Reprogramming to pluripotency does not require transition through a primitive streak-like state

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Pluripotency can be induced in vitro from adult somatic mammalian cells by enforced expression of defined transcription factors regulating and initiating the pluripotency network. Despite the substantial advances over the last decade to improve the efficiency of direct reprogramming, exact mechanisms underlying the conversion into the pluripotent stem cell state are still vaguely understood. Several studies suggested that induced pluripotency follows reversed embryonic development. For somatic cells of mesodermal and endodermal origin that would require the transition through a Primitive streak-like state, which would necessarily require an Eomesodermin (Eomes) expressing intermediate.

We analyzed reprogramming in human and mouse cells of mesodermal as well as ectodermal origin by thorough marker gene analyses in combination with genetic reporters, conditional loss of function and stable fate-labeling for the broad primitive streak marker Eomes. We unambiguously demonstrate that induced pluripotency is not dependent on a transient primitive streak-like stage and thus does not represent reversal of mesendodermal development in vivo.

During mammalian development, early cell fate decisions during the process of gastrulation lead to the formation of the three germ layers, namely ectoderm, mesoderm and endoderm. The development of mesoderm and definitive endoderm (DE) is initiated by cell rearrangements of pluripotent, epithelial cells of the posterior epiblast and a subsequent epithelial-to-mesenchymal transition (EMT) leading to the formation of the primitive streak (PS). Mesoderm and DE cells are recruited as they migrate through the PS, while epithelial cells which do not ingress through the streak give rise to neuroectodermal progeny including the epidermis and central nervous system (reviewed in1). PS formation is initiated and maintained by a complex network of transcription factors and signaling pathways. Signals include feed forward loops of WNT, TGFβ and BMP factors involving reciprocal tissue interactions of epiblast and trophectoderm (reviewed in1). Absence or misexpression of these signals in the epiblast leads to an impaired PS formation followed by disorganization or absence of the mesoderm and endoderm germ layers and embryonic lethality. Examples are null mutants for Nodal or Wnt signaling, such as Wnt32, the nuclear receptor Nr5a23, or the T-box transcription factor Eomesodermin (Eomes)4,5.

The specification of different PS-derived cell types follows a strict spatio-temporal pattern. The most anterior PS gives rise to the early transient population of mesendoderm cells that contribute to the DE and axial mesoderm. This population is followed by cells ingressing through the anterior third of the streak generating the anterior mesoderm that gives rise to head and cardiogenic mesenchyme and extraembryonic mesoderm4,5. Cells ingressing at more posterior streak levels are giving rise to paraxial, intermediate and lateral plate mesoderm. The signaling pathways regulating streak patterning include Nodal- and Wnt-activities. For example, high levels

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of Nodal activities induce the expression of mesendodermal marker genes such as Eomes, Mixl1, Tdgf1 (Cripto), Lhx1 and Foxh1 (reviewed in13). In particular, Eomes is critically required for the specification of early mesendoderm (DE and anterior mesoderm)13,14, and all cells of the early PS transiently express Eomes. Eomes-deficiency in the epiblast also dramatically perturbs PS formation due to defective EMT leading to early embryonic arrest14. Similarly, the sequential formation of different cellular subtypes of the streak can be mimicked by in vitro cell differentiation and monitored by the expression of marker genes. The in vitro differentiation of pluripotent stem cells towards the three germ layers can be guided by similar signaling stimuli and mRNA expression profiles usually reflect the in vivo situation15,16.

During reprogramming to induced pluripotency through forced expression of the core pluripotency factors SOX2, OCT4, KLF4, and C-MYC, somatic cells lose their differentiated state11,12. Several reports suggested that reprogramming follows distinct stages resembling a reversal of embryonic development13,14. Fibroblasts, as the most common starting cell type for reprogramming, represent cells of mesoderm origin. Thus, the reversal of their cellular ontology during reprogramming would most likely involve the passage through a PS-like stage14. Accordingly, it was proposed that a sequential cascade of EMT–MET facilitates the reprogramming process13,15.

