Loss of *Oct4* expression during the development of murine embryoid bodies

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Abdulrahim Abdulrahman M Sajini

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTERS OF SCIENCE

Adviser: James Dutton

May 2012

Acknowledgements

I would like to thank Dr. Jonathan Slack for his guidance and support throughout this work.
I would also like to thank James Dutton for his support and suggestions that made this work possible.

My thanks also goes out to all the members in the Slack's lab particularly, Randy Daughters and Ersin Akinci for technical support, and Anannya banga for antibodies.

My thanks also goes out to Dr. Nobuaki J Kikyo for academic and scientific discussions, Susan Keirstead for academic and imaging advice, and the university imaging center at the university of Minnesota for confocal imaging.

This work was part funded by grants U01HL100407 and R01DK080747 from the National Institutes of Health. A.S. was funded by a scholarship from the University of Tabuk, Saudi Arabia.

Dedication

This dissertation is dedicated to AL-Rahman whom without this work would not be possible. Next this dissertation is dedicated to my family especially, my mom (Susan sweet), my dad (Abdulrahman Sajini), my wife (Afnan Abdultawab), my siblings (Hend, Hanan, Taha, Azahar, and Tasneem), and my children (Rawaa, and Ziyad). Each one of you has a part in this work.

Abstract

We describe the internal organization of murine embryoid bodies (EBs) in terms of the structures and cell types formed as *Oct4* expression becomes progressively lost. This is done by making the EBs from iPS cells carrying an inducible and permanent *Oct4* reporter (*Oct4-MerCreMer;mTmG)*. When these EBs are treated with tamoxifen, the *Oct4* expressing cells switch from a red to a green fluorescence color, and this is maintained thereafter by their progeny. We show that there is no specific pattern in which *Oct4* is downregulated, rather it appears to be spatially random. The earliest cells to lose *Oct4* expression are internal and stain positive for α-fetoprotein (AFP) indicating that they are visceral endoderm. However, GATA4, characteristic of primitive endoderm, was found in *Oct4*-expressing cells at this stage. This indicates that the first formed visceral endoderm does not arise from primitive endoderm, a difference from normal embryonic development. Contrary to previous reports, our EBs did not form a layer of primitive endoderm, or visceral endoderm, around the outside. Markers of the early body axis, BRACHYURY (T) and FOXA2, behaved somewhat differently from each other. BRA, which marks the early mesoderm, node and notochord, arises in *Oct4* expressing cells on days 3-4. FOXA2, which marks the floor plate of the neural tube and definitive endoderm, as well as the node and notochord, arises at the same time but mostly in cells that have already lost *Oct4* expression. Although there is usually a concentration of T or FOXA2 cells in one region of the EB, the morphology is not predictable and there are also scattered cells expressing these markers. Several clumps of cardiomyocytes are visible by day 7 of EB development, and we show that the cells forming these clumps lose *Oct4* expression between days 3 and 5. Overall, our results indicate that EBs recapitulate normal development quite well in terms of the tempo of events and the appearance of specific markers, but they do not resemble embryos in terms of their morphology and structure, which is contrary to the previous reports.

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Introduction

Even though research on embryoid bodies dates back to 1980s, there appears to be a gap in terms of continuous research until date. In other words, there was little if any work done after the 1980s describing the biology of embryoid body development. In fact, the majority of work done after the 80's has been on deriving specific cell types from embryoid bodies. Therefore, to understand the need for further research, it is necessary to comprehensively examine the initial work done on embryoid body development in a chronological discussion.

Embryonal stem (ES) cells and embryonal carcinoma (EC) cells

Early work on embryonal carcinoma (EC) cells sequestered from murine teratrocarcinoma laid the framework for the isolation of both human and mouse embryonal stem (ES) cells (Chambers and Smith, 2004; Damjanov, 2005). Embryonal carcinoma cells are pluripotent stem cells that are found in malignant germ cell tumors known as teratocarcinomas (Andrews et al., 2005). These cells can self-renew and differentiate into all three embryonic germ layers, ectoderm, mesoderm, and endoderm, upon transplantation into immunosuppressed mice (Andrews et al., 2005; Damjanov, 2005). In addition, EC cells have been shown to contribute to different parts of a mouse embryo when they are injected into mouse blastocysts (Chambers and Smith, 2004). In 1960, Stevens found that upon flotation, EC cells aggregate to form structures that resemble mouse embryos in potency and morphology. He then investigated their differentiation abilities by transplanting single aggregates into the anterior chamber of the

eyes of mature 129 strain mice and concluded that the EC cells were pluripotent. In 1975, Martin and Evans were successful in growing EC cells in culture (Damjanov, 2005). Consequently, studying pluripotency in EC cells and knowing how to maintain them in vitro served as the backbone for the isolation of the first mouse embryonic stem cells in 1981 by Martin, and Evans, and Kaufman (Martin, 1981; Evans and Kaufman, 1981). Since then, ES cells have played a crucial role in advancing the field of pluripotency and stem cells as a whole.

Embryonic stem cells maintain full differentiative and lineage specification abilities of mouse inner cell mass cells and they can be cultured indefinitely in the presence of serum and a feeder layer of fibroblasts (Evans, 2005). However, in contrast to their counterparts, EC cells, ES cells are not malignant (Andrews et al., 2005; Damjanov, 2005). As a consequence of this factor and because ES cells can pass to the germ-line when reintroduced into mouse blastocysts, they have revolutionized the field of transgenic mice (Andrews et al., 2005; Bradley et al., 1984; Chambers and Smith, 2004).

Culturing conditions learned from maintaining EC cells ultimately led to the discovery of leukemia inhibitory factor (LIF). In the early years, when ES cells were isolated, they were cultured in conditions similar to EC cells, serum and irradiated fibroblast (Evans and Kaufman, 1981; Martin, 1981). Subsequently, it was found that medium conditioned with buffalo rat liver cells could substitute for fibroblast in maintaining ES and some EC cells in their undifferentiated state (Smith and Hooper, 1987). A year later, the same group discovered that the key ingredient maintaining ES cells in their undifferentiated states was LIF, which is a member of the interleukin-6

cytokine family (Chambers and Smith, 2004; Smith et al., 1988).

Following the discovery of LIF, many key signaling factors regulating ES cells pluripotency have been unraveled (Chambers and Smith, 2004). It was also discovered that LIF acted by activating certain genes, which formed a network of activating and repressing transciption factors that caused ES cells to maintain pluripotency and to self-renew (Chambers and Smith, 2004). In particular, three genes, *Pou5f1* also known as *Oct4* (Niwa et al., 2000), *Nanog* (Chambers et al., 2003; Mitsui et al., 2003), and *Sox2* (Masui et al., 2007) were found to be central genes for the state of pluripotency in inner cell mass (ICM) cells, epiblast cells, and ES cells (Chambers and Smith, 2004). Overall, the discovery of LIF mediated the understaing of many key regulators of pluripotency in ES cells.

