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Publication date
2015
Document Version
Final published version

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Citation for published version (APA):
Maiwald, S. (2015). *Rare genetic variants associated with early onset CVD*. [Thesis, fully internal, Universiteit van Amsterdam].

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Chapter 9

Induced Pluripotent Stem Cells

An exploration of the methodology

S. Maiwald

Introduction

A wide range of human diseases are linked to inherent and acquired defects in metabolic pathways and organelle functions. These changes may already occur in the embryonic stage but manifest later in life. The use of somatic cells bearing the specific disease phenotype will help to elucidate the pathobiology of the disease and may lead to novel therapeutic strategies.

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have the property of self-renewal and differentiation leading to the formation of an infinite quantity of different cell types [1,2]. ESC are not only used as an important research tool for the identification of pathobiology of diseases but also as unlimited resources for cell replacement therapy in regenerative medicine [1]. ESCs display a specialized transcription 'package' for keeping their pluripotent status: octamer-binding transcription factor 4 (*OCT4*), sex determining region Y-box 2 (*SOX2*), and homeobox protein *Nanog* [1]. ESCs can differentiate into all cell types represented in the human body. ESCs are derived from the inner cell mass (ICM) of a blastocyst, an early-stage embryo [3]. By mechanical isolation of the ICM the fertilized human embryo is destroyed causing ethical issues, whereas iPSCs produce ESC-like stem cells (Figure 1) without destroying the embryo overcoming ethical obstacles. Of note, it is difficult to generate patient- or disease-specific ES cells, which are required for their effective application [4]. Human iPSCs (hiPSCs) share the same pluripotent characteristics as human ESCs (hESCs).

HiPSCs can be used to generate patient-specific cells for disease modelling [5] and are becoming a frequently used model [6]. HiPSCs can be generated from skin-fibroblasts [7], keratinocytes, B-lymphocytes, hepatocytes and intestinal cells [6] and are reprogrammed by overexpressing the key transcription factors, *OCT4*, *SOX2,* Kruppel-like factor 4 (*KLF4*), and v-myc avian myelocytomatosis viral oncogene homolog (*c-Myc*) (Figure 1) [4,8,9]. Those four specific transcription factors were identified out of a pool of twenty-four factors [4] as necessary and sufficient to obtain ESC-like colonies.

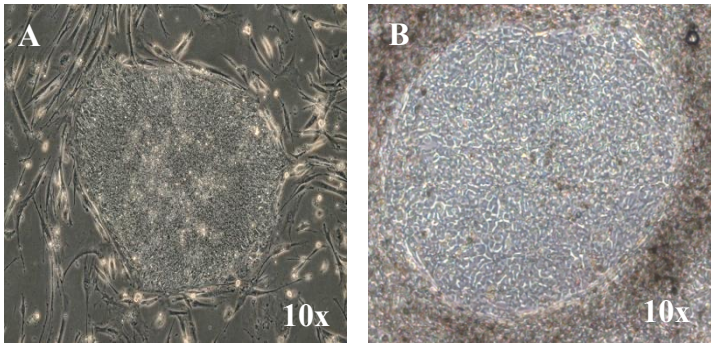
OCT4 was identified as a novel OCT family protein specifically expressed in early embryos, and germ cells [10-12]. The OCT family transcription factor proteins contain the POU domain, a ~150 amino acid sequence, conserved among pituitary-specific positive transcription factor 1 (*Pit-1*), *OCT-1*, *OCT-2*, and *Unc-86* (together named POU). Expression of *OCT4* is restricted in the blastomeres of the developing embryo, the ICM of blastocysts, the epiblast, and germ cells [13]. SOX2 protein contains a high mobility group (HMG) domain, a DNA binding domain which is conserved in abundant chromosomal proteins including HMG1 and HMG2, and binds DNA with little or no sequence specificity [13]. All SOX proteins appear to recognize a similar

binding motif, A/TA/TCAAA/TG. Like *OCT4*, *SOX2* also marks the pluripotent lineage of the early embryo. It is expressed in the ICM, epiblast, and germ cells. Unlike *OCT4 SOX2* is also expressed by the multipotential cells of the extraembryonic ectoderm [13,14]. *KLF4* is a zinc-finger protein that contains an amino acid sequence resembling the *Drosophila* embryonic pattern regulator Kruppel [13,15]. *KLF4* mRNA is found in high levels in cells during growth arrest and is nearly undetectable in cells that are in the exponential phase of proliferation [13,16]. In addition, *KLF4* is highly expressed in undifferentiated ESCs [13]. In cultured cells, the forced expression of KLF4 results in the inhibition of DNA synthesis and cell cycle progression [13]. C-Myc is one of the first proto-oncogenes found in human cancers [17]. The N terminus of c-Myc binds to several proteins, including transformation/transcription domain-associated protein (TRRAP), which are components of the histone acetyltransferase TIP60- and histone acetyltransferase GCN5-complexes, and TIP48 and TIP49, which contain ATPase domains [18]. This causes a transcriptiona repression in the cell. The C terminus of the Myc protein contains the basic region/helix-loop-helix/leucine zipper (BR/HLH/LZ) domain, through which Myc binds to a partner protein, myc-associated factor X (Max). The Myc-Max dimers bind to a DNA sequence (CACA/GTG), which is a subset of the general E box sequence (CANNTG) that is bound by all HLH proteins. In addition to binding to DNA, the C terminus of Myc is also involved in transactivation through binding to CBP (also known as CREB-binding protein) and p300 (E1A binding protein, also known as EP300), which have histone acetylase activities influencing transcriptional regulation in the cells [13]. KLF4 seems to be required to suppress p53 and c-Myc-induced apoptosis [19]. KLF4, in turn, activates *p21* and suppresses proliferation. c-Myc can improve this cytostatic effect of KLF4 by suppressing *p21*. Thus, the balance between c-Myc and KLF4 might play a critical role in the transformation process in iPSCs [13].

The advantage of hiPSCs is the production of patient-specific cell types, which are fully immune-compatible with the original donor [6,20]. The 'diseased' hiPSCs can be differentiated into a cell type that is involved in the disease pathobiology [20-23]. To date, several reports on partial modelling of CVD using human hiPSCs [24-28] have mimicked the 'disease in a dish' [23]. Raya and colleagues [29] corrected a genetic defect in somatic cells from Fanconi anaemia patients by using reprogrammed patient-specific iPSCs. Fanconi patients have a genetic defect in a cluster of proteins responsible for DNA repair, resulting in acute myelogenous leukemia and bone marrow failure. Fanconi-anaemia-specific iPSCs can create haematopoietic progenitors of the myeloid and erythroid lineages that are phenotypically normal. These data offer proof-of-concept that iPSC technology can be used for the generation of disease-corrected, patient-specific cells with potential value for cell therapy applications [29].

