will results in a lower yield of hPGCLCs.

It is important to note that these conclusions are based upon immunofluorescence analysis, which is a respectable method for analysing the expression of certain markers, but is not an

ideal method from which to draw quantitative conclusions. More suitable techniques, which were also used by Irie et al. [2], would be FACS (Fluorescence-Activated Cell Sorting) and then RNA sequencing analysis.

Another important remark is that our conclusions are based upon only one biological replicate of each culture condition. Furthermore, in each experiment, only three embryoid bodies of each culture condition were stained for hPGCLCs. When looking at the results, variation between the stainings can be observed. These observed variations could be the result of heterogeneity in the stem cell cultures. This indicates that more biological replicates are necessary before any major conclusions can be drawn. Notably, variations among hPGCLC derivation of the same culture conditions were also reported by Hayashi et al. [1,3,21] (mouse-based studies) and Irie et al. [2] (human-based studies).

Irie et al. [2] has stated that human ESCs do not require pre-induction to a more primed-like state in order to efficiently differentiate to hPGCLCs. This statement stands in direct contrast to mouse-based studies, which showed that (1) direct differentiation of mESCs into mPGCLCs is inefficient, but that (2) first converting mESCs to mouse epiblast-like cells (mEpiLCs) significantly improve that efficiency. As reported by Mitsunaga et al. [28], 4i hESCs attain a more naïve-like state due to their naïve-like morphology and similar transcriptomics as the primed condition. As stated before, derivation of hPGCLCs from naïve hESCs is rather inefficient. This has led to the question whether pre-induction could improve hPGCLC derivation from naïve hESCs [2].

However, we observed that pre-induction had no benefit in the differentiation of hPGCLCs when starting from both naïve (RSeT) and naïve-like (4i) hESCs. Rather, pre-induction resulted in a significantly lower yield of hPGCLCs compared to the non-pre-induced cells in both 4i and RSeT conditions. Markedly, these results confirm the suggestion that important molecular differences exist between species, which makes direct extrapolation of results misleading. However, it is important to note that these conclusions are based upon one biological replicate.

Notably, we observed that after two days of pre-induction, the expression of OCT4, NANOG and KLF2 on the cells was still preserved, but we could not determine whether the cells were transcriptomically altered or not. Additionally, since no high yield of hPGCLCs was obtained, we believe that expression of pluripotency markers on hESCs alone may not be conclusive proof of successful hPGCLC derivation. For further investigation, a quantitative estimation of the pluripotency markers and an analysis of the transcriptome of both conventional and pre-induced stem cells is necessary. This can be determined by using qPCR and RNA sequencing.

The hPGCLCs within pre-induced and non-pre-induced EBs stained positive for pluripotency markers NANOG and OCT4, consistent with the study of Irie et al. [2]. Late germ cell markers VASA and cKIT were not exhibited, indicating that the obtained hPGCLCs of each condition are early germ cells and that further maturation is still required. Furthermore, no expression of mouse-specific key transcription factor BLIMP1 was observed. As BLIMP1 acts downstream of SOX17, its expression during hPGCLC induction could be slightly delayed [2]. Since in our study, embryoid bodies were cultured for only 4.25 days, this could indicate why no expression of BLIMP1 was observed. Expression of PRDM14 is decreased and delayed during human PGCLC specification, in contrast to mouse PGCLC derivation [2,5]. Correspondingly, in our study, no expression of PRDM14 was observed. However, a more sensitive, quantitative measurement of these markers using qPCR or RNA sequencing might prove otherwise.

Leukaemia inhibitory factor (LIF) has an important function during stem cell culture, where it maintains the self-renewal and pluripotency features of ESCs [38]. Furthermore, it has been shown that LIF enhances induction and proliferation of the germline in mice [1,39]. It seems that LIF plays a major role in PGCLC specification, possibly by preventing non-targeted differentiation of the stem cells and by maintaining the integrity of their surface markers [1]. However, LIF is used at very high concentrations in PGCLC derivation experiments without a clear explanation. It was therefore the third aim of this study to examine the effect of the concentration of human LIF (hLIF) during hPGCLC induction. Moreover, this was also of importance for the further standardization of the differentiation protocol described by Irie et al [2]. By differentiating hESCs towards hPGCLCs in the presence of a lower hLIF concentration - 0.1 µg/ml hLIF instead of 1 µg/ml hLIF - the effect of the hLIF concentration was determined. The resulting EBs displayed no triple positive cells in both the pre-induced and non-pre-induced conditions. However, expression of OCT4 and PDPN was present. Together with the observation of the larger EBs, this could indicate that the EBs had grown as tumours. Nonetheless, these results suggests that a very high hLIF concentration is essential for differentiating hESCs into hPGCLCs, consistent with the results of mouse PGCLC derivation [1].

Overall, our research has confirmed the statement of Irie et al. [2] that pre-induction for human PGCLC derivation is not necessary. Furthermore, we also demonstrated that different pluripotent states of hESCs, induced by culture in various conditions, can have a significant effect on the germ cell competence of these hESCs, which is also in line with the study of Irie et al. [2]. Nonetheless, extensive research is still required to further clarify the effect of these

various pluripotent states and, in this way, to provide an insight into human germ cell development.

When looking at future perspectives, repeating these experiments with more replicates is key to counter variations observed in our study. In addition, the use of alternative methods of analysis are required. In addition to immunofluorescence, FACS (Fluorescence-Activated Cell Sorting) proposes an ideal method for counting the number of hPGCLCs and determining the effectiveness of the differentiation protocol. Furthermore, by using FACS, the cells of the EB can be sorted and the obtained hPGCLCs can be used for further experiments, such as maturation into gametes. RNA sequencing, on the other hand, can provide us with a detailed transcriptomic analysis of the hPGCLCs which can aid in the clarification of the molecular processes underlying human PGC development.

When looking at a more distant future, the development of a method for the maturation of hPGCLCs will be key. In the study of Duggal et al. [30], hPGCLCs were matured in an *in vitro* maturation (IVM) medium resulting in formation of germ cell like clusters and induction of meiotic gene expression. However, similar as in mice, the process of meiosis will be a challenge to overcome *in vitro*. Therefore, extrapolation of results from mouse studies can prove to be useful, although validation on human cell lines will be required.

# 5 CONCLUSION

To date, research on the mouse model has resulted in successful reconstruction of mouse *in vitro* gametogenesis starting from pluripotent stem cells (PSCs). In humans, several studies have reported *in vitro* generation of human primordial germ cell-like cells (hPGCLCs) derived from stem cells. Despite this remarkable progress, the repeatability and efficiency of these reported studies remain contentious. This research project further investigated the recent study of Irie et al. in order to assess the capability of their protocol to differentiate hPGCLCs. Since the comparison of culture conditions is essential, human PGCLCs were derived from various conditions to determine and compare their efficiency. Our results suggest that the '4i' condition has the highest competence for hPGCLC fate, which is consistent with the result of Irie et al. Correspondingly, our study provided evidence for the importance of high concentrations of hLIF during hPGCLC derivation. Furthermore, this study confirmed the hypothesis that 'pre-induction' of human ESCs is not necessary, in contrast to mouse PGCLC specification. In this manner, our study provides proof that the process of gametogenesis is not entirely conserved between species and, thus, direct extrapolation of results is not as accurate as was first thought. However, additional research on multiple biological replicates is required to confirm our results, before major conclusions can be drawn.