The study of pluripotency in ES cells inspired Yamanaka in 2006 to generate ES-like cells that he called induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). The group was able to successfully reprogram fibroblasts back to a more primitive embroinc-like state by transducing genes that were abundantly expressed in ES cells, namely *Oct4*, *Sox2*, *Klf4*, and *C-myc* (Takahashi and Yamanaka, 2006). These iPS cells were very similar to ES cell in terms of gene expression and potency as they expressed high levels of *Oct4*, *Sox2*, and *Nanog* (Takahashi and Yamanaka, 2006). A recent report by Hochedlinger's group (Ohi et al., 2011) found that early passages of iPS cells generated from hepatocytes, skin fibroblasts, and melanocytes, retained some epigenetic memories from their parent cells. However, the epigentic signature was found to fade away completely after additional passages (Ohi et al., 2011). Nevertheless, the

similarities of iPS cells with ES cells are profound and their pluripotency meets the gold standard in the field, which is the generation of chimera mice and a contribution to the germline (Takahashi and Yamanaka, 2006; Wernig et al., 2007). The importance of pluripotent cells lies in their ability to differenitiate into any type of cell in the body. The most common method of differenitating pluripotent cells in vitro is through the formation of aggregates called embryoid bodies (EBs). Therefore, articulating how EBs develop in vitro is a key step in deriving desired cells in culture.

Embryoid bodies (EBs)

Embryoid bodies are three-dimensional structures generated by an aggregate of pluripotent stem cells when they are cultured in the absence of feeder cells and plated onto low attachment surfaces. Embryoid bodies were first reported in 1959 by Stevens when he was working on the embryonic nature of teratocarcinomas as ascitic tumors and found floating structures with cavities surrounded by a layer of endoderm that resembled day 5-6 mouse embryos (Stevens, 1959). Later that year, another group also reported EBs forming from testicular tumor cells when transplanted into mice (Pierce and Dixon, 1959). The differentiation capabilities of these EBs were investigated upon transplantation into the anterior chamber of mouse eyes (Stevens, 1960). These EBs form tissue from all three germ layers of an embryo and retain a subset of pluripotent cells that gave rise to new tumors when serially transplanted (Stevens, 1960). Thus, EBs formed from testicular tumors were said to be similar to normal mouse embryos in venues of embryonic morphology as well as potency (Stevens, 1960).

It was later shown that EBs formed in ascitic conditions in vivo could efficiently develop in vitro into all three germ layers (Pierce and Verney, 1961). Their study further indicated that the mode of EB development in vitro was indistinguishable to EB development in vivo, thus concluding that EB development in vitro followed similar patterns to normal mouse embryos (Pierce and Verney, 1961). However, it was unclear how EBs were formed and what cells were forming them since the means of obtaining EBs was through explants of testicular tumors transplanted into the peritoneal cavity of mice (Pierce and Dixon, 1959; Pierce and Verney, 1961; Stevens, 1959, 1960).

In 1974, pluripotent stem cells from mouse teratocarcinomas were isolated, propagated, and differentiated in vitro as a homogenous population and were called EC cells (Martin and Evans, 1974). Subsequently, these EC cells were found to form cystic EBs in vitro that differentiated in a specific tempo of events, as whole cells participated in forming the EBs (Martin and Evans, 1975). Consequently, morphological studies in cystic EBs, formed in vitro by EC cells, indicated that developmentally, cystic EBs and isolated ICM cells grown in vitro are more a like, because the initial differentiation event in both cases, is the formation of an outer endoderm layer (Martin et al., 1977). However, the type of endoderm cells formed in cystic EBs was different from those formed in isolated ICM cells grown in vitro, as cystic EBs form visceral endoderm whereas ICM cells form parietal endoderm (Martin et al., 1977). Furthermore, the location of cells at the outer surface of EBs was speculated to act as the main determinant for endoderm fate, which is contrary to normal development (Martin et al., 1977). Conversely, cystic EBs were observed to form mesoderm differently than normal mouse embryos, which is by a

third layer between the endoderm and ectoderm (Martin et al., 1977). Overall, results by Martin illustrated that cystic EBs formed in vitro resemble ICM cells rather than whole mouse embryos (Martin et al., 1977). Their finding also indicated that EBs could mimic normal mouse development in endoderm, and ectoderm, but not in mesoderm. In addition, it showed that the first cells to differentiate in EBs were destined to form the outer endoderm layer, which in the case of cystic EBs was parietal endoderm and in isolated ICM cells was the visceral endoderm (Martin et al., 1977).

It was later shown that mouse ES cells can also form EBs in vitro (Doetschman et al., 1985; Keller, 1995; Leahy et al., 1999) as can mouse iPS cells (Takahashi and Yamanaka, 2006; Wernig et al., 2007), and human ES and iPS cells were also found to form EBs when cultured in suspension (Rust et al., 2006a; Thomson et al., 1998; Yu et al., 2007). However, little is known about human EB morphological changes and how much they recapitulate normal embroyonic development, as human development itself is poorly charachterized (Rust et al., 2006b). Early studies done on mES cells confirmed the previous interpretation that EBs resemble day 6-8 egg cylinder stage mouse embryos (Doetschman et al., 1985). However, EBs made from mES cells were found to differ from EBs made from EC in that they develop mesoderm as a third layer between endoderm and ectoderm and thereby resembling normal development (Doetschman et al., 1985). Furthermore, EBs made from mES cells were reported to form an outer layer of enderm as soon as they begin to differentiate, thus behaving like ICM cells (Doetschman et al., 1985). Therefore, EBs formed from mES cells seemed to demonstrate a higher level of development organization compared to EBs formed from EC cells

(Doetschman et al., 1985). Thus, mES cell EBs are considered to good models for in vivo development in terms of cell differentaition. In conclusion, early studies in EBs formed from mES cells suporrted the convention that EBs resemble mouse embryos in their morphology and differenaiton and hence can be used as models to study in vivo development.

In a recent study, EBs generated from mES cells were reported to form Wnt signalling centers early during their development (ten Berge et al., 2008). The study was conducted using a reporter that expresses EGFP when 7xTCF, a Wnt targeted gene, was activated (ten Berge et al., 2008). Thus, by using this system the group claimed that globally, EBs almost always formed Wnt centers that posteriorize them (ten Berge et al., 2008). The study, therefore, suggests that EBs downregulate *Oct4* expression through a gastrulation-like process during their development, thus displaying a pattern of pluripotency suppression (ten Berge et al., 2008). However, the study utilized insensitive methods to image the reporter during EB development, therefore making their results questionable (ten Berge et al., 2008). Clearly showing whether EBs do form Wnt centers early in their development will greatly enhance our understanding of EB development. Thus, revisiting this area with our system would decisively show whether there is a certain pattern of *Oct4* repression during EB development as our system provides a more sensitive measure for *Oct4* expression at the cellular level.