To initiate the transcription factor networks and signaling pathways that are characteristic for pluripotent cells, extensive alterations in the epigenetic landscape take place such as broad changes of chromatin modifications, chromatin architecture, and gross changes in the cellular transcriptome16. Although several studies have explored mechanisms and stages during the reprogramming process17, the question concerning an analogy of the reprogramming process as reversal of physiological embryonic development, including gastrulation is controversial18. Moreover, it is debatable why also cells derived from ectodermal lineages, such as astrocytes or keratinocytes would show a PS-like global gene expression pattern during reprogramming19, given that ectodermal cells developmentally never ingress through the PS. Thus, it is questionable, whether these events indeed reflect reverted embryonic development or might rather represent changes in transcriptional programs induced by the forced expression of reprogramming factors. To address these developmental aspects of reprogramming, we used different reprogramming approaches including somatic cells from different germ layers and organisms, namely murine and human cells as well as different reporter alleles and fate analysis tools. Thereby, we provide evidence that somatic cell reprogramming neither follows a reversed mesendoderm development nor that occurring mesendodermal gene signatures reach physiological and functionally relevant levels during differentiation.

Results
Gene expression patterns during reprogramming of human somatic cells of mesoderm and ectoderm origin. To investigate if cells during human reprogramming follow stages of reversed embryonic development, we transduced keratinocytes and fibroblasts which have ectodermal and mesodermal origin, respectively, with a polycistronic OKSM (OCT3/4, KLF4, SOX2, c-MYC) construct to monitor and directly compare gene expression signatures during reprogramming (Fig. 1A, Supplemental Fig. 1A). Consistent with previous reports14, a transcriptional signature resembling a PS-like and mesendodermal program was observed during reprogramming of both cell types representing different germ layer origin (Fig. 1B, Supplemental Fig. 1B,C). Expression patterns of key markers of PS formation and subsequent early mesendoderm differentiation (EOMES, T, CER, LHX1, FGF4, FGF8, MIXL1) were similarly regulated in both keratinocytes and fibroblasts. However, reprogramming of keratinocytes appeared delayed compared to fibroblasts as shown by the expression profile of the pluripotency marker NANOG (Fig. 1B). Of note, particularly NANOG expression, previously shown to reinforce mesendoderm differentiation during pluripotency exit19, coincides with the mesendodermal signature (Fig. 1B, Supplemental Fig. 1B,C). Both mesodermal fibroblasts and ectodermal keratinocytes displayed an increase in mesendoderm and primitive streak markers starting at day 6–8 (fibroblasts) or 9–12 (keratinocytes) with a peak between day 12–14 (fibroblasts; except for CER1) or 15–18 (keratinocytes), followed by the downregulation of these genes (except FGF8) until the induced pluripotent stem cell (iPSC) state (Fig. 1B, Supplemental Fig. 1B,C). Next, we aimed to determine the expression range of this mesendodermal gene signature by comparing mRNA levels of cells during reprogramming with cells undergoing directed mesendoderm differentiation in vitro. (Fig. 1C). This direct comparison showed that mRNA levels of mesendoderm markers (EOMES, LHX1, CER1) were several magnitudes higher in differentiating cells compared to the expression during reprogramming (Fig. 1D). Given that EOMES expression is critical for PS and subsequent mesendoderm formation14,18, we evaluated EOMES protein levels during reprogramming. However, no EOMES protein was detected, neither by immunocytochemistry nor by Western blot (in fibroblasts), during reprogramming of fibroblasts and keratinocytes (Fig. 1E–G). As a control, expression of the pluripotency marker NANOG was analyzed in parallel showing increasing protein levels during reprogramming (Fig. 1E–G). As control, mesendodermal differentiation of hiPSCs displayed the expression of EOMES protein on day 3 as analyzed by immunofluorescent staining (Fig. 1H). In summary, these results indicate that PS and mesendoderm markers are significantly upregulated during the course of reprogramming. At the same time NANOG reaches its expression peak, suggesting that the establishment of pluripotency networks triggers a PS-like expression phenotype. However, mRNA levels are detected at much lower levels compared to those found during mesendodermal differentiation (Fig. 1D).