Despite their great promising applications, there is still a lot of research necessary until stem cell-derived gametes can be applied safely in the future. It is important to further elucidate the differences between species and to map the complex network of processes that govern human gametogenesis.

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# APPENDIX

## APPENDIX I: Cell culture

**Mitomycin C**

2 ml Dulbecco's phosphate-buffered saline (1x DPBS)

2 mg mitomycin C

200 ml Dulbecco's Modified Eagle Medium (1x DMEM)

**Gelatine 0.1%**

0.5 g gelatine powder

500 ml sterile water

## APPENDIX II: Immunofluorescence analysis

**1x PBS**

50 ml 10x PBS

500 ml embryo transfer water

**100x Triton**

500 µl Triton

49.5 ml 1x PBS

**200x Tween**

250 µl 10x Tween

49.75 ml 1x PBS

**DABCO mounting medium**

1% DABCO

90% Glycerol

10% FBS

## APPENDIX III: Protocol of cryosection and positive controls

**Solutions:**

- Fixation of cryosections: 100% methanol
- Washing solution: 1x PBS
- Washing solution: 5mM ammonium chloride
- Permeabilization solution: 1x PBS/0.1% Triton X-100
- Blocking solution: 1x PBS/0.5% BSA/10% Fetal calf serum (FCS)

**Positive control of the following antibodies:**

**Table S1** Positive control was performed for following primary antibodies

| Primary antibody | Species | Dilution factor |
|---|---|---|
| **Oct3/4** | Mouse | 1:175 |
| **SOX17** | Goat | 1:175 |
| **VASA** | Goat | 1:175 |
| **NANOG** | Goat | 1:175 |
| **PRDM14** | Rabbit | 1:175 |
| **cKIT** | Rabbit | 1:175 |
| **BLIMP1** | Rat | 1:175 |
| **PDPN** | Rat | 1:175 |
| **KLF2** | Rabbit | 1:175 |

**Table S2** Positive control was performed for following secondary antibodies

| Secondary antibody | Species | Dilution factor |
|---|---|---|
| **Alexa 488** | Donkey Anti Goat | 1:250 |
| **Alexa 488** | Donkey Anti Rabbit | 1:250 |
| **Alexa 594** | Donkey Anti Goat | 1:250 |
| **Alexa 594** | Donkey Anti Rat | 1:250 |
| **Alexa 647** | Donkey Anti Mouse | 1:250 |

**Followed protocol:**

1. Cryosections of 10µM of mouse testis are cut using a microtome, put on a +/+ glass slide and dried for 60 minutes.
2. The sections are fixed using 100% technical methanol for 3 minutes.
3. A wash step is performed with 1x PBS for 5 minutes – 3 times
4. The sections are permeabilized with Permeabilization solution for 10 minutes.
5. A wash step is performed with 1x PBS for 5 minutes – 3 times
6. A wash step is performed using 50mM ammonium chloride for 5 minutes.

7. A wash step with 1x PBS is performed for 5 minutes – 3 times
8. The sections are blocked with Blocking solution for 30 minutes.
9. The sections are incubated with Primary antibodies overnight at 4°C.
10. After incubation, a wash step is performed with 1x PBS for 5 minutes – 3 times
11. The sections are incubated with Secondary antibodies for 60 minutes (work in the dark).
12. DAPI staining and mounting of the sections is done using 1 drop of Vectashield.
13. Seal the section with a coverslip and keep in the dark.
14. Images are taken with a Leica SP8 Confocal microscope.
15. All the images are processes according to 2.4.6.

## APPENDIX III: Supplementary results

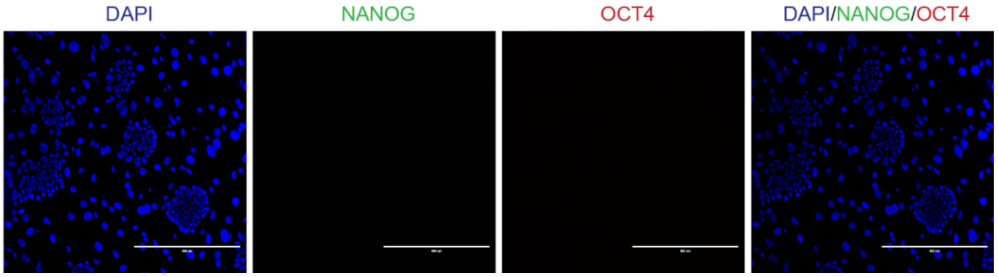


**Figure S1 Negative control of OCT4 (red) and NANOG (green) staining.** Nuclear staining is performed using DAPI (4',6-diamidino-2-phenylindole, blue). In the last column, an overlay of the stainings is shown. Scale bar = 400 µm

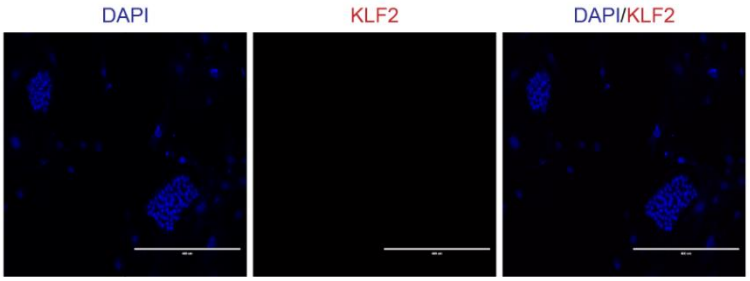


**Figure S2 Negative control of KLF2 (red) staining.** Nuclear staining is performed using DAPI (4',6-diamidino-2-phenylindole, blue). In the last column, an overlay of the stainings is shown. Scale bar = 400 µm

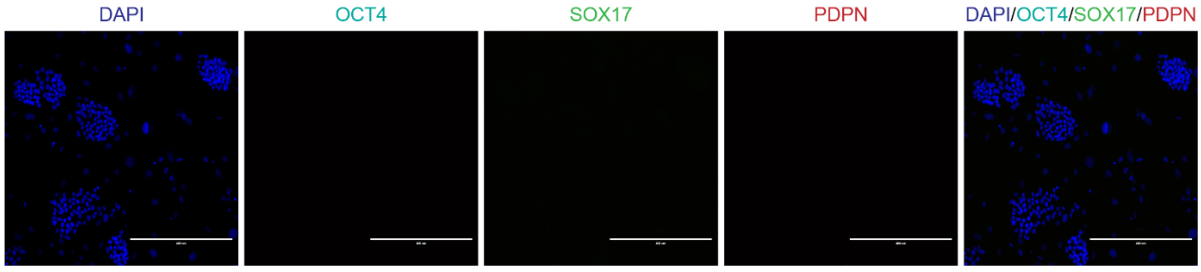


**Figure S3 Negative control of OCT4 (cyan), SOX17 (green) and PDPN (red) staining.** Nuclear staining is performed using DAPI (4',6-diamidino-2-phenylindole, blue). In the last column, an overlay of the stainings is shown. Scale bar = 400 µm