The early proposal, that EBs differentiate in parallel to mouse embryos, caused scientists to focus their efforts on differentiating EBs towards cell types rather than stuyding how EBs developed. Therefore, by direct or indirect methods, many terminally

differentiated cell types have been derived from EBs, such as pancreatic beta cells, cardiomyocytes, dopaminergic neurons, and germ cells (Geijsen et al., 2004; Murry and Keller, 2008). However, studies describing the biology in which EBs develop are quite limited. Perhaps the only descriptive characterization of EB development currently available stems from the study conducted in 1985 (Doetschman et al., 1985). Therefore, with the constant pressure of translating stem cell research, more efforts are moving towards EB differentiation, and as a result leaving research on EB development an overlooked area. Therefore, despite the enormous practical use of EBs in todays directed differentiation protocols, relatively little is known about their internal signaling, structure, and development. In addition, the current perception of EB development dates way back to work done on tumor EC cells and/or poorly maintained ES cells (Doetschman et al., 1985). Therefore, the current belief that EBs initially differentiate to an outer endoderm layer could be biased by weakly maintained ES cells or by alterations in potency of EC cells due to genomic alteration. Furthermore, the idea that EBs form Wnt signaling centers and thereafter suppress *Oct4* in a gastrulation-like pattern is also ambiguous, due to the use of insensitive imaging methods. Hence, studying how EBs develop would answer the questions of whether or not the first cells to differentiated in EBs form an outer layer of endoderm, and whether EBs truly adopt a certain pattern of *Oct4* suppression that is dependent on Wnt signaling. Illustrating such findings would enhance our basic knowledge of EB development, which would increase our ability to derive desired cells from differentiating EBs. Therefore, inspired by the lack of basic knowledge in EB development, we employed a novel cell line that enabled us to exogenously label,

track, and distinguish *Oct4* expressing cells from non-*Oct4* expressing cells during the development of EBs.

In this study we describe the internal organization of mouse EBs in terms of the structures and cell types formed as *Oct4* expression becomes progressively lost. To do this, we have employed a line of iPS cells made from a mouse containing a knocked-in *Oct4-CreER* gene together with the *mTmG* reporter, which is a cassette in which expression of the red fluorescent protein td-Tomato is replaced by enhanced green fluorescent protein (EGFP) following Cre mediated DNA excision (Muzumdar et al., 2007). Treatment of these cells with tamoxifen activates the CreER and thereby label all *Oct4*-expressing cells with a permanent, membrane bound, EGFP label. If the specimens are fixed at a later time point, then depending on whether the structures of interest are green or red, it is possible to determine whether their precursor cells were or were not expressing *Oct4* at the time of tamoxifen administration.

OCT4 is well known as the core member of a group of pluripotency-conferring transcription factors which also include SOX2 and NANOG (Niwa, 2007). In the mouse embryo, it is expressed at a high level in the entire early preimplantation embryo. During expansion of the blastocyst, expression is enhanced in the inner cell mass and declines in the trophectoderm. It continues to be expressed in the primitive endoderm but is downregulated as this differentiates into the visceral and parietal endoderm. During the postimplantation stages, *Oct4* is highly expressed in the epiblast of the egg cylinder stage and becomes downregulated from the anterior end during gastrulation. By the somite formation stage, it is lost from all parts of the embryo except the primordial germ cells

(Kehler et al., 2004; Scholer et al., 1990; Yeom et al., 1996). OCT4 both upregulates expression of the other transcription factors needed for pluripotency, and represses expression of the transcription factors involved in regional specification during embryonic development (Bernstein et al., 2006; Ying et al., 2008). The knockout of *Oct4* has shown that it is essential for preimplantation development and for the establishment of embryonic stem cell lines (Nichols et al., 1998). Conditional knockout has shown that it is essential for normal germ cell development (Kehler et al., 2004). However, despite many claims to the contrary, careful examination of the expression and requirement for Oct4 in postnatal mouse development indicates that it has no role in the function of the various types of tissue-specific stem cell other than spermatogonia (Lengner et al., 2007).

In this study we have investigated EB development from the aspect of identifying which cell populations lose *Oct4* expression at which times. We have used a standard size of EBs and standard culture conditions to maximize reproducibility. Our study shows several findings. The first cells to lose *Oct4* express AFP and thus are said to be visceral endoderm. However, we find that the outer endoderm layer does not arise from the first cells that lose *Oct4,* as formerly believed. We find that the main axial structures arise between days 3-4 of EB differentiation and although there is often a single major concentration of cells, there are also many scattered cells expressing the relevant marker genes. The heart arises from days 4-5 and usually appears as multiple blocks of cardiac muscle. In general, the time course of development of many cell types in EBs is similar to what is seen in embryos, while the structural organization is very different. The EBs do not show large-scale structures similar to embryos, but are like micro-mosaic regions.

Materials and Methods

Retroviral production, fibroblast infection and iPS cell reprogramming

Retrovirus production and infection was performed according to lab protocols. One day before infection, Plat-E cells were seeded at 2.5 x 10^6 cells per 10 cm dish. The cells were then infected with 9 μg of pMX-based retroviral vectors expressing *Oct4*, *Sox2*, *Klf4*, and *c-MycT58*. Each vector was individually transfected into Plat-E cells using 27 μl of Fugene 6 transfection reagent (Roche). 24h after transfection, the medium was replaced with growth medium containing 10% FBS (HyClone). The virus supernatant was collected at 48h and 72h post infection and passed through a 0.45 μm PVDF filter. 24h after transfection, the target cells were plated at 1.25 x 10^5 cells per well *(do it per area)* of a gelatin-coated tissue culture plate. The next day, the target cells were infected with a cocktail of equal volumes of the viral supernatants and supplemented with 8μg/ml hexadimethrine bromide (Sigma). The target cells were incubated with the viral supernatants for 24h. Subsequently, the virus containing media was replaced with growth media. The cells were either left on the tissue culture plate for the duration of the experiment or alternatively after 5 days were trypsinized and divided onto irradiated embryonic fibroblast cells in ES media containing mouse LIF (Greder et al., 2012).

Working Media

Mouse embryonic fibroblast (MEF) medium was made by supplementing knockout DEMEM, high glucose (Gibco) with 10% fetal bovine serum (FBS) (HyClone), 1% L-

glutamine 200mM (Gibco), 1% penicillin/streptomycin (Gibco) and 1% MEM Non-Essential amino acids (Gibco). Prepared MEF medium was stored at 4°C and discarded 3 weeks after preparation date.

Embryonic stem cell (ES) medium was made by supplementing knockout DMEM (Gibco) with 10% (FBS) (HyClone), 10% knockout serum replacer (Gibco), 1% L-glutamine 200mM (Gibco), 1% penicillin/streptomycin (Gibco), 1% MEM Non-Essential amino acids (Gibco), 0.1 mM 2-Mercaptoethanol (Sigma) and 1000 Units/ ml ESGRO-mLIF (Millipore). Prepared ES medium was stored at 4°C and discarded 10 days after mLIF addition.

Embryoid Body Differentiation (EBD) medium was made by supplementing knockout DMEM (Gibco) with 15% (FBS) (HyClone), 1% L-glutamine 200mM (Gibco), 1% penicillin/streptomycin (Gibco), 1% MEM Non-Essential amino acids (Gibco), 0.1 mM 2-Mercaptoethanol (Sigma) and 1000 Units/ ml ESGRO-mLIF (Millipore). Prepared EBD medium was stored at 4C and discarded 3 weeks after preparation date.

Cells Freezing (CF) medium was composed of 90% FBS (HyClone) and 10% DMSO (Sigma). Prepared CF medium was stored at -20°C and discarded 10 days after preparation date.

All above described media were filtered using 0.22 µl Stericup and steritop membrane filters (Millipore) before utilizing them.