This chapter describes the basic protocol I have used to reprogram specific human skin fibroblasts, derived from patients with early onset atherosclerosis, into hiPSCs and analyse their pluripotent status. The first paragraph describes a method to harvest of skin fibroblasts, followed by the generation of hiPSCs and their growth conditions. This is accomplished by using moloney murine leukaemia virus-derived vectors each containing one of the four human transcription factors. The resulting hiPSCs were analysed for the pluripotent status using real-time quantitative polymerase chain reaction (qPCR) and immunohistochemistry. Next, the differentiation potential of hiPSCs is described. Finally, I will show the results from the generation of iPSC from affected relatives of a pedigree with premature atherosclerosis.

Figure 1: Comparison of the phenotypical appearance of pluripotent stem cells.



Human embryonic stem cells (A), Image by Junying Yu, University of Wisconsin-Madison) and human induced pluripotent stem cells (B) on a layer of γ-irradiated mouse embryonic fibroblasts under phase contrast microscope.

Fibroblast Cell Culture

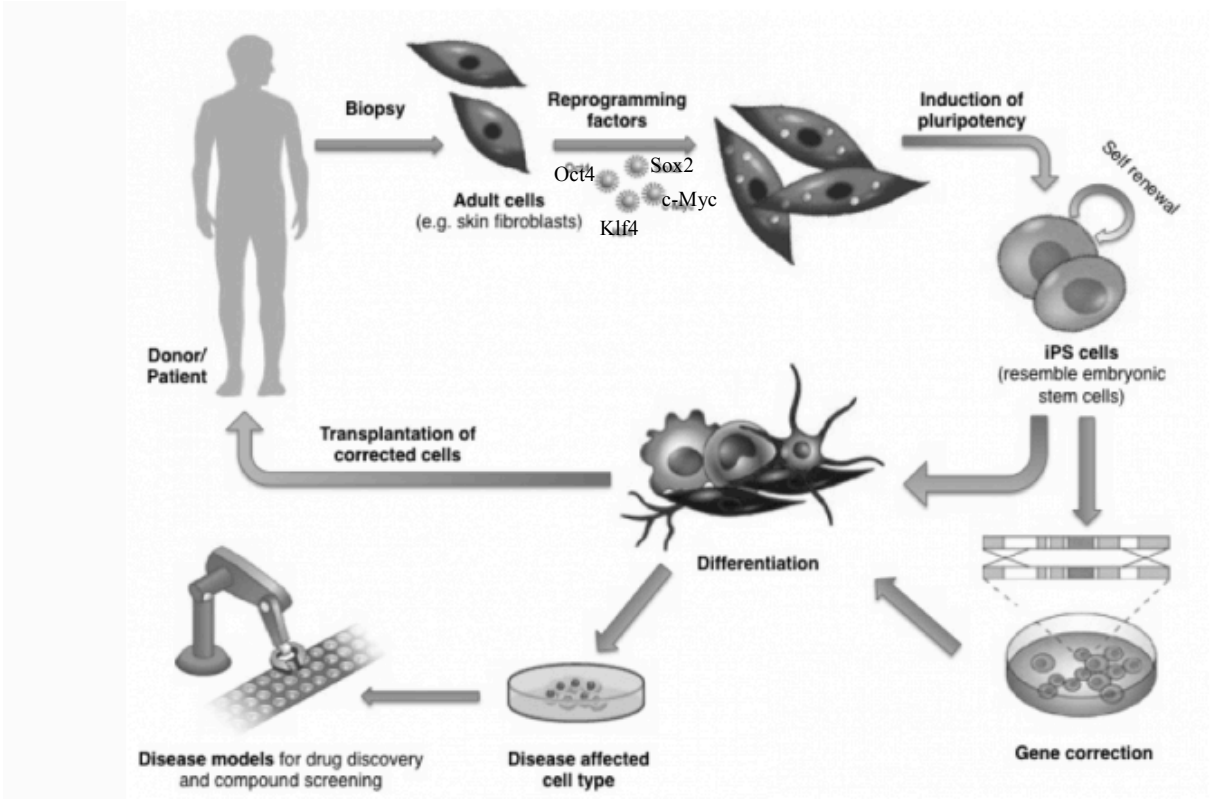
Explants of 3-mm dermal biopsies were minced and plated in a 60-mm tissue culture dish under a sterile coverslip held down by sterilized silicon grease. Nutrient mixture F-10 (Ham's F-10; Life technologies, Carlsbad, CA, USA) supplemented with 1% L-glutamine (Life technologies) was added and incubated at 37°C in a humidified 5% CO_2 atmosphere with media exchange. Fibroblast outgrowths were harvested by trypsinization, expanded in a T25 flask in Ham's F-10 medium, and allowed to reach ~90% confluence. The cells were either frozen in liquid nitrogen or used for a reprogramming experiment (Figure 2)

Generation of hiPSCs

Prior to the generation of the hiPSCs, mouse embryonic fibroblasts (MEFs) (CF-1 IRR (γ-irradiated), passage 3; Global Stem Inc., Rockville, USA) have to be seeded in the cell culture

plate. The plate was coated with 0.01% gelatine type A (Sigma Aldrich, St. Louis, MO, USA) dissolved in 'water for embryo transfer' (Life technologies) and incubated for 1h under sterile conditions at room temperature. After aspiration, 2×10^3 cells/cm^2 of γ-irradiated MEFs were plated and incubated at 37°C in a humidified 5% CO_2 atmosphere for at least 24h. The plates can be kept for a maximum of seven days in MEF medium (Life Technologies), supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, and 1% penicillin/streptomycin (all Life Technologies)

Figure 2: HiPSC-derivation and applications.