Murine fibroblasts undergoing reprogramming do not express Eomes protein. To corroborate the finding that PS markers are frequently expressed only at low mRNA level during reprogramming, independent of the parental germ layer origin, we investigated the expression of Eomes as one of the key TFs for PS and mesendoderm development14,18. Since we didn’t detect EOMES protein during human somatic cell reprogramming (Fig. 1E–G), we sought to apply mouse embryonic fibroblasts (MEFs) harboring a EomesGFP/+ reporter allele19 to track eventually arising Eomes-expressing, GFP-positive cells using very sensitive FACS techniques (Fig. 2A). EomesGFP/+ MEFs were transduced with a polycistronic OKS (OCT3/4, KLF4, SOX2) construct harboring a Td-tomato expression cassette to visualize cells that undergo reprogramming20 (Fig. 2A,B). During 21 days
Figure 1. Upregulation of primitive streak and mesendoderm markers during reprogramming of human somatic cells of fibroblast and keratinocyte origin, but absence of EOMES protein. (A) Schematic overview of the reprogramming experiment for the somatic cells of ectodermal – keratinocytes, and mesodermal origin – fibroblasts. (B) Expression patterns of indicated genes during reprogramming of human keratinocytes (upper row) and human fibroblasts (lower row). All mRNA levels are expressed relative to the housekeeping gene HMBS and values have been normalized to iPSCs, which have been set to 1 to illustrate fold induction. (C) Schematic overview of the experimental setup of mesendoderm differentiation. (D) Comparison of marker gene expression for definitive endoderm (CER1) and mesendoderm/primitive streak (LHX1, EOMES) are lower in magnitude for the reprogrammed cells compared to the differentiated cells. (E,F) Protein expression of EOMES and NANOG during the time course of human keratinocytes (E) and human fibroblasts (F) reprogramming. The scale bar represents 100 µm for all images. (G) Western Blot analysis EOMES and NANOG protein expression during reprogramming of fibroblasts. Actin was used as loading control. Lane 1: human foreskin
of reprogramming, FACS analyses and immunocytochemistry for both the GFP and Tomato signal were conducted at intervals of 2 or 3 days. FACS analysis did not reveal any GFP-positive cells, while the red tomato-signal from the reprogramming cassette expectedly got silenced when reaching the iPSC state on day 21 (Fig. 2B,C). In line with human data, we observed Eomes up-regulation on mRNA level but in a far lower range than observed in spontaneous, differentiating mouse iPSC cultures (Supplemental Fig. 2; Fig. 2D,E). To control for the efficiency of the EomesGFP/+ reporter, we differentiated resulting EomesGFP/+ iPSCs using high doses (50 ng/ml) of Activin A to drive mesendoderm formation and could detect high levels of GFP-expression. Thus, we could successfully validate the functionality of the reporter allele during differentiation (Fig. 2F–H). Despite the inability to detect EomesGFP/+ reporter expression during reprogramming, transient Eomes expression cannot be entirely excluded, as cell samples were harvested at time-intervals of 2–3 days.

**Eomes expression remains undetectable during cell lineage tracking.** To rigorously test if Eomes is significantly expressed at any stage during reprogramming, we used MEFs carrying a 4-hydroxytamoxifen (4-OHT)-inducible CreERT in the Eomes locus (EomesCreERT) and a Cre-inducible fluorescent reporter cassette (Rosa26Tom/GFP) to permanently lineage-label Eomes-expressing cells (Fig. 3A,B). Ssea1 was used in FACS analysis to mark pluripotent cells and assess the efficiency of reprogramming. Among the detected Ssea1-positive cells, no GFP-positive cells were detected after tamoxifen treatment on days 3–15 during reprogramming (Fig. 3C). The absence of GFP-positive cells in successfully reprogrammed cells was further confirmed by immunofluorescence in picked and expanded iPSC cultures (Fig. 3D, upper image), indicating the lack of Eomes expression during and at the end of the reprogramming process. To validate the lineage labeling tool used in these experiments, we induced mesendoderm differentiation of resulting iPSCs in the presence of tamoxifen and Activin A, which resulted in the appearance of GFP-positive, Eomes expressing cells within the differentiating Tomato-positive embryonic bodies (Fig. 3D, lower image).