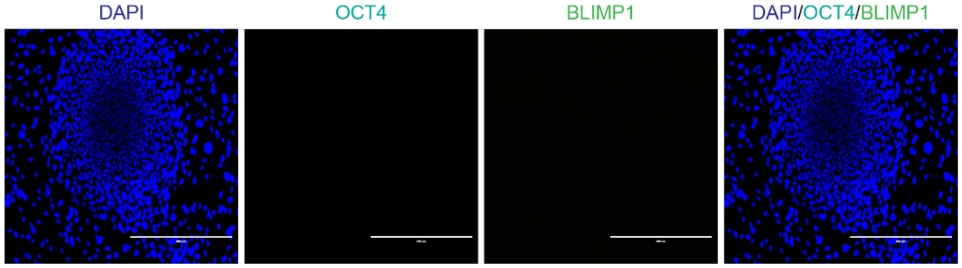


**Figure S4 Negative control of BLIMP1 (green) and OCT4 (cyan) staining.** Nuclear staining is performed using DAPI (4',6-diamidino-2-phenylindole, blue). In the last column, an overlay of the stainings is shown. Scale bar = 400 µm

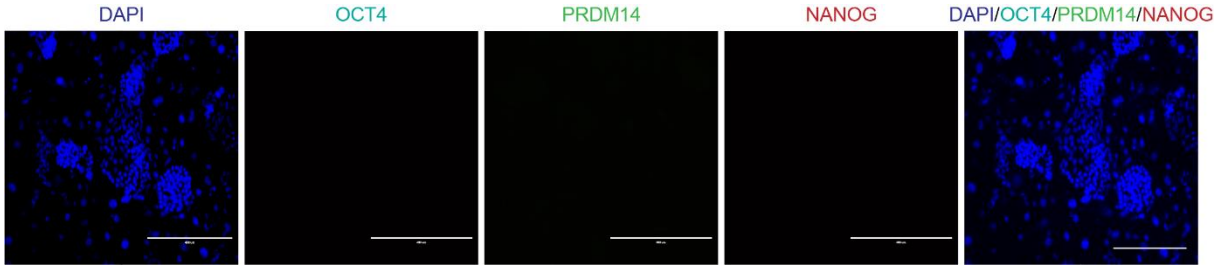


**Figure S5 Negative control of PRDM14 (green), NANOG (red) and OCT4 (cyan) staining.** Nuclear staining is performed using DAPI (4',6-diamidino-2-phenylindole, blue). In the last column, an overlay of the stainings is shown. Scale bar = 400 µm

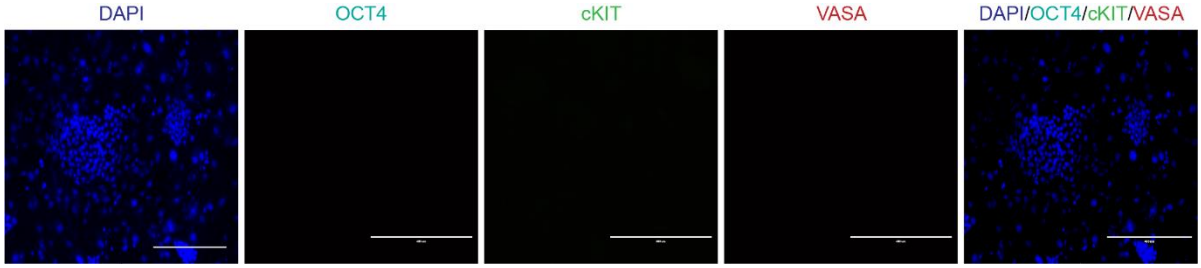


**Figure S6 Negative control of cKIT (green), VASA (red) and OCT4 (cyan) staining.** Nuclear staining is performed using DAPI (4',6-diamidino-2-phenylindole, blue). In the last column, an overlay of the stainings is shown. Scale bar = 400 µm