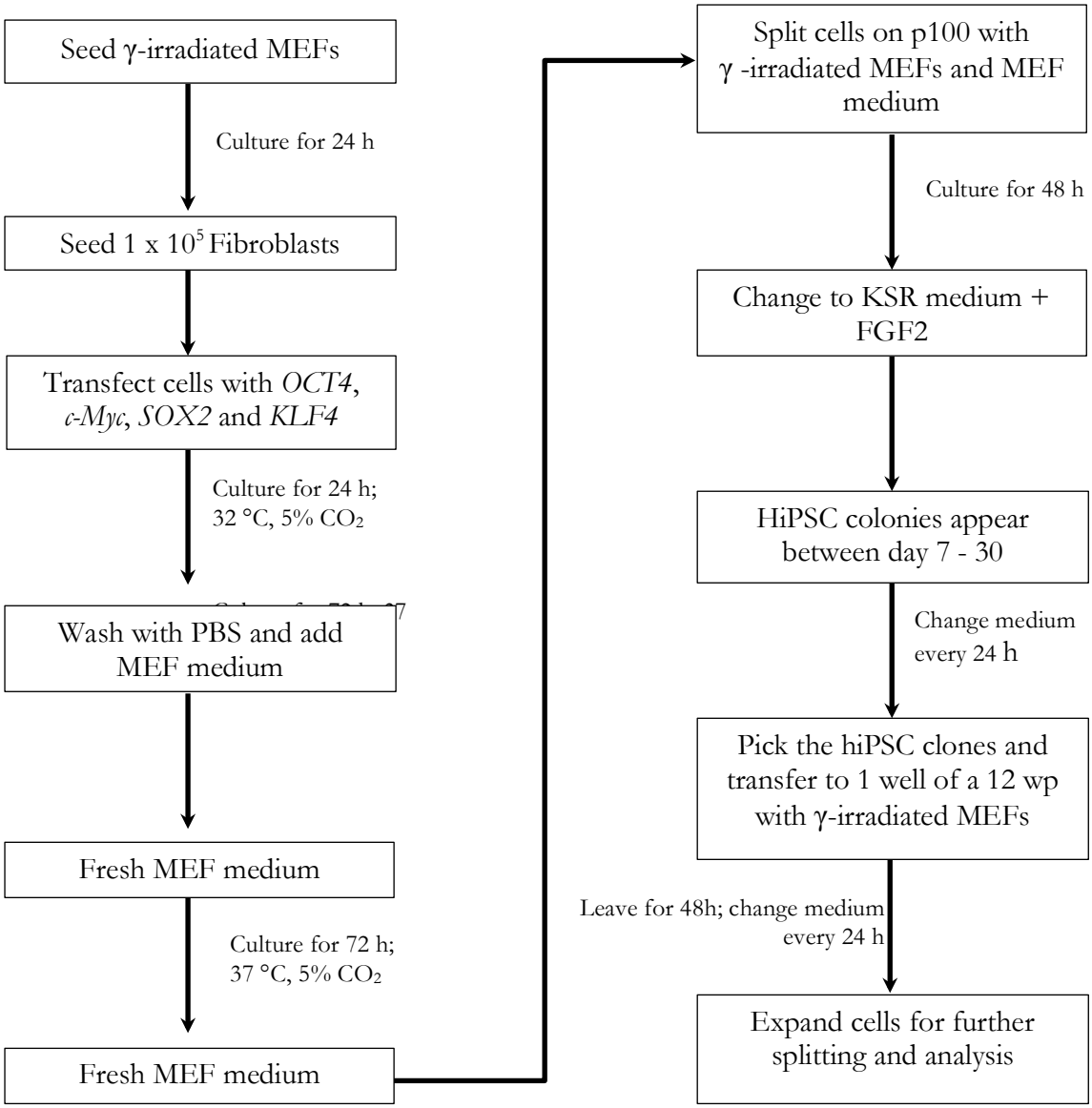


This figure illustrates the workflow to create hiPSC. Reprogramming of the isolated skin fibroblast cells will be achieved by the addition of 4 specific transcription factors, which introduces pluripotency of the cells. The resulting hiPSCs resemble ESC and can be differentiated into any type of cell to study the disease of interest, or to test future cell therapies using gene correction. Legend: *Oct4*: octamer-binding transcription factor 4, *Sox2*: sex determining region Y-box 2, *Klf4*: Kruppel-like factor 4, and *c-Myc*: v-myc avian myelocytomatosis viral oncogene homolog. (www.eurostemcell.org).

For the generation of hiPSC, MEF medium was added to a total of 1 x 10^5 fibroblasts in a 6 well-plate and incubated at 37°C in a humidified 5% CO_2 atmosphere overnight. Moloney murine leukaemia virus-derived vectors, each containing the coding sequence of one of the four human genes, *OCT4*, *SOX2*, *c-Myc* and *KLF4* (Vectaly, Toulouse, France) were transduced using the linker molecule polybrene at a multiplicity of infection (MOI) of 10 (for each virus) for 24 h at 32°C in a humidified 5% CO_2 atmosphere. The next day, cells were washed three times with phosphate buffered saline (PBS) followed by the addition of fresh MEF medium and further incubation at 37°C in a humidified 5% CO_2 atmosphere for another three days. Then again cells were washed and medium was replaced by fresh MEF medium, followed by another three days of culture. On day 7, cells were passaged on plastic plates containing γ-irradiated MEFs and grown for two additional days in MEF medium. To obtain hiIPSC, the medium was changed to human embryonic stem cell (hESC) culture conditions, i.e. advanced Dulbecco's modified eagle medium with Ham's F12 (ad. DMEM-F12), supplemented with 20% knockout™ serum replacement (KSR), 1% penicillin/streptomycin, 1% L-glutamine, 1% non-essential amino acids (all Life Technologies), 0.0007% β-mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA), and 4 ng/ml fibroblast growth factor 2 (FGF2; R&D systems Inc, Minneapolis, MN, USA)(KSR medium). HiPSC colonies will start to grow between the next 7 - 30 days. When a hiPSC colony was observed, cells should be picked after five additional days of culture. Individual colonies were picked and transferred into a single well of a 12 well plate containing γ-irradiated MEFs in KSR medium + 4 ng/ml FGF2 and incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells are left undisturbed for 48h, followed by the addition of fresh KSR medium + FGF2 medium the following day. Henceforth, KSR medium + FGF2 was replaced daily. After 7- 10 days colonies are large enough to be splitted and expanded (Figure 3).

A fresh 6 well plate with γ-irradiated MEFs was used to further split the cells. MEF medium was aspirated and replaced with KSR medium + FGF2 medium. HiPSCs were washed with PBS and detached with 1 mg/ml collagenase IV and 1 mg/ml dispase (both Life Technologies) during 1h incubation at 37°C in a humidified 5% CO_2 atmosphere. Detached colonies should be cracked during resuspension in KSR medium. The cracking should not result in single cells, but reduce the size of the colony. The reduced colony must not be centrifuged but must settle down by gravity, followed by aspiration of the medium and an additional washing with KSR medium. After resuspending, the colonies are transferred into KSR medium + FGF2 medium in a 6 well plate, showing a sub-confluence of 50% and incubated at 37°C in a humidified 5% CO_2 atmosphere.

Figure 3: Experimental scheme for the generation of hiPSC.



Legend: MEFs: mouse embryonic fibroblasts; *OCT4*: octamer-binding transcription factor 4, *SOX2*: sex determining region Y-box 2, *KLF4*: Kruppel-like factor 4, *c-Myc*: v-myc avian myelocytomatosis viral oncogene homolog, PBS: phosphate-buffered saline, KSR: knockout serum replacement, and FGF2: fibroblast growth factor 2.