**Eomes is dispensable for reprogramming of somatic cells of mesoderm origin.** To exclude that very low amounts of Eomes protein are being expressed at levels undetectable via FACS or immunofluorescent staining, we used MEFs carrying the EomesGFP reporter allele and a floxed Eomes allele (EomesCreERT) in combination with a ubiquitously expressed 4-hydroxytamoxifen (4-OHT)-inducible CreERT (Rosa26CreERT) to inducibly delete Eomes function during reprogramming (Fig. 4A,B). Given the critical role of Eomes for PS formation in the early embryo, we reasoned that the reprogramming of cells lacking Eomes expression would be impaired if the transition of cells through the PS-like intermediate state would be a crucial step during the reprogramming process. Cells were transduced with the OKS construct29 and treated with tamoxifen at different time points to induce the genetic deletion of Eomes (orange letters; Fig. 4B,C). Efficient reprogramming was assessed by alkaline phosphatase staining (Fig. 4D), FACS staining for Ssea1-positive cells (Fig. 4E) and Oct3/4 expression (Fig. 4F). The loss of Eomes did not result in any significant change in number, morphology, or marker expression of arising iPSC colonies, irrespective of the time-point of induced deletion (Fig. 4D–F), albeit the slight Eomes expression peak (Supplemental Fig. 2) could be ablated upon tamoxifen treatment (Fig. 4G). This indicates that Eomes is functionally entirely dispensable for the reprogramming to pluripotency, despite its prominent role during PS formation and gastrulation initiation.

**Discussion**

Cellular events during reprogramming were extensively studied over the past years. However, the different stages of reprogramming need to be further defined and exact molecular mechanisms remain to be resolved. Several studies of changes in gene expression during reprogramming have suggested that cells undergo a reversal of embryonic development including MET-EMT events and transiently acquire a PS-like gene expression signature3,21. Gene expression patterns of PS formation, as well as EMT-MET events, occur independently of an epithelial or mesenchymal origin of the starting cell population during reprogramming21,23,14,15. Thus, it remains questionable whether reprogramming indeed follows stages of “reverse embryonic development”, or if observed gene signatures solely represent the spurious activation of developmental programs, or if genetic programs are indeed necessary to establish the pluripotency network. The latter view was recently supported by studies indicating that EMT-related transcription factors cooperate with core factors of the pluripotency circuitry to induce pluripotency22,23. Here, we apply different genetic tools including fate-analysis and reporter alleles at the Eomes gene locus, as one of the central transcription factors with important functions in the gastrulating mouse embryos for PS formation, EMT and specification of the mesendoderm lineages. None of the applied genetic tools and analyses indicated significant expression of Eomes on the route to iPSC reprogramming. Additionally, we tested if Eomes was functionally required during reprogramming by genetically deleting Eomes in starting cells. Indeed, the genetic deletion of Eomes had no effect on reprogramming efficiency, suggesting that the induction of a transient PS-like state is no crucial step during reprogramming.