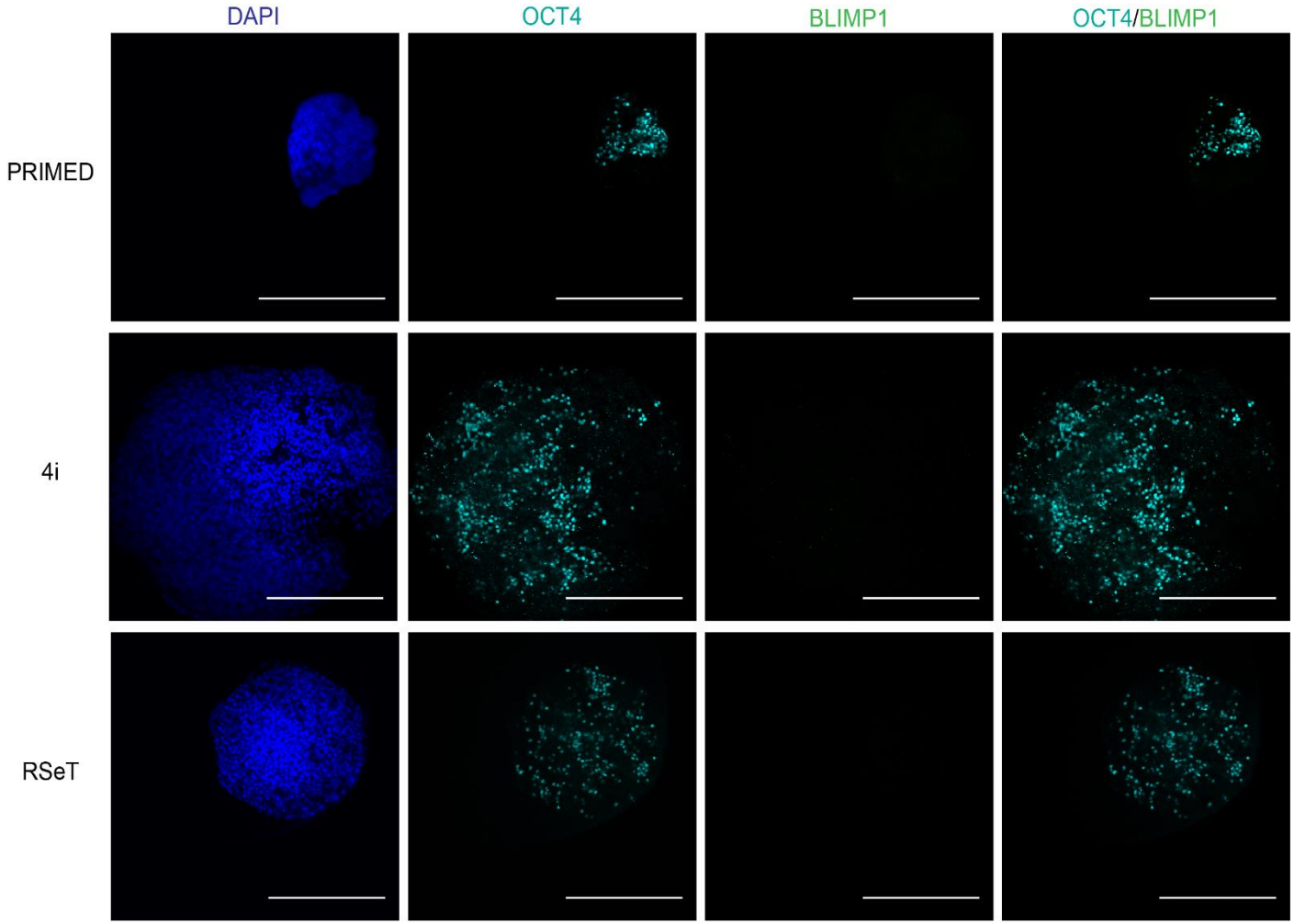


**Figure S7 Immunofluorescence analysis of embryoid bodies for the markers OCT4 and BLIMP1.** Embryoid bodies of the 4i, RSeT and primed condition were stained for OCT4 (cyan) and BLIMP1 (green) expression. Nuclear staining was performed using DAPI (4',6-diamidino-2-phenylindole, blue). In the last column, an overlay of the stainings is shown. Scale bar = 100 µm

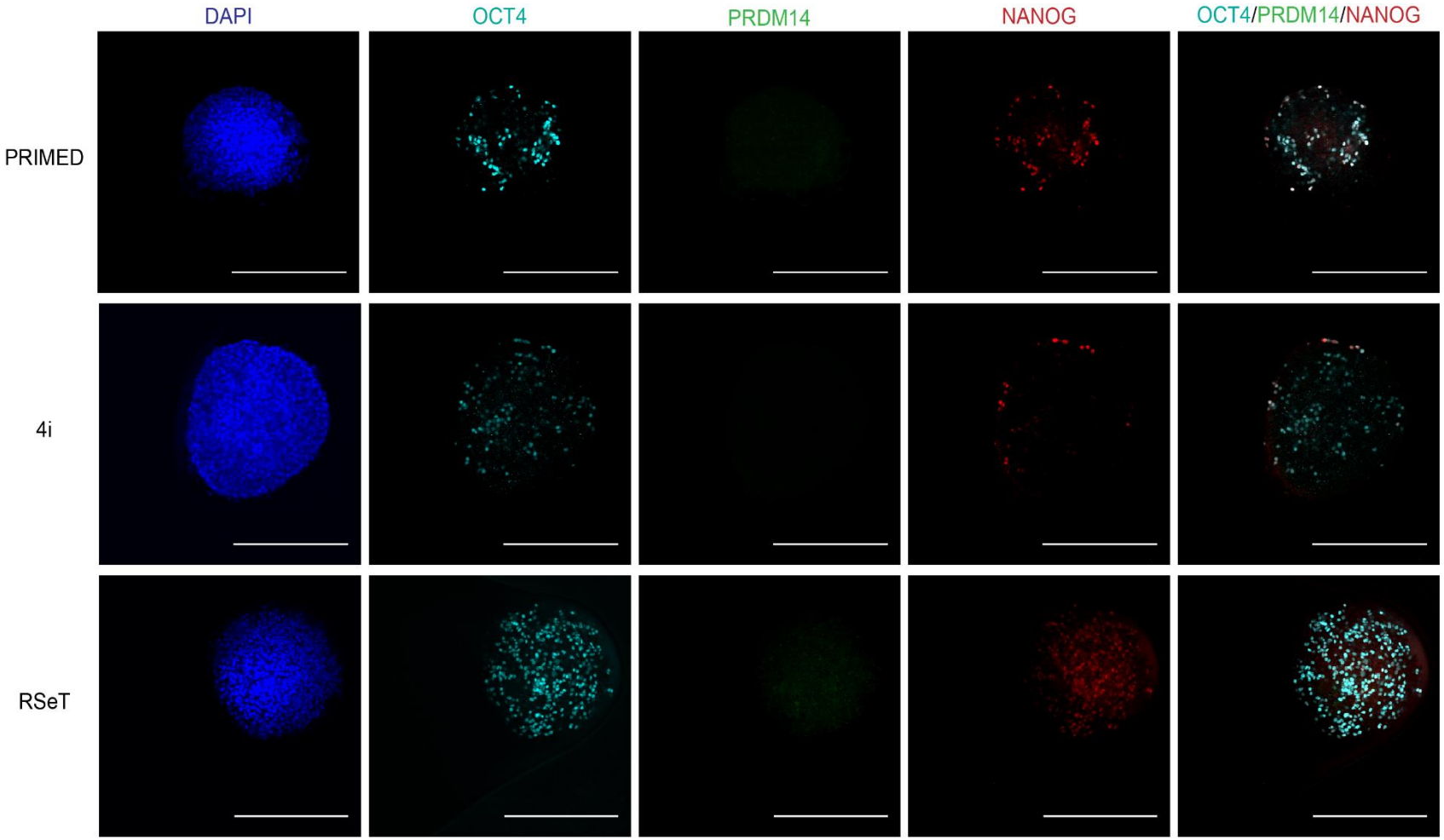


**Figure S8 Immunofluorescence analysis of embryoid bodies for the markers OCT4, PRDM14 and NANOG.** Embryoid bodies of the 4i, RSeT and primed condition were stained for OCT4 (cyan), PRDM14 (green) and NANOG (red) expression. Nuclear staining was performed using DAPI (4',6-diamidino-2-phenylindole, blue). In the last column, an overlay of the stainings is shown. Scale bar = 100 µm