Application to generate hiPSC of patients with premature atherosclerosis

Atherosclerosis is complex and multifactorial, and is the primary underlying pathology of coronary artery disease (CAD), the leading cause of mortality in the western society. We identified 5 pedigrees with Mendelian forms of premature atherosclerosis and identified novel non-synonymous variants in candidate genes co-segregating with the disease phenotype via next generation sequencing (NGS) methods and linkage analysis. To potentially gain more inside into the mechanism we reprogrammed patient skin fibroblasts into hiPSCs. In the follow up stage we

could differentiate the patient-specific hiPSCs into endothelial cells, smooth muscle cells or cardiomyocytes, to understand the pathophysiology of the identified candidate genes.

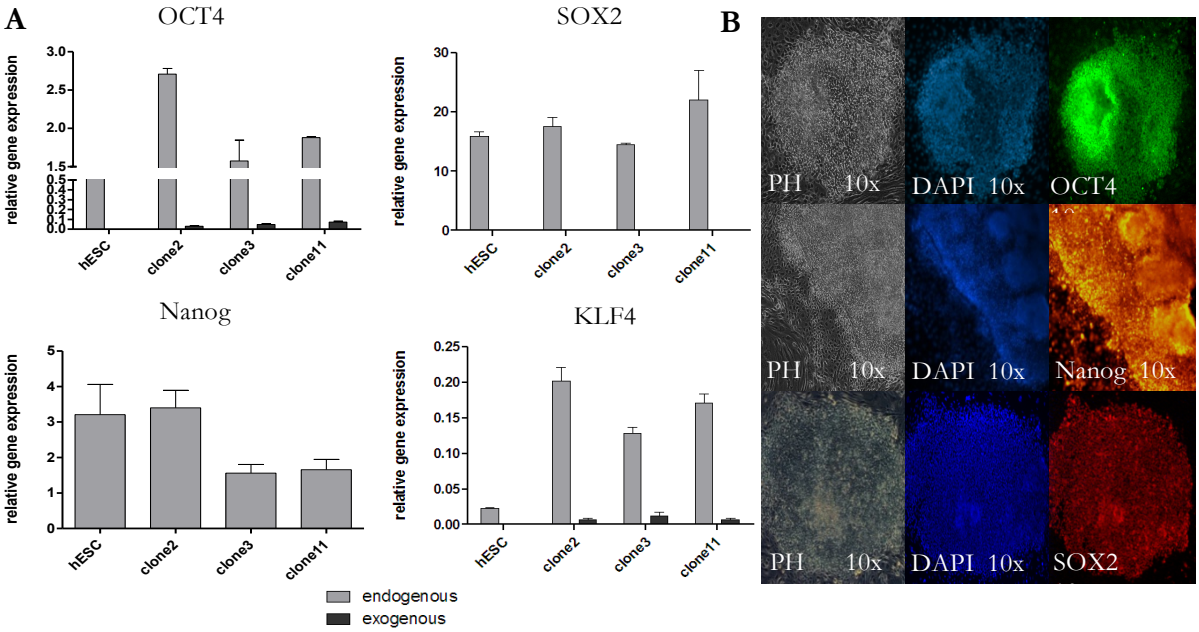
Reprogramming of patient skin fibroblasts into hiPSCs.

The fibroblast were obtained from one affected member per pedigree and cultured as described above. After transfection with the four transcription factors, hiPSCs colonies were tested on pluripotency status and their capability to differentiate into the three germ layers of extraembryonic tissue, mesendoderm and neurosctoderm.

Testing hiPSCs for pluripotency status

To analyse the pluripotency status of the reprogrammed hiPSC, the known pluripotency marker expressed by ESCs are analysed on gene expression (Supplementary table 1) and protein expression level (Supplementary table 2). Both, exogenous and endogenous gene expression of the transfected transcription factors were analysed. The exogenous transgenes have to be strongly silenced in fully reprogrammed cells (Figure 4A). Furthermore Nanog gene expression gives information about upregulation of the activin/nodal signaling [7]. Activin/Nodal signaling is necessary to maintain pluripotency and to induce differentiation toward endoderm. However, the mechanisms by which Activin/Nodal signaling achieves these opposite functions remain unclear [30]. Figure 4 illustrates the results for 1 patient comparing different hiPSC clones.

Figure 4: Status of pluripotency in patient specific hiPSCs.



A: Gene expression of the pluripotency marker *OCT4*, *SOX2*, *KLF4*, and *NANOG*. The hiPSCs clones were generated from skin fibroblast from a patient with premature atherosclerosis. Human ESCs were used as positive control. RNA was extracted and gene expression was assayed using qPCR as described. The exogenous gene expression depicts the gene expression of the transfected transcription factors whereas the cells own gene expression is shown as endogenous gene expression. All qPCR reactions were normalized against PBGD and run in triplicates in multiple runs. **B:** Immunostaining analysis was performed to detect the expression of the pluripotency marker OCT4, Nanog and SOX2. PH: phase contrast, DAPI: 4',6-Diamidin-2-phenylindol, *OCT4*: octamer-binding transcription factor 4, *SOX2*: sex determining region Y-box 2, *KLF4*: Kruppel-like factor 4, *c-Myc*: v-myc avian myelocytomatosis viral oncogene homolog, and PBGD: Porphobilinogen deaminase.

We then continued to test the pluripotency of the cells by their ablility to differentiate into the three germ layers (extraembryonic tissue, mesendoderm, and neuroectoderm) by analysing gene (Supplementary table 3) and protein expression (Supplementary table 4).

First we tested the differentiation into extraembryonic cells by testing the ability to differentiate under defined in-vitro medium conditons expressing specific markers on gene level (heart- and neural crest derivatives-expressed protein 1 (*Hand1*), Homeobox protein CDX-2 (*CDX-2*), and GATA Binding Protein 4 (*GATA4*)) and protein level (GATA4, Sox7, and Cdx2). Figure 5

illustrates the results for 1 patient. Similar results were seen for all the cellular preparations derived from different patient.

Extraembryonic tissue

For the differentiation into extraembryonic cells the hiPSCs were cultured in chemically defined medium (CDM) medium, containing of 50% Iscove's modified Dulbecco's medium (IMDM) plus 50% F-12 GlutaMax (both Life Technologies), supplemented with 7 μg/ml of insulin, 15 μg/ml transferrin (both Roche Diagnostics, Risch, Switzerland), 450 μM monothioglycerol (Sigma Aldrich) and 5 mg/ml bovine serum albumin fraction V (BSA-V) (Sigma Aldrich). HiPSCs were grown for 7 days in CDM + BSA-V medium in the presence of 10 ng/μl bone morphogenic protein 4 (recombinant human *BMP4*; R&D System Inc). Medium was changed every day. BMP4 drives differentiation of hiPSCs into extraembryonic tissue due to inhibition of the actin signaling.