We propose that observed mesendodermal/PS-like expression profiles in cells that are undergoing reprogramming reflect a non-physiological transcriptional response to the reprogramming factors. Thus, it is likely that high level expression of reprogramming factors by the lentiviral transduction and subsequent re-activation of the pluripotency network triggers the transcription of mesendodermal genes. In addition to maintaining the pluripotent state, transcription factor of the pluripotency network such as Nanog, Oct3/4, Klf4, and Tbx3 share functions
Figure 2. EOMES protein is not detectable during several stages of murine fibroblast reprogramming. (A) Schematic illustration of the Eomes alleles used in (B–H). MEFs carry a GFP knock-in at the Eomes\(^{GFP/+}\) locus (upper and middle panel) allowing for quantification of Eomes\(^{GFP/+}\) positive cells by putative GFP expression\(^9\). A lentiviral 3-factor (OKS) reprogramming construct includes a tdTomato reporter to track expression of pluripotency markers during reprogramming\(^2\). (B) FACS-based quantification of GFP- and tdTomato-positive cells during reprogramming at the indicated days. GFP-positive cells arise only late, after the reprogramming process indicating differentiation of formed iPSCs (day 21). (C) Corresponding phase contrast images (upper panel) and fluorescence images of the MEF cultures during reprogramming (red, middle panel) and the Eomes\(^{GFP/+}\) reporter signal (green, lower panel). (D) Scheme for spontaneous in vitro differentiation of WT-iPSCs towards embryoid bodies representing early germ layer formation mirrored in the three colors. (E) Comparison of marker gene expression for mesendoderm expression peaks during reprogramming of murine fibroblasts in comparison to iPSCs and cells differentiated in EBs. All mRNA levels are expressed relative to the housekeeping gene Hmbs and values have been normalized to day 10 reprogramming cultures, which have been set to 1 to illustrate fold induction. (F) Scheme for in vitro differentiation of Eomes\(^{GFP/+}\) reporter iPSCs isolated from (A) in embryoid bodies toward mesendoderm using high doses of Activin A. Germ layer formation is mirrored in the three colors, while high doses of Activin A favor endoderm formation (green). (G) Eomes\(^{GFP/+}\) reporter iPSCs are differentiated towards mesendoderm. Expression of GFP validates the functionality of the Eomes\(^{GFP/+}\) reporter. (H) FACS-based quantification of independent experiments from (G).
during the early phase of exit from pluripotency. Thus, they contribute to the initiation of transcriptional programs to guide mesendoderm cell fate determination, e.g. by regulating Eomes expression\textsuperscript{5,24–26}. In that way, core pluripotency factors govern the first steps of differentiation and cell fate determination\textsuperscript{5,26,27}. This hypothesis is underlined by the fact that mesendoderm transcriptional signatures can be likewise found in cells of ectodermal origin during reprogramming, although these cells never go through a mesendodermal/PS-like state during \textit{in vivo} embryogenesis. Finally, the extent of mesendodermal gene transcription levels did not reach the range of physiological lineage differentiation as shown for murine and human somatic cell reprogramming. In summary, our data confirm the previously described mesendodermal fingerprint arising during reprogramming in cells of mesoderm and ectoderm origin\textsuperscript{14}. While previous reports interpreted these findings as a reversed process of embryonic development\textsuperscript{13–15}, our data instead favors a non-physiological transcriptional response resulting from the forced induction of the pluripotency network, which does not reflect the magnitude of gene expression as seen during mesendodermal lineage commitment \textit{in vivo}\textsuperscript{5,24–26}.

**Material and Methods**

**Cell cultures.** Rat embryonic fibroblasts (REFs) from embryonic day 14 Sprague Dawley rats were generated according to the protocol previously described in\textsuperscript{28} and were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma Aldrich) containing 10% fetal bovine serum (FBS, Sigma Aldrich/Biochrom), 1% GlutaMAX, 1% nonessential amino acids (NEAA), and 1% antibiotic-antimycotic (all from Life Technologies). REFs were treated with 7.5μg/mL mitomycin C (Biomol) for 2.5 hours for mitotic inactivation. All animal experiments were performed in compliance with the guidelines for the welfare of experimental animals issued by the Federal Government of Germany, the National Institutes of Health and the Max Planck Society. The experiments in this...
Mouse embryonic fibroblasts (MEFs) were cultured according to standard methods at 5% CO₂ and 37 °C as described previously. Briefly, DMEM was supplemented with 15% FBS, 1% P/S, 1% GlutaMAX, 1% NEAA, 1 mM Sodium Pyruvate (Sigma Aldrich), 1% β-Mercaptoethanol (Merck Millipore) and 0.05 mg/ml Vitamin C. The cultivation of keratinocytes from plucked human hair was performed according to. In brief, keratinocytes were cultured on 20 μg/mL collagen IV (Sigma Aldrich) coated dishes in EpiLife medium with HKGS supplement (Gibco® Life Technologies) until they reached about 70% confluency. Human foreskin fibroblasts (HFFs) (System Biosciences) were cultivated in DMEM supplemented with 10% FBS and 1% GlutaMAX, 1% NEAA, and 1% antibiotic-antimycotic.