IPS cell culture

Liquid nitrogen frozen *Oct4-CreER*, *mTmG* iPS cells (line 3F10, passage 13-26) were

thawed in 37C pre-warmed waterbath then resuspended with MEF medium and centrifuged at 1000 rpm for 5 minutes. This step is crucial to remove the DMSO in CF medium, which can reduce cell viability when remained with cultured cells. The cell pellet was then suspended with ES medium and the cells were plated on a single layer of irradiated embryonic mouse fibroblast (feeder) plated on 6-well plates. Each well contains approximately 210,000 plated feeder cells, whose main purpose is to condition the environment to help maintain iPS cells in their undifferentiated state. Plated iPS cells were culture at 37°C in 5% CO2. Medium was changed the next day and iPS cells were always passaged at day 2 to avoid cell differentiation. At day 2 of culture iPS cells are washed once with PBS (Cellgro) and incubated with 0.25% Trypsin- EDTA (Gibco) at 37°C for 2-3 minutes. The cells were then washed with MEF medium and the single cell suspension was either further cultured or frozen by re-suspending the cells in CF medium and stored at -80°C for a day then at liquid nitrogen.

Embryoid body generation and culture

3F10 iPS cells were cultured for 48h in ES media. And were then dissociated to single cells by using 0.25% Trypsin- EDTA (Gibco). Cells were re-suspended with 10% MEF medium and centrifuged at 1000 RPM for 5 minutes. A feeder depletion step was then carried-out by culturing the cells on tissue culture plates for 45 minutes in ES medium at 37°C and 5% CO2. Cells were then centrifuged down and re-suspended in EBD medium and counted with a hemocytometer (Bright-line). 500 cells were dispensed into 96 well Round Bottom, Ultra-Low Attachment plates (Costar) then centrifuged at 3000 RPM for

4 minutes. It is important to note that the same batch of FBS was used for all EB differentiation experiments to avoid factor variability.

Tamoxifen treatment

Stock Tamoxifen 10mM (Sigma) was diluted using DMSO to 0.1mM, which was further diluted in ES or EBD medium to100nM. Treatment with Tamoxifen was carried out either at the time of cell aggregation (day 0) or on the indicated days of EB differentiation in the text. Embryoid bodies were fixed for analysis either 12h following tamoxifen addition, or at later time points as indicated in the text.

Immunohistochemistry

Embryoid bodies were collected from low attachment 96 well plates and washed once with PBS (Cellgro) before being fixed with 4% PFA for 5 minutes at room temperature. Embryoid bodies were then permabilized for 30 minutes 3 times with PBS/ 0.5% Triton (Sigma) (PBS-T), and blocked for 2h at RT with 5% normal donkey serum (NDS) in PBS-T. Blocked EBs were incubated overnight at 4°C with primary antibodies diluted in blocking buffer. Oct-4 (SC-9081 1:200; Santa Cruz), Brachyury (SC-17743 1:200 Santa Cruz), Foxa2 (SC-9187 1:200; Santa Cruz), Gata-4 (61170 1:500; Abcam), AFP (3H8 1:100; Cell Signaling) Cardiac Troponin (CT-3C 1:500; DSHB). Embryoid bodies were then washed 3 times with PBS-T for 30 minutes and re-blocked with the blocking buffer for 2h at RT. Subsequently, EBs were incubated overnight at 4°C with secondary

antibodies. Alex Fluor 647 donkey anti-mouse IgG (A31571 1:1000; Invitrogen), goat IgG (A21447 1:1000; Invitrogen) and rabbit IgG (A31573 1:1000 Invitrogen), then washed 3 times with PBS-T for 30 minutes before imaging. Processed embryoid bodies where seeded onto depression slides and mounted with Vectashield and analyzed using the Olympus FluoView FV1000 IX2 Inverted Confocal with FLIM detector or Leica DMRB6000 equipped with a Retiga 2000 camera using IP Lab software.

Quantitative Real-Time PCR

Total RNA was prepared from 3F10 cells and EBs via RNeasy Mini Kit (Qiagen). Genomic DNA was removed from RNA by DNase (Promega) treatment for 1h at 37°C. After DNase treatment a 35-cycle PCR amplifying beta- actin, a house keeping gene, was done on the samples to ensure no remnants of genomic DNA was present. cDNA was then generated by reverse transcription from 2 μg total RNA using SuperScript III Reverse Transcriptase, oligo(dT)20 and dNTP (10 mM) (Invitrogen). Generated cDNA was validated by a 30-cycle PCR beta-actin reaction. Gene expression profiles of developing EBs included the following genes: *Oct4*, *Brachyury*, *FoxA2*, *Sox1*, *Sox17*, *Flk1*, *Gata4*, *Gata6*, *Ctnt*, *AFP* and the housekeeping gene *GAPDH*. Gene expression for sorted cells included *Oct4*, *Gata4*, *Gata6*, *AFP*, *Sox17*, Fgf5, and *Cdx2*. All qRT-PCR experiments were carried out on three biological replicates and results were scored as means with standard error bars on all results. Quantitative RT-PCR was preformed on an the Eppendorf lightcycler using IDT predesigned primers see Table.1

Fluorescent activated cell sort (FACS)

Embryoid bodies were dissociated using 0.25 Trypsin-EDTA (Gibco) to single cells and centrifuged at 1000 RPM for 5 minutes. Dissociated cells were then re-suspended with 700 µl of PBS (Cellgro) and filtered using 5ml polystyrene Round-Bottom Tubes with Cell-strainer Caps (BD Falcon). Filtered cells were then counted using FACS Calibur. For sorting, tamoxifen treated EBs were processed as described above and dissociated cells were separated using FACS Aria. For photochrome controls, uncolored iPS cells were used to gate uncolored cells. No tamoxifen treated day 3 EBs were used to gate Td-Tomato (red). Day 3 EBs made from tamoxifen treated iPS cells were used to gate EGFP (Green). RNA from separated cells was isolated using RNeasy Mini Kit (Qiagen) and cDNA was made from isolated RNA as described above.

16- cell stage embryo isolation

Pregnant female mice (2.5 d.p.c) were sacrificed and embryos were flashed through their oviducts into a 35-mm petri dish containing room temperature M2 medium. Flushed embryos were then transferred to pre-wormed KSOM (Millipore) medium and cultured at 37°C in 5% CO2 for 2 days. After the culture period blastocysts were transferred to KSOM medium containing 100nM of tamoxifen and analyzed 12h later.

Results

Transgenic cell line faithfully recapitulates in vivo *Oct4* expression

Before using the 3F10 cell line, we wanted to ensure that the transgenic mouse, which 3F10 cells were derived from, can recapitulated *Oct4* expression in vivo developmental events and whether the system was sensitive enough to capture the earliest events of *Oct4* loss. In mouse development the first cells to lose *Oct4* expression are trophoblast cells. Thus, we isolated 16 cell stage embryos from transgenic pregnant mice (see methods) and cultured them in vitro. After 2 days of culture tamoxifen was added to medium and the blastocysts were fixed 12h later. Our results show that ICM cells activated Cre and therefore were EGFP positive, whereas trophectoderm cells did not and therefore were Td-Tomato positive (Fig.1). Therefore, our data indicates that the system is able to distinguish the earliest events of *Oct4* loss, hence is reliable for in vitro studies.