Then we tested the differentiation potential into mesedoderm and neurocetoderm by using defined in-vitro medium conditions. The gene expression of specific mesenoderm markers (sex determining region Y-box 17 (*Sox17*), C-X-C chemokine receptor type 4 (*CxCr4*), forkhead box A2 (*FoxA2*), and Mix paired-like homeobox 1 (*Mixl1*)) and protein markers (Sox17, HNF3b/FoxA2, CXCR4, Eomes, and Brachyury) were evaluated. Figure 6 illustrates the results for 1 patient. Similar results were seen for all the cellular preparations derived from different patient. The gene expression of specific neuroectoderm markers (sex determining region Y-box 1 (*Sox1*), Paired box protein Pax-6 (*Pax6*), and Gastrulation Brain Homeobox 2 (*Gbx2*) and protein marker (SOX2, Sox1, and Pax6) were analysed. Figure 7 illustrates the results for 1 patient. Similar results were seen for all the cellular preparations derived from different patient.

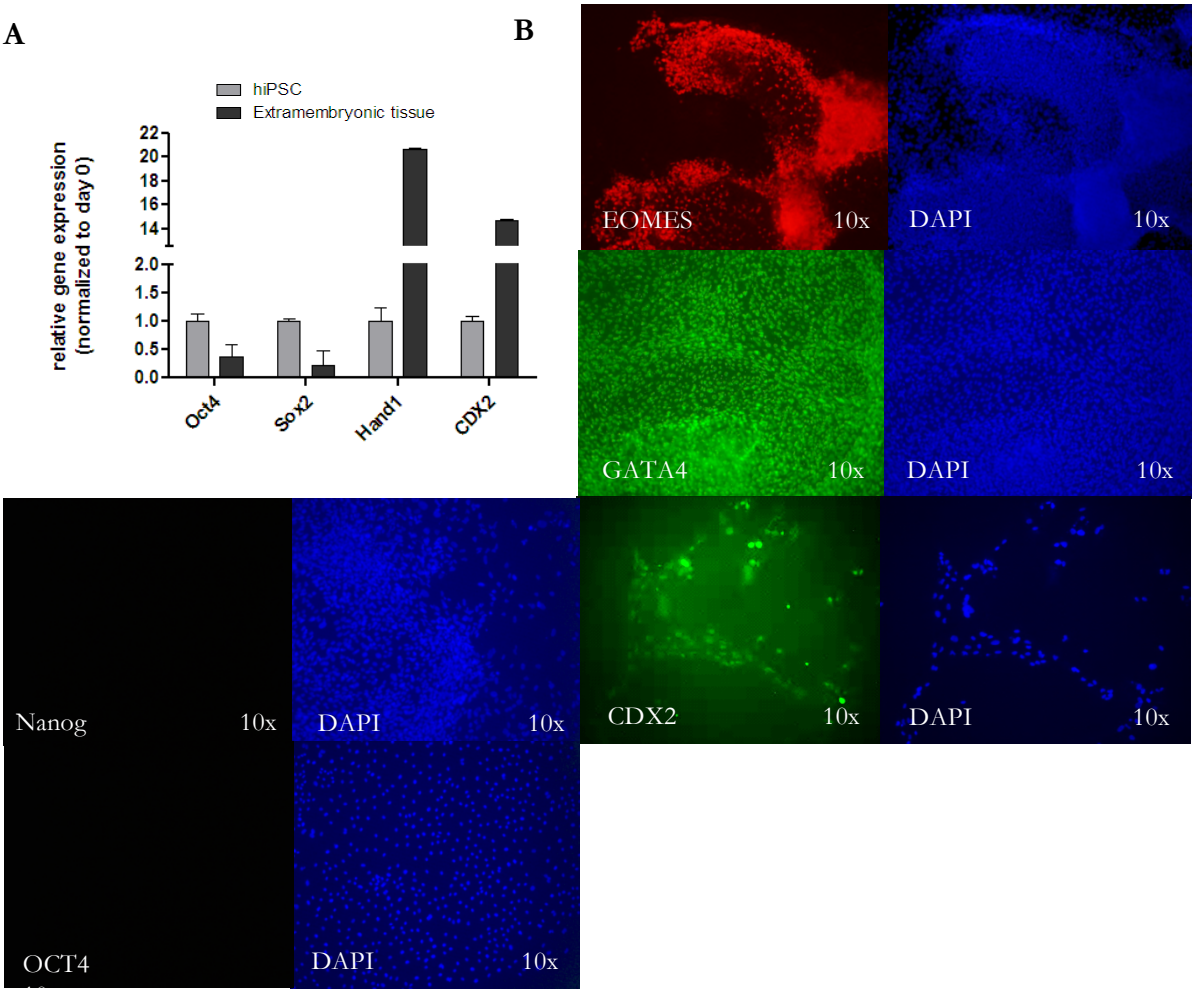
Mesendoderm and Neuroectoderm

For the differentiation into mesendoderm and neuroectoderm the hiPSCs were cultured in CDM supplemented with 1 mg/ml polyvinyl alcohol (PVA) (Sigma Aldrich).

For the differentiation into mesendoderm, hiPSCs were grown for 3 days in CDM-PVA medium in the presence of 10 ng/ml *BMP4*, 20 ng/ml FGF2, 100 ng/ml activinA (recombinant human/mouse/rat activinA; R&D Systems), and 10 μM LY294002 (Promega, Madison, WI, USA). On the second day, 3 μM ChiR (Stemgent) was added for 24 hours.