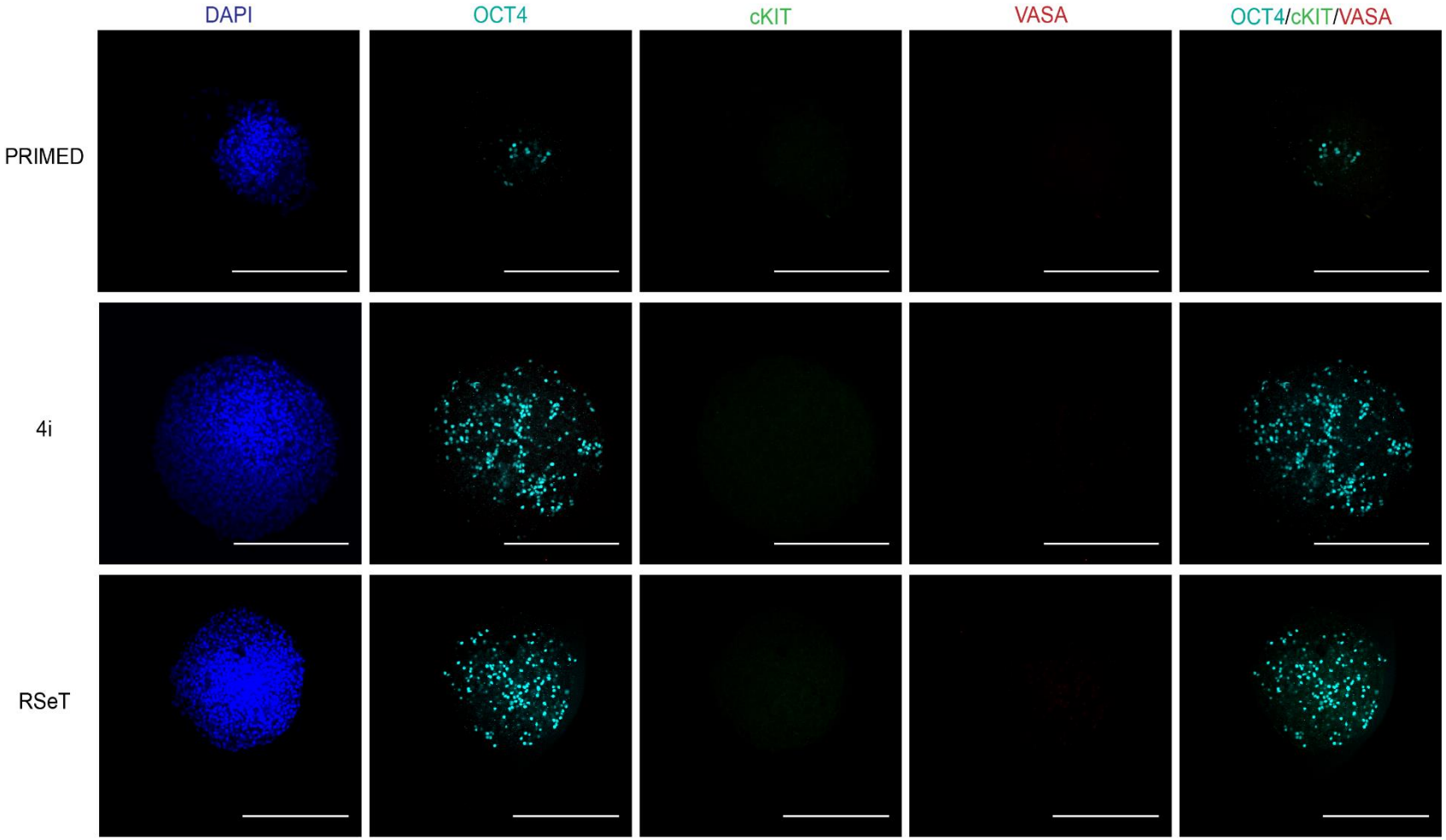


**Figure S9 Immunofluorescence analysis of embryoid bodies for the markers OCT4, cKIT and VASA.** Embryoid bodies of the 4i, RSeT and primed condition were stained for OCT4 (cyan), cKIT (green) and VASA (red) expression. Nuclear staining was performed using DAPI (4',6-diamidino-2-phenylindole, blue). In the last column, an overlay of the stainings is shown. Scale bar = 100 µm

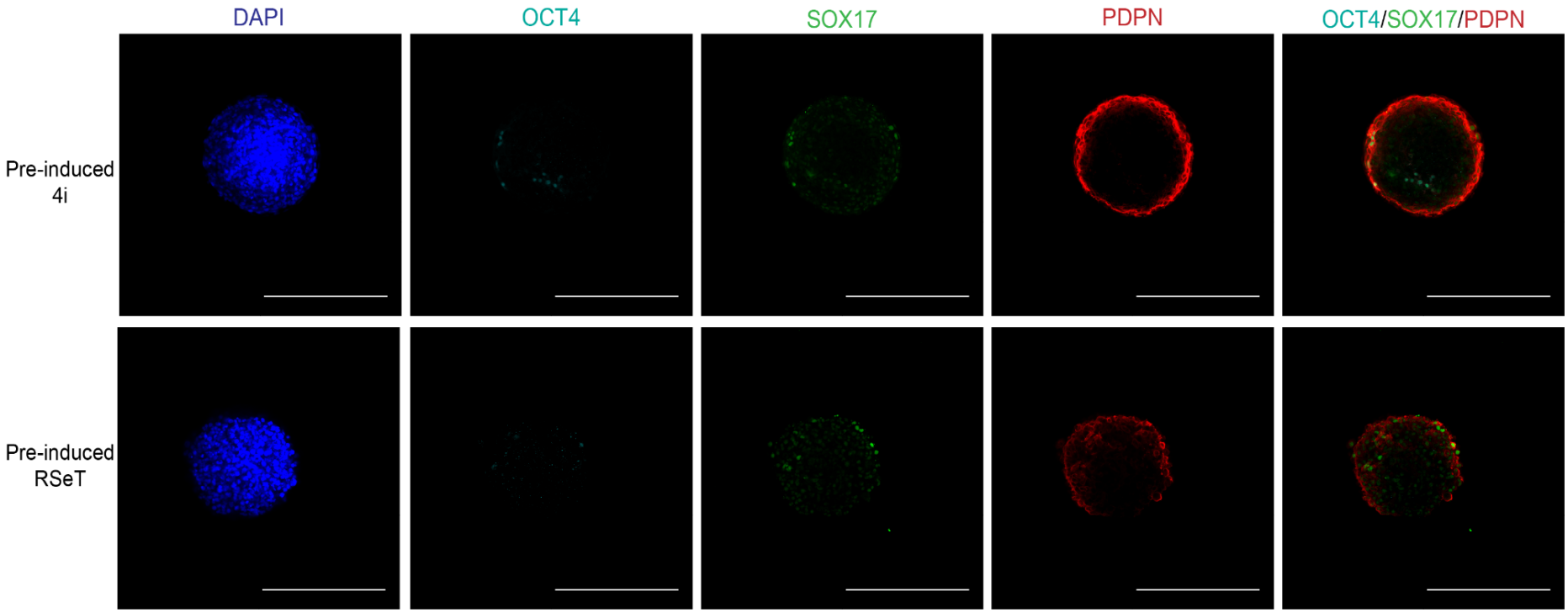


**Figure S10 Immunofluorescence analysis of 'pre-induced' embryoid bodies for the markers OCT4, SOX17 and PDPN.** Embryoid bodies of the pre-induced 4i and RSeT condition were stained for OCT4 (cyan), SOX17 (green) and PDPN (red) expression. Nuclear staining was performed using DAPI (4',6-diamidino-2-phenylindole, blue). In the last column, an overlay of the stainings is shown. Scale bar = 100 µm