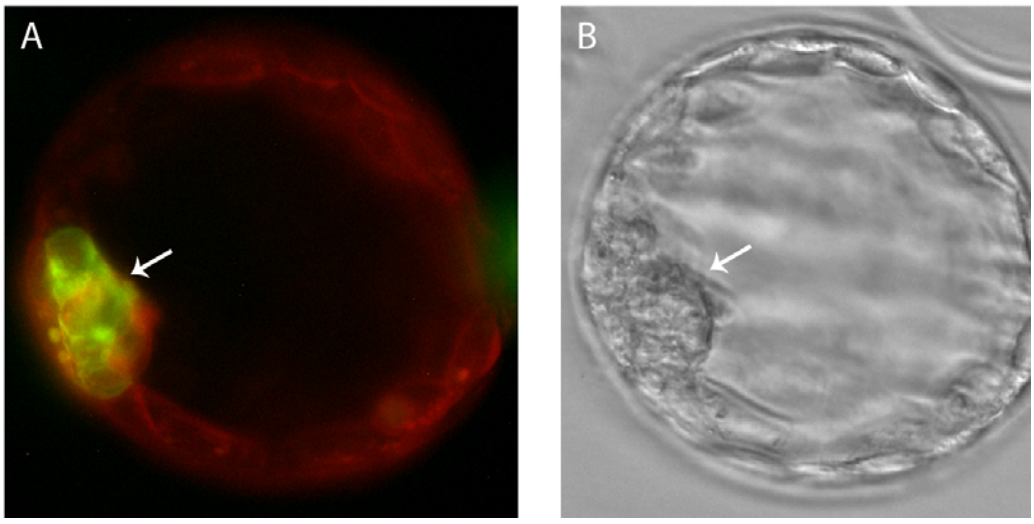


Figure 1. Transgenic system detects the earliest loss of *Oct4* expression in blastocysts.

16 cell stage embryos were flushed out of pregnant transgenic mice and left to culture in vitro for 2 days before tamoxifen was added. A) Successful activation of Cre in ICM cells (EGFP) and unsuccessful activation of Cre in trophectoderm cells (Td-Tomato). B) Phase image of the blastocyst. Errors are point at an aggregate of ICM cells expressing EGFP.

Morphological characterization of formed EBs

After ensuring that the cell line can reliably be used to analyze *Oct4* expression, iPS cells were generated from fibroblast of transgenic mice (Greder et al., 2012). Many cells line were made, however 3F10 was chosen for this study, because its pluripotency was validated in (Greder et al., 2012). In the current study 500 cells was chosen as the starting number, hence the initial diameter is about 9-10 cells. We chose the spin method for EB generation (Ng et al., 2005), due to feasibility in interrogating individual EBs for unintended Cre activation. When cultured for one week in differentiation medium the EBs remain spherical for some days although could become irregular by day 7. For spherical EBs, the diameter increases about 2-2.5x fold during the culture period, corresponding to an increase of cell number, of about 10 fold (Fig.2).

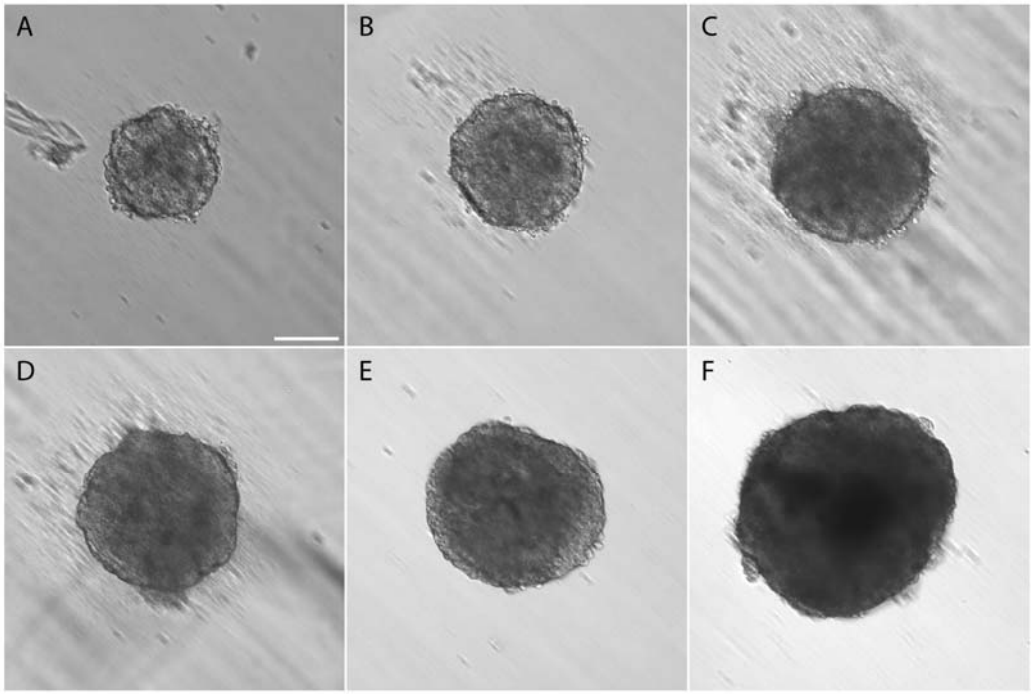


Figure 2. Growth of EBs during the period of 7 days.

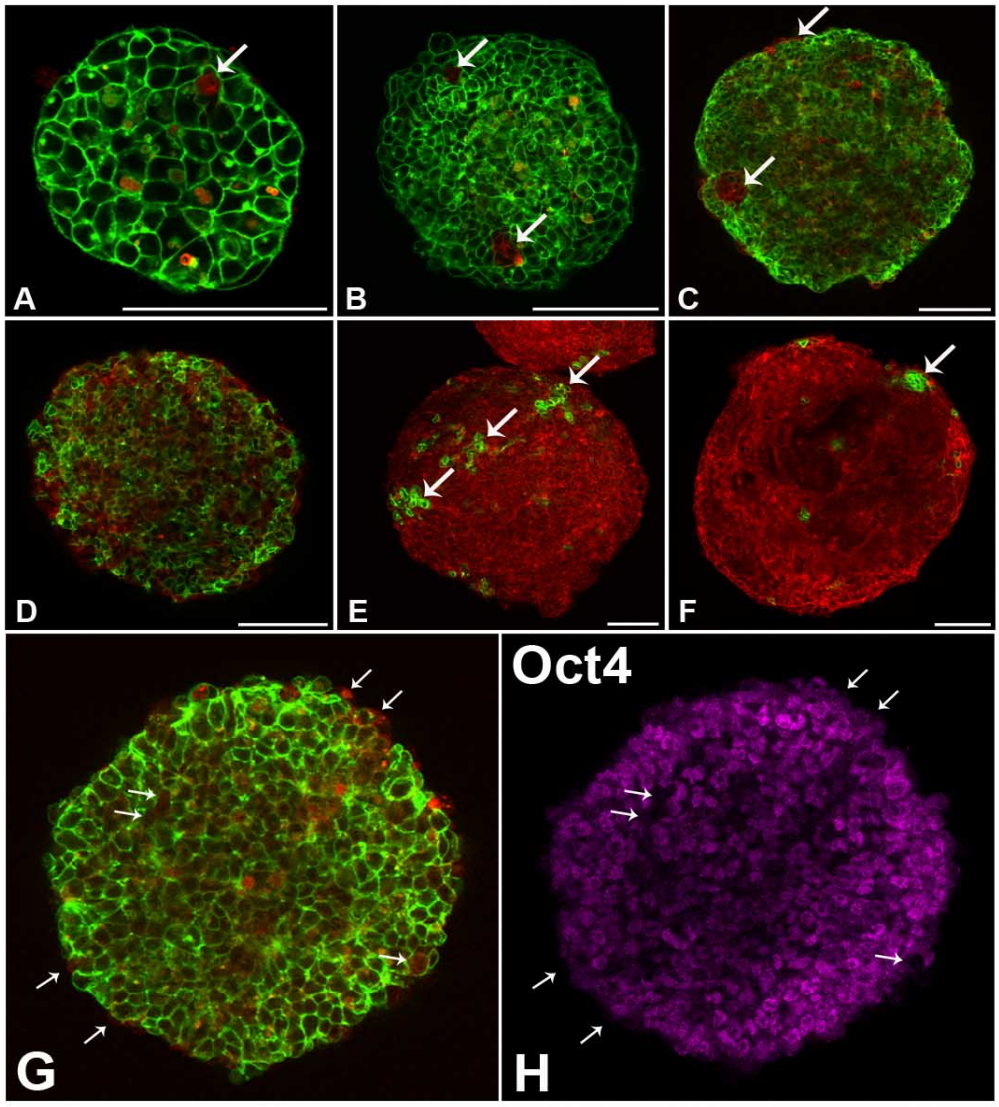
500 cell EBs were generated by the spin method and imaged on each consecutive day of development. (A-F) Days 1, 2, 3, 4, 5, 7. Scale bars 100μm.