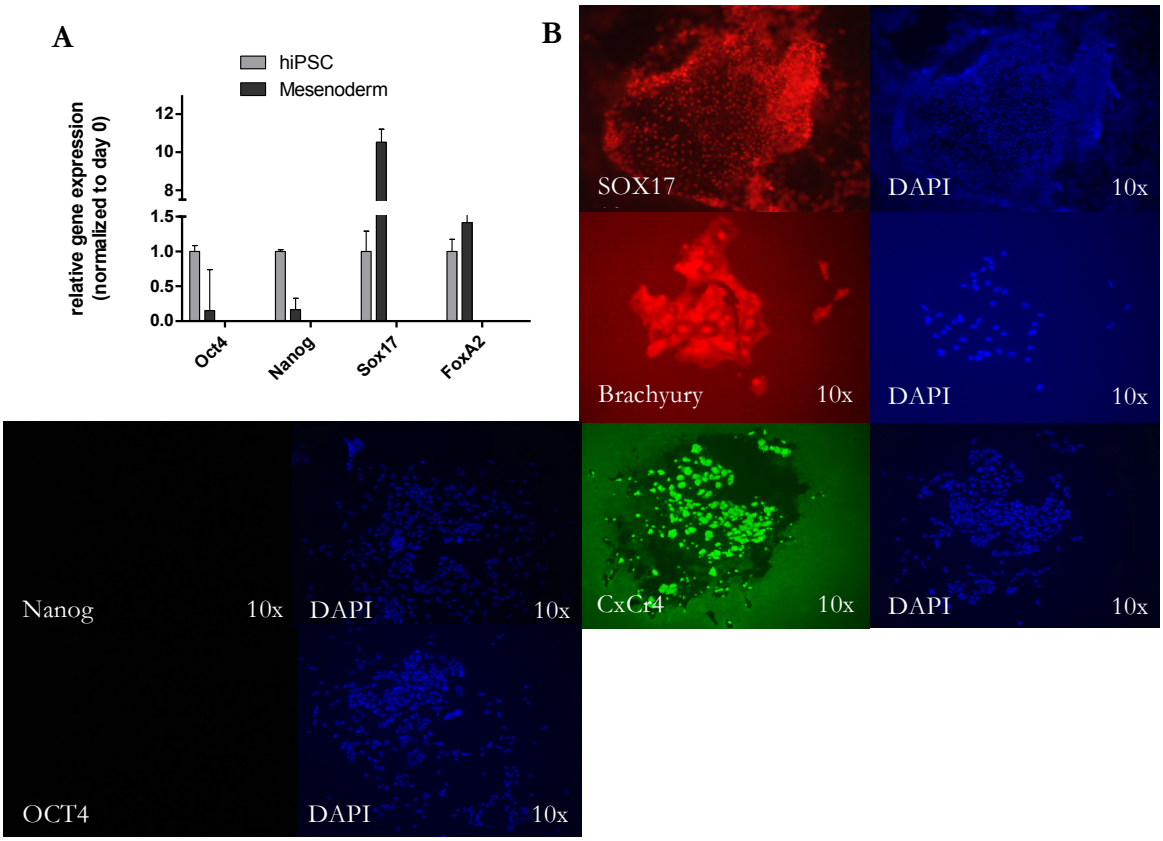
For the differentiation into neuroectoderm, hiPSCs were grown for 7 days in CDM-PVA medium in the presence of 10 μM SB431542 (Tocris Bioscience) and 12 ng/ml FGF2.

Figure 5: Differentiation potential of patient specific hiPSCs into extraembryonic cells.



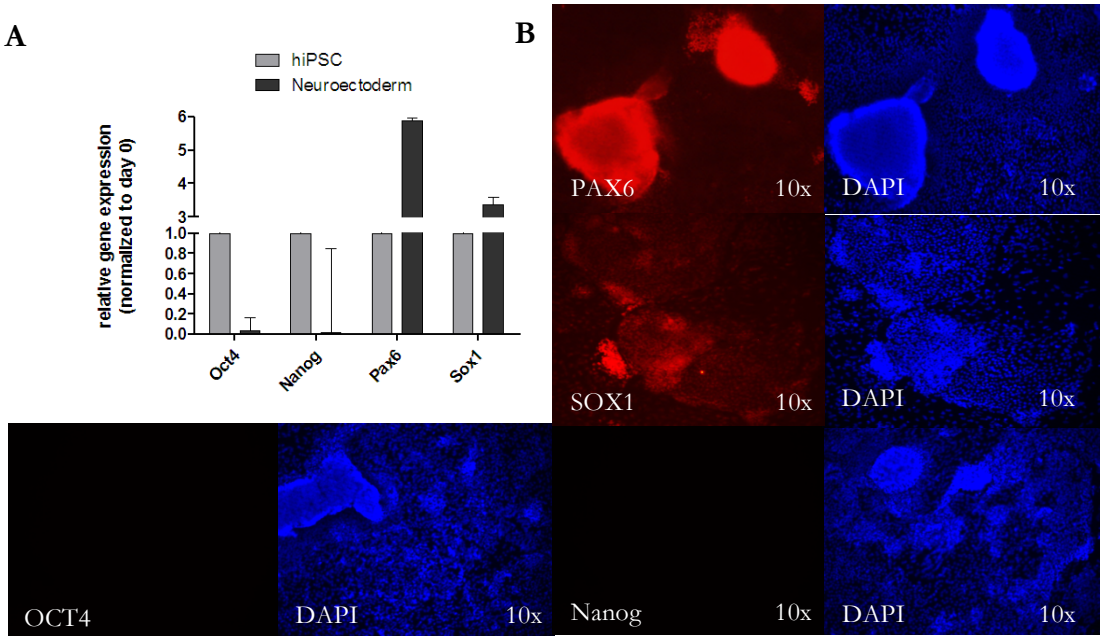
BMP4 drives differentiation of hiPSCs into extraembryonic tissue. **A:** hiPSCs grown in the presence of *BMP4* (and in the absence of activin) differentiate into extraembryonic cells Absence of the pluripotency marker (*OCT4* and *SOX2*) and induction of the extraembryonic marker (*Hand1* and *CDX2*) was observed. RNA was extracted and all qPCR results are normalized against PBGD and set relative to day 0 of the differentiation protocol. **B:** Immunostaining analysis was performed to detect expression of pluripotency markers OCT4 and Nanog, primitive endoderm marker GATA4, and trophoectoderm markers EOMES and CDX2.

Figure 6: Differentiation potential of patient specific hiPSCs into mesendodermal cells.



A combination of activinA, BMP4, and FGF2 drives hiPSCs into mesendoderm. **A:** Absence of pluipotency marker (*OCT4* and *Nanog*) and induction of mesendoderm marker (*Sox17* and *FoxA2*). RNA was extracted and gene expression was assayed using qPCR as described. All qPCR reactions were normalized against PBGD and set relative to day 0 of the differentiation protocol. **B:** Immunostaining analysis for the expression of mesendoderm marker Brachyury, and the definitive endoderm marker Sox17 and CxCr4, and absence of pluripotency marker OCT4 and Nanog.

Figure 7: Differentiation potential of patient specific hiPSCs into neuroectodermal cells.



Inhibition of activin/nodal signaling and bone morphogenic protein (BMP) signaling in the presence of FGF2 leads to neuroectoderm differentiation. **A:** Absence of pluipotency marker (*OCT4* and *Nanog*) and induction of neuroectoderm marker (*Pax6* and *Sox1*). RNA was extracted and gene expression was assayed using qPCR as described. All qPCR reactions were normalized against PBGD and set relative to day 0 of the differentiation protocol. **B:** Immunostaining analysis for the expression of neuroectoderm marker Pax6 and Sox1, and the absence of pluripotency marker OCT4 and Nanog.