Cultivation of induced pluripotent stem cells (iPSCs). Human iPSCs were cultured on Matrigel-coated (Corning) 6-well plates in FTDA culture medium at 5% CO₂, 5% O₂ as described in. Mouse iPSCs were cultured either in feeder-dependent conditions or in feeder-free conditions (2i) . For feeder-dependent conditions (ES feeder medium) Knockout™ DMEM (KO-DMEM; Life-Technologies) was supplemented with 15% FBS, 1% P/S, 1% GlutaMAX, 1% NEAA, 1% Sodium Pyruvate, 1% β-Mercaptoethanol and 240 U/ml leukaemia inhibitory factor (LIF, Cell guidance systems). In case of feeder-free (2i) culture mouse KO-DMEM with 15% Knockout Serum Replacement (KOSR, Life Technologies), 1% P/S, 1% GlutaMAX, 1% NEAA, 1% Sodium Pyruvate, 1% β-Mercaptoethanol, 240 U/ml LIF and 1 μM PD0325901 (Calbiochem) and 3 μM GSK3β-inhibitor CHIR99021 (Axon Medchem) was used.

Lentivirus production. Lentivirus production and vector systems encoding for human and mouse variants were described previously.

Figure 4. Eomes is dispensable for reprogramming of murine fibroblasts. (A) Schematic illustration of the Eomes alleles used in (D–G). MEFs carry one functional null allele with a GFP knock-in at the Eomes locus and a second conditional allele, where exons 2–5 are flanked by loxP sites. The tamoxifen (4-OHT)-inducible CreER-recombinase is expressed from the Rosa26 locus and used to induce the complete genetic deletion of Eomes by 4-OHT administration. (B, C) 4-OHT treatment regimen used for timed Eomes ablation during reprogramming. Orange lines indicate tamoxifen treatment intervals: A: d-3 to d-1 (48 h), B: d5–9 (96 h), C: d10–d14 (96 h). (D) Representative images of Alkaline phosphatase staining of iPSC colonies at different timepoints of 4-OHT treatment as indicated. (E, F) FACS-based quantification of (E) Ssea1 and (F) Oct3/4 positive cells at day 20 of reprogramming following with and without 4-OHT administration. Scale bars in all images: 50 μm. Representative experiments from n = 3 in triplicates are shown.
Generation of induced pluripotent stem cells (iPSCs). Human. Human iPSCs were generated from plucked human hair keratinocytes and from human foreskin fibroblasts (System Biosciences). Keratinocytes were cultured and infected as described in [2]. Fibroblasts were cultured in DMEM, 10% FBS, 1% antibiotic-antimycotic, 1% NEAA, and 1% GlutaMAX. For reprogramming 1×10^6 fibroblasts were plated on coated 6-well plates and were infected with 5×10^8 viral copies of STEM.CCA[29] OKSM lentivirus on two subsequent days in culture medium supplemented with 10μM Rock inhibitor/Y-27632 (Selleckchem), 8μg/ml polybrene (Sigma Aldrich). On the third day infected keratinocytes and fibroblasts were distributed equally into 6-well plates on mitomycin-inactivated rat embryonic fibroblast (REF) feeder cells. 1.5×10^6 REFs were mitotically inactivated with 7.5μg/ml mitomycin C for 2.5h. During reprogramming cells were cultured in KO-DMEM, 20% KOSR, 1% antibiotic-antimycotic, 100μM NEAA, 1% GlutaMAX, 50 mM β-mercaptoethanol, 50μg/ml L-Ascorbic acid (Carl Roth), 10ng/ml FGF2 (Cell Guidance Systems), 10μM Rock inhibitor/Y-27632 (Selleckchem) at 5% CO2, 5% O2, and 37 °C, and medium was changed every second day. iPSC colonies were mechanically transferred onto Matrigel coated (Corning) 6-well plates after three weeks.