***Oct4* expression in developing EBs**

To test the array of *Oct4* down regulation in developing EBs, tamoxifen was given commencing on each day of the culture after EBs were made. Then they were fixed 12h later, immunostained and mounted, and optical sections were recorded by confocal microscopy. In figure.3 are shown sections of typical specimens through the equator. It is known from previous work that EGFP expression becomes visible about 4h after administration of the tamoxifen, and that in vitro the recombination occurs in essentially 100% of the *Oct4*-expressing cells (Greder et al., 2012). The results show that the loss of *Oct4* begins right after the cells are aggregated (Fig.3A). Interestingly, day 1 red cells (i.e. those that had ceased expressing *Oct4* before the day 1 tamoxifen treatment) are not located on the exterior but are scattered through the interior of the EB (Fig.2B). Although we cannot prove clonal growth in this situation it seems likely that the clumps represent clonal growth of the earlier formed red cells, while the scattered ones have newly lost *Oct4* expression. By 3 days, the red patches are a bit bigger and now some appear on the exterior of the EB (Fig.3C). By 4 days about half of the cells have lost *Oct 4* expression in a random fashion (Fig.3D). From day 5 onwards some green cells remain scattered as small clumps or single cells, but the EB is predominantly red (Fig.3E). By day 7, some EBs become irregular in shape and some structures are beginning to form in the interior, and a connected epithelial layer can be seen surrounding some of the EB (Fig.3F).

To make sure that the green fluorescence really does report *Oct4* expression, we examined day 3 tamoxifen-treated EBs, fixed 12h later, by immunostaining for OCT4 protein (Fig.3 G and H). Typical of a day 3 EB this shows some single red cells and some small clumps scattered through the specimen. The green cells correspond exactly with the presence of OCT4, however red cells did not. Therefore, indicating that the system does effectively report the expression of Oct4 at the transcription level.

We preformed flow cytometry on EBs treated with tamoxifen at indicated time-points, to obtain a more quantitative venue of the proportion of green and red cells (Fig.3 I). In this experiment tamoxifen treated EBs were fixed 24h later to provide more time for green cells to degrade the Td-Tomato protein for better segregation of the 2 populations. The results confirm that the main loss of *Oct4* expression occurs between day 3 and day 5, with about equal proportions of green and red cells being present at day 4. The last two bars shown on this figure represent essential controls. The first bar shows flow cytometry of day 3 EBs that had been tamoxifen- treated while they were still iPS cells. Theoretically 100% of cells should be green. The <1% that are red have lost *Oct4* expression while still in the iPSC culture and, as shown below, they do not participate in forming the EB. The second bar shows day 3 EB cells which were never exposed to tamoxifen. This shows that there is no background of spontaneous reporter activation over the time scale of this type of experiment, although there is a low background during late passages of culture.


A
B
C
D
E
F
G
Oct4
H

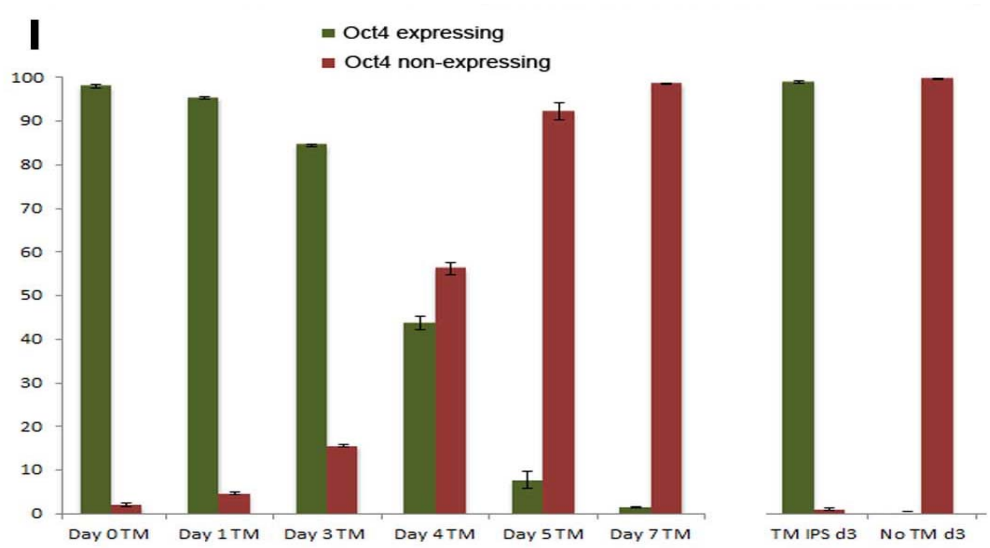


Figure 3. *Oct4* down-regulation during development of embryoid bodies**.**

The pictures are optical sections through EBs. They were labeled by exposure to tamoxifen at the indicated time and fixed 12h later. Green (EGFP) indicates *Oct4* expression at the time of treatment, red (tdTom) indicates no *Oct4* expression. (A) day 0 (i.e. added to the medium during aggregation of the iPS cells). (B) day 1. (C) day 2. (D) day 3. (E) day 4. (F) day 6. (G) day 7. Arrows indicate red cells or patches in (A-C) and green ones in (E-F). Scale bars 100μm. (G,H) OCT4 protein. EBs were treated with tamoxifen at day 3, fixed 12h later, and stained for OCT4. (G) Green and red fluorescence. (H) OCT4 immunostain (magenta). Arrows are pointing at red cell in the EB. (I) Flow cytometry of red and green cells from EBs treated with tamoxifen from day 0 to day 7. Treated EBs were left in culture for 24h before dissociation and counting.