In conclusion we were able to successfully reprogramme patient-specific fibroblast into stable hiPSCs. These patient-derived hiPSCs have the potential for differenatiation into disease related cell types, e. g. smooth muscle cells.

Remarks for reprogramming hiPSCs

HiPSCs could be instrumental in the field of regenerative medicine, disease modelling and understanding the development of diseases. Several examples of successfully reprogramming of patient-derived hiPSC have been reported using material from patients with spinal muscular atrophy (SMA) [22], patients with liver disease [28,31], patients with dilated cardiomyopathy (DCM) [32], patients with diabetes type 1 and type 2 [33], and patients with maturity onset diabetes of the young type 2 (MODY2) [34].

However, hiPSCs do have similar safety issues as the use of hESCs therapies. This includes karyotypic instability and post-transplant tumor formation [35]. Improvement of the quality of hiPSC and hiPSC-derived cells is required to make disease models using hiPSCs more faithful [36].

To start the reprogramming process in the selected cell type, the transcription factors have to be translocated into the nucleus. Therefore the retrovirus system was chosen by Yamanka et al. [4] to allow a high infection rate of the cells and to enable the exogenous transcription factors to be expressed at high levels for longer time to induce the reprogramming. The cell-own transcriptome of the pluripotency network has to be activated and should show stable expression. The integrating retroviral-delivery systems are a handicap with prospect for future clinical applications, especially the use of the oncogenes *c-Myc* and *KLF4*. Therefore establishing a transgene-free reprogramming system would be an advantage. Possible alternative approaches are mimicking transcription factors with small compounds [37], integration-free vectors (plasmids or adenovirus) [38,39], RNA molecules [40,41]. The main issue with these methods is that they tend to be much less efficient compared to retroviral methods [37,38]. Additionally some problems such as genetic mutations during reprogramming, incomplete epigenetic reprogramming, and undesired gene expression should be controlled and standardized [36]. More sophisticated differentiation, maturation, and purification protocols will be indispensable to create physiologic cellular conditions that reflect the actual disease phenotype [36]. So far especially the direct differentiation into hepatic cells has improved with more efficient and functional methods [33,42-45]. Furthermore the MODY2 patietns specific hiPSCs were successfully differentiated into β cells, and DCM patient specific hiPSCs into cardiomyocytes [32]. Furthermore neutrophils [46], neural stem cells [47], and intestinal tissue [48] have been derived from healthy hiPSC. And further protocols are coming up.

Although human skin fibroblasts are the most common used cell type for reprogramming, they might not be the ideal cells. The isolation requires a surgical procedure for the donor, which is especially unwanted for children, patients with skin disorders or patients with abnormal wound healing or bleeding disorders [5].

Furthermore it is important to use low passage numbers for the transduction of the transcription factors. It has been shown that reprogramming of fibroblasts in higher passage numbers became less efficient [4,49]. Therefore alternative cell material like endothelial cells harvested from the blood of a patient or keratinocytes might be an attractive option.

To analyse the successful reprogramming further expansion of the hiPSC colonies into higher passage numbers gives more stable hiPSCs. The potential of differentiation into the germ layers

was improved. It has been shown that reprogrammed hiPSCs can be cultured in a feeder free system [7] but not every hiPSC colony does like it and should be tested as uncontrolled differentiation can occur.

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Supplementary Information

RNA Extraction and quantitative (real-time) polymerase chain reaction

Total RNA was extracted from hiPSCs using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the instruction manual. For reverse transcription polymerase chain reaction 500 ng of total RNA was transcribed using Superscript II Reverse Transcriptase (Invitrogen). Quantitative real-time polymerase chain reaction (qPCR) mixture were prepared as described (SensiMix protocol, Quantace, London, UK) [7] using 3 mM Sensi Mix, 100 nM SYBR green I solution, 200 nM of each primer in a final volume of 15 µl, followed by denaturation at 94°C for 5 minutes and cycled at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension of 72°C for 10 minutes after completion of 40 cycles. All rimer sequences are displayed in supplementary table 1 and 3. QPCR reactions were performed on a Mx3005P system (Stratagene, La Jolla, CA, USA) in triplicates and normalized to porphobilinogen deaminase (*PBGD*).

Immunohistochemistry

HiPSCs and differentiated hiPSCs were fixed for 20 minutes at 4 °C in 4% paraformaldehyde and washed three times with PBS. Cells were incubated for 20 minutes at room temperature in PBS with 10% donkey serum (Serotec Ltd., Kidlington, UK). The fixed cells were incubated overnight at 4 °C with primary antibody for hOCT3/4 (1:100; mouse monoclonal IgG, Santa Cruz sc-5279), hSOX2 (1:200; goat polyclonal IgG, R&D AF2018), hTRA-1-60 (1:100; mouse monoclonal IgM, Santa Cruz sc-21705), and hNanog (1:100; goat polyclonal IgG, R&D AF1997) diluted in 1% donkey serum in PBS and 0.1% triton. All primary antibodies are listed in supplementary table 2. Cells were washed three times in PBS and incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (1:200; Sigma Aldrich) in 1% donkey serum in PBS or rabbit IgG (1:400; Jackson Laboratory, Bar Harbor, ME, USA) in 1% donkey serum in PBS or goat IgG (1:400; Jackson Laboratory) in 1% donkey serum in PBS for 2 hours at room temperature. Unbound secondary antibody was removed by several washes in PBS. Hoechst 33258 (1:10.000; Sigma Aldrich) was added to the first wash (Figure 4B). The primary antibodies for the extraembryonic tissue were hGATA-4 (1:100; mouse monoclonal IgG, Santa Cruz sc-25310), hSox7 (1:100; rabbit polyclonal IgG, Abcam Cat#ab22584), and hCdx2 (1:100; mouse monoclonal IgG, BioGenex MU392A-UC) (Supplementary table 4). The primary antibodies for the mesendoderm were hSox17 (1:200; goat polyclonal IgG, R&D AF1924), hHNF3b/FoxA2 (1:100; goat polyclonal IgG, R&D AF2400), hCXCR4 (1:100; mouse monoclonal IgG, R&D MAB173), hEMOES (1:600; rabbit polyclonal IgG, Abcam AB23345), and hBrachyury (1:100;

mouse monoclonal IgG, R&D AF2085) were detected (Supplementary table 4). The primary antibodies for the neuroectoderm were hSOX2 (1:200; goat polyclonal IgG, R&D AF2018), hSox1 (1:100; goat polyclonal IgG, R&D AF3369), and hPax6 (1:100; rabbit polyclonal IgG, Covance (Cambridge Bio Science) PRB-278P-100) (Supplementary table 4).