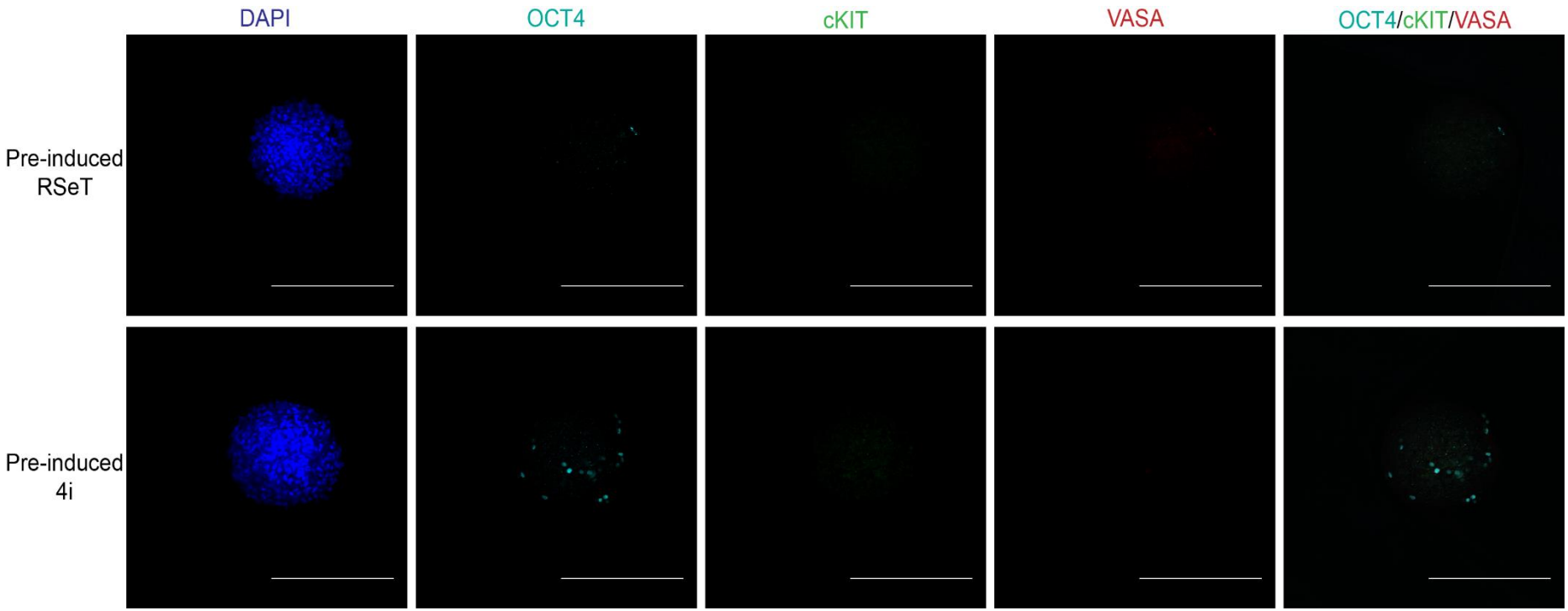


**Figure S11 Immunofluorescence analysis of 'pre-induced' embryoid bodies for the markers OCT4, cKIT and VASA.** Embryoid bodies of the pre-induced 4i and RSeT condition were stained for OCT4 (cyan), cKIT (green) and VASA (red) expression. Nuclear staining was performed using DAPI (4',6-diamidino-2-phenylindole, blue). In the last column, an overlay of the stainings is shown. Scale bar = 100 µm

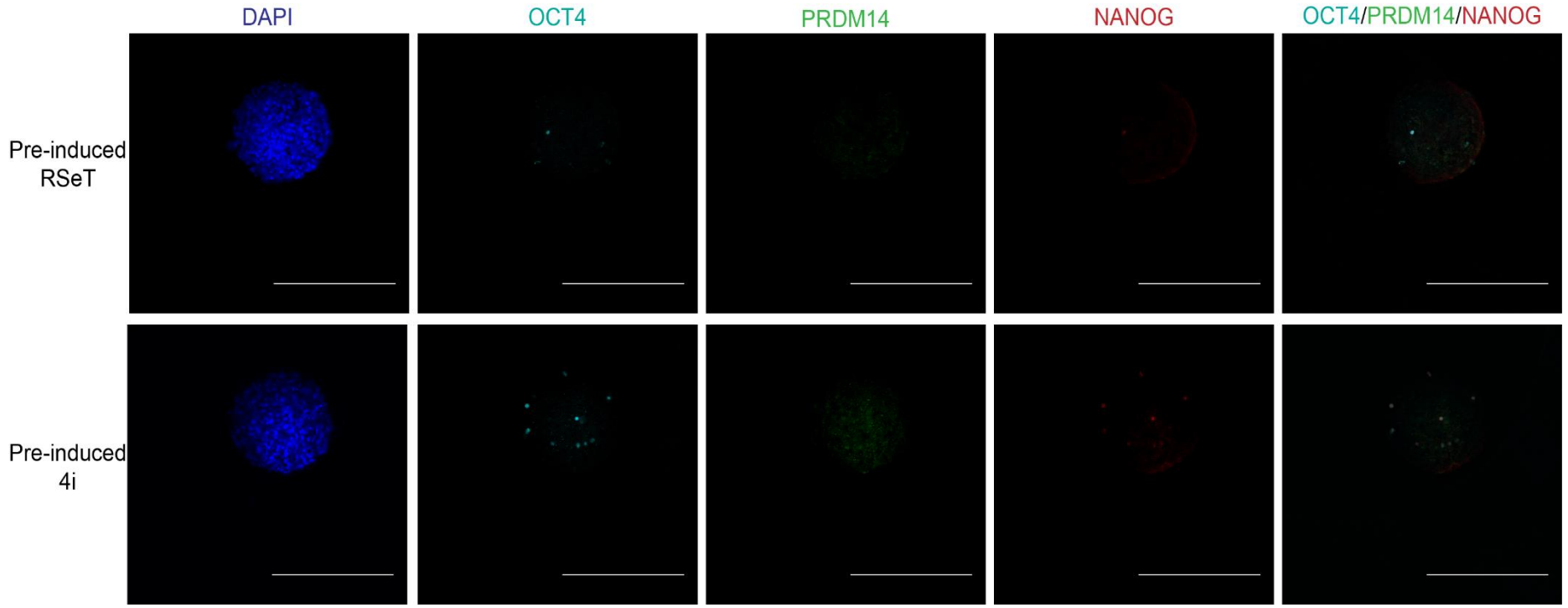


**Figure S12 Immunofluorescence analysis of 'pre-induced' embryoid bodies for the markers OCT4, PRDM14 and NANOG.** Embryoid bodies of the pre-induced 4i and RSeT condition were stained for OCT4 (cyan), PRDM14 (green) and NANOG (red) expression. Nuclear staining was performed using DAPI (4',6-diamidino-2-phenylindole, blue). In the last column, an overlay of the stainings is shown. Scale bar = 100 µm