Embryoid bodies are only formed by *Oct4* expressing cells

We were concerned that the small number of *Oct4* negative cells located in the iPS cell cultures before aggregation could bias our results. Therefore, we chose to address this issue by generating EBs from iPS cells that were treated with tamoxifen while cultured is ES medium. Generated EBs were analyzed at day 1, 2, and 3 to monitor clonal growth of any red cell contaminants. It can be seen from (Fig. 4) that very few number of red cells can be detected in the EBs, and they are predominantly found on the exterior.

Moreover, their numbers do not multiple relative to the rest of the EB, if anything they are growing less slowly. Our results demonstrate that only *Oc4* expressing cells go on to form EBs as the cells are aggregated. Therefore, the lose of *Oct4* expression seen at later time points during EB development must be cells switching off *Oct4* and not contaminants from the culture.

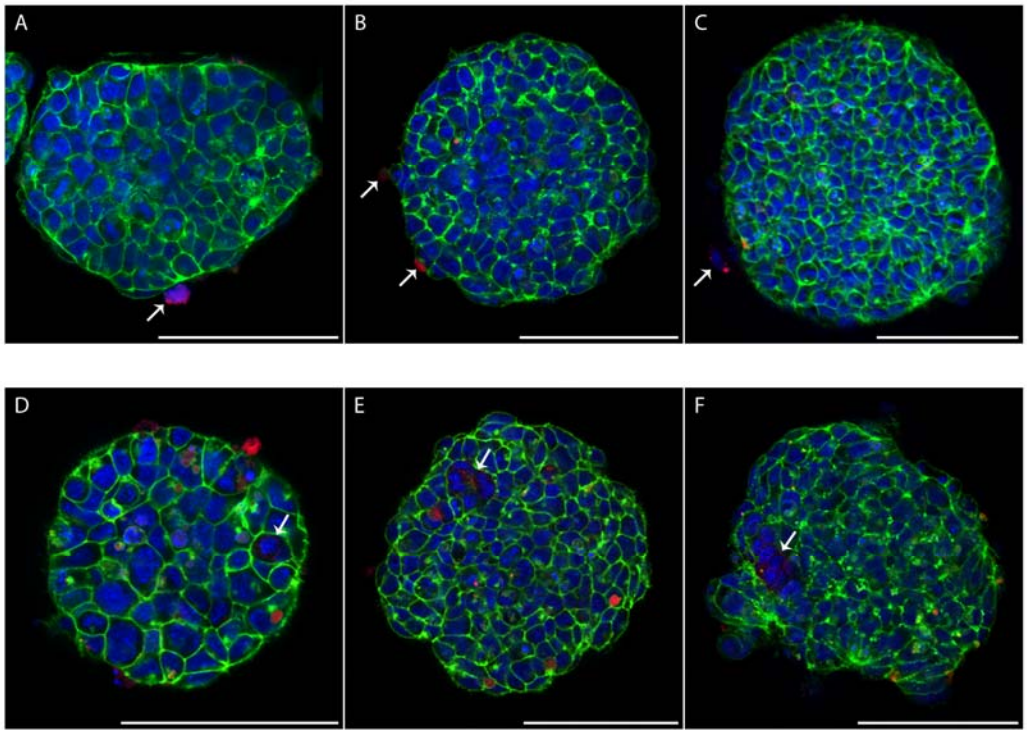


Figure 4. EBs are formed by *Oct4* cells and internal cells are first to differentiate.

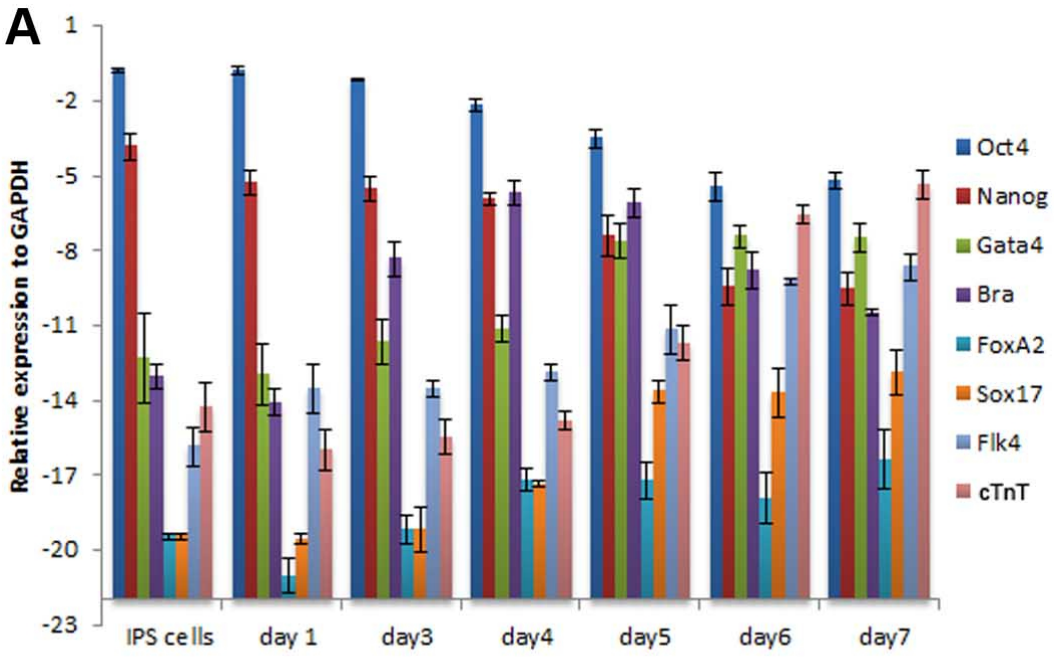
Tamoxifen was given to iPS cells (day-1) or day 0 EBs then analyzed. A-C) 1, 2 and 3 days of development. Green fluorescence shows EGFP, blue shows DAPI, red shows tdTomato. There are very few red cells (arrowed). They are not seen inside the EB and they do not expand as the EB develops. D-F) 1, 2, and 3 days of day 0 tamoxifen treated EBs showing internal clonal expansion of first cells to lose *Oct4* (arrowed). Scale bars 100μm.

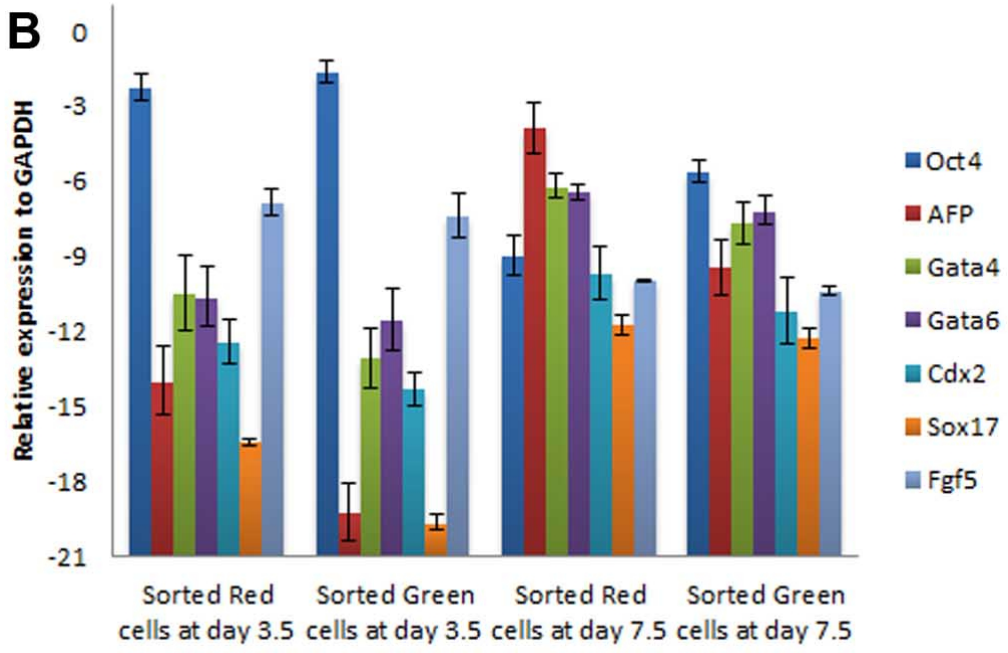
Normal EB differentiation

To test whether 3F10 iPS cells differentiate appropriately, we quantified various