Freezing hiPSCs

The hiPSCs were frozen in knockout™ serum replacement, supplemented with 10% dimethyl sulfoxide (DMSO; Sigma Aldrich). Cells should be transferred from -80 °C into liquid nitrogen as soon as possible.

Supplementary table 1: Primer sequences for qPCR analysis of transgene and endogenous gene expression.

	Name	Sequence [5' – 3']	Tm [°C]
Transgene expression	OCT4 forward	CCTCACTTCACTGCACTGTA	60
	KLF4-forward	GATGAACTGACCAGGCACTA	60
	SOX2-forward	CCCAGCAGACTTCACATGT	60
	c-Myc-forward	AAGAGGACTTGTTGCGGAAA	59
	Trans 3'-reverse	TCCTGTCTTTAACAAATTGGACT	59
Endogenous expression	OCT4-forward	CCTCACTTCACTGCACTTGTA	60
	OCT4-reverse	CAGGTTTTCTTTCCCTAGCT	60
	SOX2-forward	ATGTCCCAGCACTACCAGAG	60
	SOX2-reverse	GCACCCCTCCCATTTCCC	60
	c-Myc-forward	CTGAAGAGGACTTGTTGCGGAAAC	59
	c-Myc-reverse	TCTCAAGACTCAGCCAAGGTTGTG	59
	KLF4-forward	GGTCGGACCACCTCGCCTTACAC	61
	KLF4-reverse	CTCAGTTGGGAACTTGACCA	61
	PBGD-forward	GGAGCCATGTCTGGTAACGG	60
	PBGD-reverse	CCACGCGAATCACTCTCATCT	60

OCT4: Octamer-binding transcription factor 4, *SOX2*: sex determining region Y-box 2, *KLF4*: Kruppel-like factor 4, *c-Myc*: v-myc avian myelocytomatosis viral oncogene homolog, and PBGD: Porphobilinogen deaminase.

Supplementary table 2: List of primary antibodies for characterization of iPSCs.

Name	Specification	Company	Dilution
hOCT-3/4 (C-10)	mouse monoclonal IgG	Santa Cruz sc-5279	1:100
hSOX2	goat polyclonal IgG	R&D AF2018	1:200
hTRA-1-60	mouse monoclonal IgM	Santa Cruz sc-21705	1:100
hNanog	goat polyclonal IgG	R&D AF1997	1:100

HOCT4: Octamer-binding transcription factor 4, hSOX2: sex determining region Y-box 2, and hTRA-1-60: keratan sulfate antigen, which confirms the absence of spontaneous differentiation in culture conditions.

Supplementary table 3: Primer sequences for qPCR analysis for characterization of extraembryonic tissue, mesendoderm and neuroectoderm.

	Name	Sequence [5' – 3']	Tm [°C]
Extraembryonic tissue	CDX2_Forward	GGGCTCTCTGAGAGGCAGGT	62
	CDX2_Reverse	CCTTTGCTCTGCGGTTCTG	62
	GATA4_Forward	GCTCTACAGCAAGATGAACGGC	65
	GATA4_Reverse	ATGTAGAGCCCATCTTGACCCG	65
	Hand1_Forward	GTGCGTCCTTTAATCCTCTTC	60
	Hand1_Reverse	GTGAGAGCAAGCGGAAAAG	60
Neuroectoderm	Sox1_Forward	ATGCACCGCTACGACATGG	60
	Sox1_Reverse	CTCATGTAGCCCTGCGAGTTG	60
	Pax6_Forward	AGCCAGGTTGCGAAGAACTC	60
	Pax6_Reverse	CTTTGCTTGGGAAATCCGAG	60
	Gbx2_Forward	GTTCCACTGCAAAAAGTACCTCT	61
	Gbx2_Reverse	GGGACGACGATCTTAGGGTTC	61
Mesendoderm	Sox17_Forward	CGCACGGATTTGAACAGTA	60
	Sox17_Reverse	GGATCAGGGACCTGTCACAC	60
	CxCr4_Forward	CACCGCATCTGGAGAACCA	60
	CxCr4_Reverse	GCCCATTTCCTCGGTGTAGTT	60
	FoxA2_Forward	GGGAGCGGTGAAGATGGA	60
	FoxA2_Reverse	TCATGTTGCTCACGGAGGAGTA	60
	Mixl1_Forward	GGTACCCCGACATCCACTTG	60
	Mixl1_Reverse	TAATCTCCGGCCTAGCCAAA	60

CDX2: Homeobox protein CDX-2, *Hand1*: heart- and neural crest derivatives-expressed protein 1, *GATA4*: GATA Binding Protein 4, *Sox1*: sex determining region Y-box 1, *Pax6*: Paired box protein Pax-6, *Gbx2*: Gastrulation Brain Homeobox 2, *Sox17*: sex determining region Y-box 17, *CxCr4*: C-X-C chemokine receptor type 4, *FoxA2*: Forkhead box A2 and *Mixl1*: Mix paired-like homeobox 1.

Supplementary table 4: Primary antibodies for immunohistochemistry staining of extraembryonic tissue, mesendoderm and neuroectoderm.

	Name	Specification	Company	Dilution
Extraembyronic tissue	hGATA-4	mouse monoclonal IgG	Santa Cruz sc-25310	1:100
	hSox7	rabbit polyclonal IgG	Abcam Cat#ab22584	1:100
	hCdx2 (CDX2-88)	mouse monoclonal IgG	BioGenex MU392A-UC	1:100
Neuroectoderm	hSOX2	goat polyclonal IgG	R&D AF2018	1:200
	hSox1	goat polyclonal IgG	R&D AF3369	1:100
	hPax6	rabbit polyclonal IgG	Covance(Cambridge Bio Science) PRB-278P-100	1:100
Mesendoderm	hSox17	goat polyclonal IgG	R&D AF1924	1:200
	hCXCR4 (Fusin, Clone 44717)	mouse monoclonal IgG	R&D MAB173	1:100
	hEomes	rabbit polyclonal IgG	Abcam AB23345	1:600
	hHNF3b/ FoxA2	goat polyclonal IgG	R&D AF2400	1:100
	hBrachyury	mouse monoclonal IgG	R&D AF2085	1:100

hGATA4: human GATA Binding Protein 4, hSox7: human sex determining region Y-box 17, hCDX2: Homeobox protein CDX-2, hSOX2: human sex determining region Y-box 2, Pax6: Paired box protein Pax-6, hSox17: human sex determining region Y-box 17, hCxCr4: human C-X-C chemokine receptor type 4, EOMES: Eomesodermin and hHNF3B/FoxA2: human forkhead box A2.