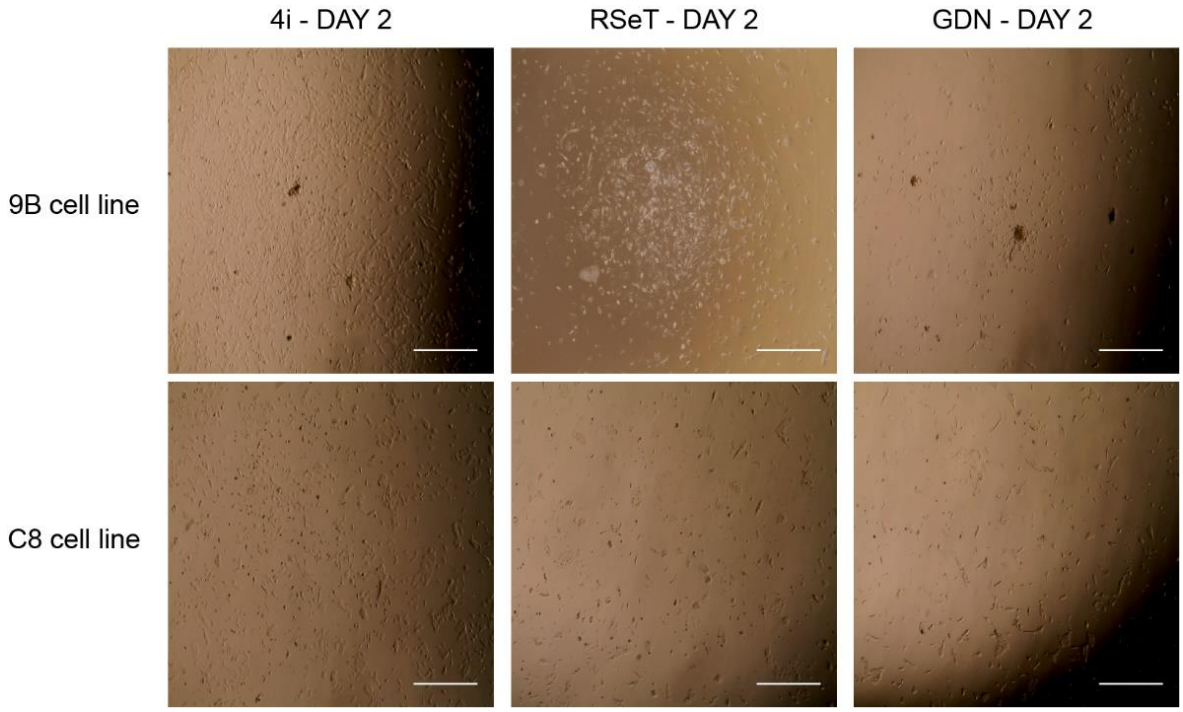


**Figure S13** Morphology of 'pre-induced' hESCs that were afterwards derived to hPGCLCs in a lower hLIF concentration. Scale bar = 400 µm

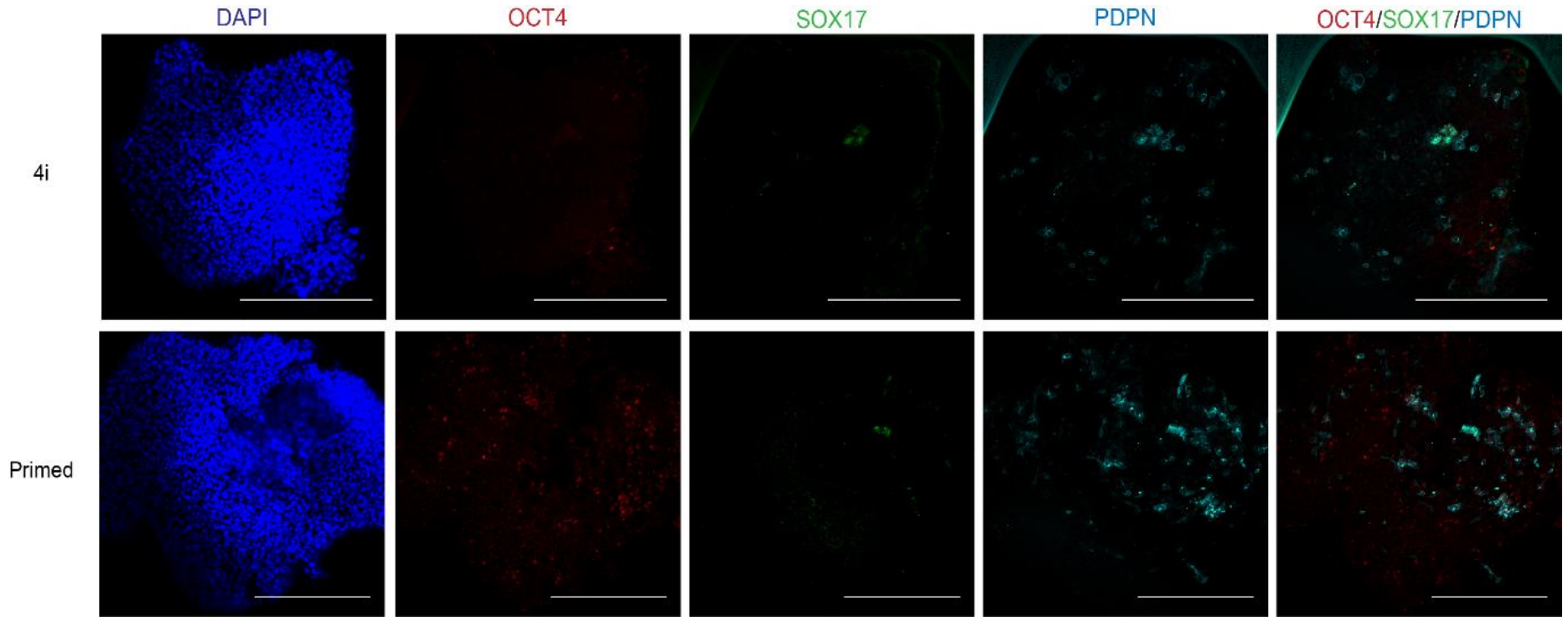


**Figure S14 Immunofluorescence analysis of embryoid bodies cultured in the presence of a lower hLIF concentration.** Embryoid bodies of 4i and primed condition were stained for OCT4 (cyan), SOX17 (green) and PDPN (red) expression. Nuclear staining was performed using DAPI (4',6-diamidino-2-phenylindole, blue). An overlay of all the stainings is shown in the last column. Scale bar = 100 µm

# APPENDIX IV: Products and equipment

**Table S3** Used materials

| Products | Company | Cat. N° |
|---|---|---|
| 1 ml pipette | VWR International | 612-3707 |
| 2 ml pipettes | VWR International | 612-3704 |
| 5 ml pipettes | VWR International | 734-0350 |
| 10 ml pipettes | VWR International | 734-0352 |
| 25 ml pipettes | VWR International | 734-0343 |
| 50 ml pipettes | VWR International | 734-0351 |
| 15 ml falcon tubes | VWR International | 734-0450 |
| 50 ml falcon tubes | VWR International | 734-0453 |
| Eppendorf 1.5 ml | VWR International | 211-0015 |
| Cryovial | VWR International | 479-0801 |
| 6-well plates | VWR International | 734-1599 |
| 12-well plates | VWR International | 734-1598 |
| 96-well plate | VWR International | 732-2719 |
| Corning Costar 96-well plate, ultra-low attachment U-bottom | Sigma-Aldrich | CLS7007-24EA |
| Nunc Microwell Mini Trays | Sigma-Aldrich | M0815-100EA |
| T12,5 culture flask + filter top | VWR International | 734-0043 |
| T25 culture flask + filter top | VWR International | 734-0044 |
| T25 culture flask without filter top | VWR International | 734-0045 |
| T75 culture flask + filter top | VWR International | 734-0046 |
| T75 culture flask without filter top | VWR International | 734-0049 |
| Yellow tip boxes | VWR International | 613-2329 |
| Blue tip boxes | VWR International | 613-2332 |
| Filter 250 ml | VWR International | 513-1571 |
| Filter 500 ml | VWR International | 513-1621 |
| Parafilm | VWR International | 291-1212 |
| Coverslips (13 mm) | VWR International | 631-1578 |
| Coverslips (staining) | VWR International | 630-1846 |
| Superfrost Glass slides | Thermo Scientific | 10504182 |
| Glass slides (+/+) | VWR International | PR-P-001 |
| Glass beads | Sigma-Aldrich | Z265926-1EA |