Mouse. MEFs were seeded on a gelatine coated plate (4 × 104 cells/12-well) one day prior to infection. Next day, 5 μl concentrated polycistronic OKS (OCT3/4, KLF4, SOX2) lentivirus harboring a Td-tomato[22] together with 8 μg/ml polybrene (Sigma Aldrich) in 1 ml ES Feeder Medium was added to each 12-well. After 8h of incubation at 37 °C, medium was removed, cells were washed with PBS and ES-Feeder medium was added and refreshed daily. At day 6, medium was changed to ES Feeder KOSR, where FCS was exchanged by KOSR. On day 20, cells were either stained for alkaline phosphatase (AP) expression according to standard protocols or cells were analyzed by flow cytometry.

Differentiation of human iPSCs. For mesendodermal differentiation, iPSCs (70% confluency) were incubated with basal medium RPMI Media 1640, 1% antibiotic-antimycotic, 1% GlutaMAX, 2% B-27 Supplement (all Thermo Fisher) supplemented for the first day with 500 nM IDE1 (StemCell Technologies), 50ng/ml BMP4 (PeproTech), 3μM CHIR-99021 (Selleckchem), 5μM LY294002 (Selleckchem) at 5% CO2, 5% O2 and 37 °C. For the next three 500 nM IDE1, 50ng/ml BMP4, 5μM LY294002, 20ng/ml FGF2 (Cell Guidance Systems) was added to the basal medium.

Differentiation of mouse iPSCs. Cells were seeded in hanging drops (400cells/20μl) in N2B27 medium. N2B27 medium was produced by adding 37.5 ml IMDM, 12.5 ml Ham’s F12 medium, 0.5x B27, 0.5x N2 (all Gibco® Life Technologies), 1% P/S, 0.05% BSA, 1% GlutMAX, 2 mM Ascorbic acid, and 450μM 1-thioglycerol (Sigma). After two days, drops were washed off with 5 ml N2B27 Medium containing 50ng/ml Activin A (PreproTech) and transferred to non-adherent plates. Medium was changed every 48h by carefully centrifuging the cells at 800 rpm for 2 min, discarding the supernatant up to about 500 μl and carefully resuspending the embryoid bodies in fresh medium (N2B27 + Activin A) on a new plate.

Tamoxifen treatment. 4-Hydroxytamoxifen (Sigma Aldrich) was added to the cell culture medium to a final concentration of 1 μg/ml for the respective time frames. After tamoxifen treatment cells were washed with PBS once and received fresh medium.

Immunocytochemistry. Cells were fixed using 4% paraformaldehyde, 10% sucrose for 15 min on ice. All subsequent steps were performed at room temperature. After washing twice 5 min with PBS (Thermo Fisher) cells were permeabilized with 0.2% TritonX (Carl Roth) for 5 min. Following blocking for 1.5 h with 5% normal donkey serum (NDS, Sigma Aldrich), samples were incubated with primary antibodies for 2h. The primary antibody Sheep α-Eomes (R&D Systems AF6166) was diluted 1:28, Rabbit α-Nanog (Cell Signaling 9656) 1:200. After washing with PBS, samples were incubated with 1:1000 diluted Alexa Fluor® labeled secondary antibodies α-Sheep 488 nm (Abcam ab15077) and α-Rabbit 568 nm (Abcam ab175470) for 1 h. Following a final washing step with PBS, samples were mounted using ProLong® Gold Antifade with DAPI (Thermo Fisher). All images were captured using Axio Imager M2 microscope and analyzed using AxioVision software (Zeiss).

Western Blot. Western Blotting was performed according to standard protocols. In brief, cell pellets were lysed in cold RIPA-Puffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 1% IGEPAL® (Sigma Aldrich), 0.5% Na-Deoxycholat, 0.1% SDS, 1X Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher)). The samples were added to a 12% SDS Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad) and subsequently blotted on a nitrocellulose membrane (Protran). Before adding the primary antibodies (Sheep α-Eomes (1:28) (R&D Systems AF6166), Rabbit α-Nanog (1:200) (Cell Signaling 9656), Chicken αnti β-Actin (1:1000) (Abcam ab13822), samples were blocked using 6% porcine serum in 1x TBS buffer for 2h. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C. For subsequent washing steps 0.05% Tween20 in 1x TBS was used. Secondary antibodies (α-Sheep, α-Rabbit, α-Chicken 800 CW or 680RD (LI-COR)) were diluted as recommended from the manufacturer and incubated for 1.5 h in the dark. Membrane was developed using a near-infrared fluorescence system, Odyssey® FC and analyzed using Image Studio Lite software (LI-COR).

Gene expression Analysis. Total RNA was isolated from cell lysates using RNaseasy Mini Kit according to the manufacturer’s instructions (Qiagen). First, cDNA synthesis was performed using 80 ng RNA with RT Buffer (Promega), dNTPs (GE Healthcare), Hexanucleotide Mix (Roche) and MMLV RT (Promega). For the preamplification step PreAmp Master Mix, SuperScript III First-Strand Synthesis SuperMix (both Thermo Fisher), TE buffer (Ambion) was used according to the manual. To quantify the amount of the genes of interest QuantiTect
Primer Assays (Qiagen) were used on the BioMark HD System with 96.96 Dynamic Arrays (both Fluidigm). Relative gene expression was calculated as a ratio of target gene concentration to the housekeeping gene concentration. Details have been described in 24-26.

**FACS analysis.** While Ssea1 surface staining and Tomato/GFP auto-fluorescence staining was performed on living cells, cells were fixed in 4% paraformaldehyde, 10% sucrose for 20 minutes on ice for intranuclear Oct3/4 staining. Stainings were performed according to standard methods. Briefly, adherent cells were washed with PBS and dissociated into single cell suspension by incubation with 0.25% trypsin/EDTA (Millipore). For staining of cells were blocked in PBS supplemented with 10% FBS, incubated with primary antibody α-Ssea1 (1:1600) (Cell Signaling MC480) for 1.5h on ice in the dark, and incubated with secondary antibody α-mouse AlexaFluor 647 nm (1:600) (Invitrogen A21238) for 30 min on ice in the dark. Washing steps were performed with PBS with 2% FBS, and 1% P/S (FACS Buffer). For staining of Oct3/4 paraformaldehyde-fixation was followed by permeabilization of cells for 30 min in 0.5% Saponin (Sigma Aldrich) in FACS Buffer, blocking in 5% normal goat serum (Sigma Aldrich), and 0.5% Saponin in PBS for 20 min on ice. Antibodies were diluted in the blocking dilution, the primary antibody α-Oct3/4 (Santa Cruz sc-5279) 1:100, and the secondary antibody α-mouse AlexaFluor 488 nm (Invitrogen A11029) 1:200. Washing steps were performed with FACS Buffer supplemented with 0.5% Saponin. Cells were analyzed with a FACSARia II or III flow cytometer (BD). All events were gated with forward scatter and side scatter profiles.

**Statistical analysis.** All experiments were independently repeated at least 3 times and Error bars in the graphs show calculated Standard Error if not otherwise stated. Statistical significance was calculated using Students t-test. p-values have been calculated where appropriate and now illustrated by asterisks according to the following definitions: *p < 0.05; **p < 0.01; ***p < 0.001. GraphPad Prism 5 was used for statistical and graphical data evaluations.

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