**Table S4** Used equipment

| Equipment | Specifications |
|---|---|
| Fast Read Slides (10 chamber) | Dominique Dutscher SAS |
| Centrifuge | Eppendorf Multipurpose Centrifuge 5804R |
| $CO_2$ incubator 5% $O_2$ | Binder, VWR International |
| EVOS light microscope | EVOS XL4 – Life Technologies |
| EVOS fluorescence microscope | EVOS FL – Life Technologies |
| Leica Confocal microscope | Leica SP8 Confocal microscope |
| Type 2 Safety Cabinet | MSC-Advantage™ Class II Biological Safety Cabinets, Thermo Fisher Scientific |
| Warm water bath | Julabo SW20 Analis |

**Table S5** Used products

| Products | Company | Cat. N° |
|---|---|---|
| Activin A (10µg) | R&D Systems | 338-AC010 |
| Ascorbic Acid | Sigma-Aldrich | A8960 |
| B27 Supplement (50X) | Thermo Fisher Scientific | 17504-044 |
| Basic Fibroblast Growth Factor (bFGF, 50µg) | Peprotech | 100-18B |
| BLIMP1 Antibody (6D3 clone) | Thermo Scientific | 14-5963-80 |
| BIRB0796 | Axon Medchem | 1358 |
| Bovine serum albumin (BSA) (5g) | Calbiochem | 12657-5 |
| CHIR99021 (5mg) | Axon Medchem | Axon 1386 |
| cKIT Antibody (CD117) | Agilent Technologies | A450229-2 |
| Collagenase Type IV (1g) | Thermo Fisher Scientific | 17104-019 |
| DABCO mounting medium | Made in house (GFAST) | - |
| DABCO | Sigma | D27802-100G |
| Defined lipid concentrate | Thermo Fisher Scientific | 11905031 |
| Dimethylsulfoxide (DMSO) | SERVA | 20385 |
| Donkey Anti-Goat IgG H&L (Alexa Fluor® 488) | Abcam | AB150129 |
| Donkey Anti-Goat IgG H&L (Alexa Fluor® 594) | Abcam | AB150132 |
| Donkey Anti-Mouse IgG H&L (Alexa Fluor® 594) | Abcam | AB150105 |
| Donkey Anti-Mouse IgG H&L (Alexa Fluor® 647) | Thermo Fisher | 31571 |
| Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 488) | Abcam | AB150073 |
| Donkey Anti-Rat IgG (Alexa Fluor® 594) | Abcam | AB150157 |
| Dulbecco's Modified Eagle Medium (DMEM) | Thermo Fisher Scientific | 41965-039 |
| Dulbecco's Modified Eagle Medium F-12 Nutrient Mixture (DMEM/F-12) | Thermo Fisher Scientific | 21041-025 |
| Dulbecco's Phosphate-Buffered Saline (DPBS-/-) | Invitrogen | 14190144 |
| Ethanol 70% in Water solution | Chem-Lab | CL02.0539 |
| Foetal Bovine Serum (FBS) | VWR | 20816298 |
| Forskolin (10mg) | Thermo Fisher Scientific | 10270-106 |
| Gelatine powder | Sigma-Aldrich | G-1890 |
| Glasgows Modified Eagle Medium (GMEM) | Thermo Fisher Scientific | 21710025 |

| | | |
|---|---|---|
| Glycerol | Novolab | A20052 |
| Gö6983 | Sigma-Aldrich | G1918 |
| Human serum albumin | Rode Kruis Vlaanderen | - |
| Insulin (50 mg) | Sigma-Aldrich | 2643 |
| IWR-1 | Sigma-Aldrich | I0161 |
| KLF2 Antibody | Abcam | AB203591 |
| Knock Out Dulbecco's Modified Eagle Medium (KO-DMEM) | Thermo Fisher Scientific | 10829-018 |
| Knock Out Serum Replacement (KOSR) | Thermo Fisher Scientific | 10828028 |
| L-Glutamine | Thermo Fisher Scientific | 25030-024 |
| LIF (human) (100µg) | Peprotech | 300-05 |
| Matrigel | VWR International | 734-1440 |
| Minimum Essential Medium Non Essential Amino Acids (MEM NEAA) | Thermo Fisher Scientific | 11140035 |
| Mitomycin C (2mg) | Sigma-Aldrich | M4287 |
| N2 Supplement (100X) | Thermo Fisher Scientific | 17502-048 |
| NANOG Antibody | R&D Systems | AF1997 |
| Neurobasal | Thermo Fisher Scientific | 21103049 |
| OCT-3/4 Antibody | Santa Cruz Biotechnology | SC-5279 |
| Paraformaldehyde (PFA) powder | Sigma-Aldrich | 158127 |
| PD0325901 (5mg) | Cayman | 13034 |
| PDPN Antibody | Thermo Fisher Scientific | 14-9381-80 |
| Penicillin/streptomycin (P/S) | Thermo Fisher Scientific | 15140122 |
| Phosphate-Buffered Saline (PBS) 10X | Thermo Fisher Scientific | AM9624 |
| ROCKi Y27632 (5mg) | Stem cell technologies | 72304 |
| PRDM14 Antibody | Merck Millipore | AB4350 |
| SB203580 | Biotechne | 1202/10 |
| Sodium Pyruvate | Thermo Fisher Scientific | 11360070 |
| SOX17 Antibody | R&D systems | AF1924 |
| SP600125 (10mg) | Tocris Bioscience | 1496 |
| TGFβ | Peprotech | 100-21C |
| Triton X-100 (50ml) | Sigma-Aldrich | T8787 |
| Trypan blue 0,4% | Sigma-Aldrich | T8154 |
| TrypLE™ Express Enzyme (1X), phenol red | Life Technologies | 12605010 |
| Trypsin/EDTA 0.05% | Thermo Fisher Scientific | 25300054 |
| Trypsin/EDTA 0.25% | Thermo Fisher Scientific | 25200056 |
| Tween 20 (0,05%) (100ml) | Sigma-Aldrich | P7949 |
| VASA Antibody | Abcam | AB13840 |
| Vectashield mounting medium with DAPI | Vector laboratories Inc. | H-1200 |
| Water for embryo transfer | Sigma-Aldrich | W1503 |
| β-mercaptoethanol (50mM) | Thermo Fisher Scientific | 31350010 |