genes markers that are often used for monitoring ES cell differentiation by qRT-PCR. Antibodies to the protein products of several of these genes were used for the morphological studies later in the study. The gene expression data over time (Fig.5A) indicates that the process of EB differentiation in our system is very similar to what has been reported in murine ES cell differentiation studies, e.g. (Ensenat-Waser et al., 2006; Mogi et al., 2009) In the figure the mRNA levels are all plotted relative to that of *Gapdh*. *Oct4* and *Nanog* expression, characteristic of pluripotent cells, drop gradually. *Gata4*, normally expressed in primitive, visceral and definitive endoderm and in the heart, rises at first, then plateaus from day 5. *Brachyury (T)*, indicative of early mesoderm, rises and falls, peaking at day 4. The expression of the various other markers: *FoxA2* (axial structures), *Sox17*: (definitive endoderm), *Flk4* (VEGF receptor, endothelium), rise continuously over the 7 day period. *Cardiac troponin (cTnT)*, a terminal differentiation marker for cardiomyocytes, rises steeply over the last three days. The level of *Oct4* expression is still high at the end of the culture period. However the vertical scale is logarithmic, so the final level represents about a 32 fold reduction from the start, consistent with the presence of perhaps 3% Oct4 positive cells at the end. For a developmental transcription factor, *Oct4* has an unusually high absolute level of expression which explains why the mRNA abundance exceeds that of all the other genes except *cTnT* and the *Gapdh* standard. Furthermore, figure.5B shows gene expression in green and red cells separated by FACS and then subjected to RNA analysis by qRT-PCR. On the left are shown samples treated with tamoxifen at day 3 and analyzed 12h later. As expected, this shows that the green cells at this stage retain a profile similar to iPS cells.

The only significant difference with the red cells is seen for the *Afp*, which is about 32x more abundant in the red cells. In the immunostaining studies listed below, it is shown that AFP protein is detected in the first cells to lose Oct4 expression; thus, validating the PCR data. On the right side of the figure are shown the same measurements conducted on EBs that were tamoxifen treated at 3 days and then allowed to develop to 7.5 days. This means that most of the green cells have subsequently lost *Oct4* and differentiated. Although there is little difference between gene expression of green and red cells, the *Oct4* level is higher in green cells, possibly due to the remaining *Oct4* expressing cells at 7.5 days. In addition, the *Afp* level is still much higher in red cells, indicating that most *Afp* positive cells lost Oct4 before 3 days. Finally, the gating for isolating green and red cells via FACS at day 3.5 and 7.5 can be seen in (Fig. 5C).


A
Relative expression to GAPDH
1
-2
-5
-8
-11
-14
-17
-20
-23
IPS cells
day 1
day3
day4
day5
day6
day7
Oct4
Nanog
Gata4
Bra
FoxA2
Sox17
Flk4
cTnT


B
Relative expression to GAPDH
0
-3
-6
-9
-12
-15
-18
-21
Sorted Red cells at day 3.5
Sorted Green cells at day 3.5
Sorted Red cells at day 7.5
Sorted Green cells at day 7.5
Oct4
AFP
Gata4
Gata6
Cdx2
Sox17
Fgf5

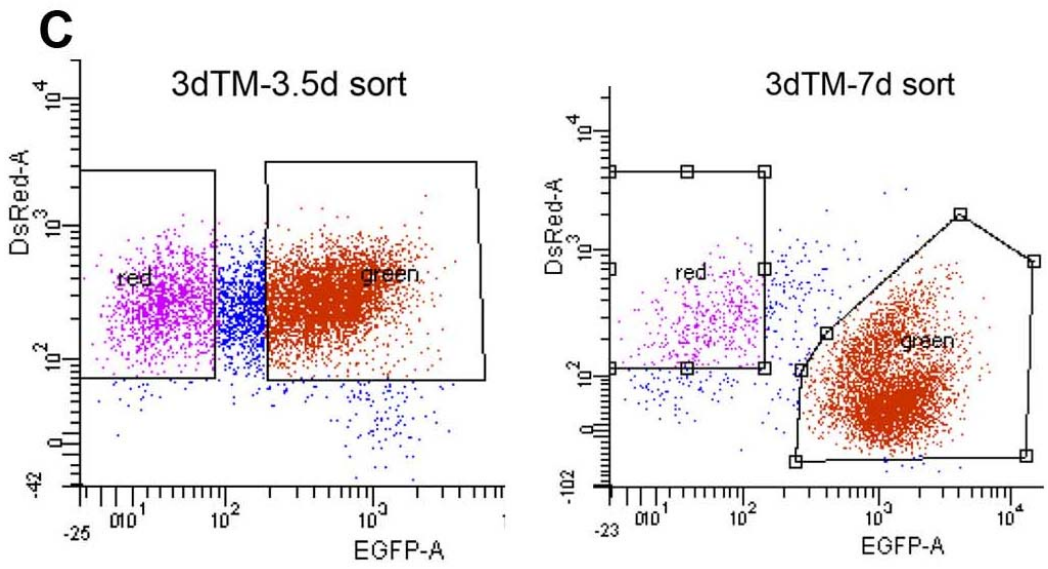


Figure 5. Expression of key marker genes in iPS cells and during EB differentiation.

Levels were measured by qRT-PCR and are expressed relative to *Gapdh* mRNA. The vertical scale is logarithmic. (A) Gene expression in whole EBs on the indicated days. (B) EBs were tamoxifen treated on day 3 and were dissociated and sorted into red and green cells either 12h later (left) or 4.5 days later (right). C) FACS Gating plots to sort red and green cells from day 3 tamoxifen treated EBs, left at day 3.5 and right at day 7.5.

Formation of early extra-embryonic endoderm

Previous studies of EB formation have usually described an endoderm layer forming around the exterior. It is often implied that this is the first structure to be determined in EBs, as does the primitive endoderm on the blastocoelic surface of the inner cell mass of the mouse embryo. In the embryo, the primitive endoderm expresses *Oct4*, but its derivatives, the visceral and parietal endoderm, do not. However, Alpha-fetoprotein (AFP) is expressed in the visceral endoderm, but not in the primitive endoderm of the normal mouse embryo (Dziadek and Adamson, 1978). We investigated

AFP protein in EBs that were tamoxifen treated at day 3 and fixed 12h later. In our EBs, AFP immunostaining was found mainly in red cells, particularly red cell clumps, of day 3-treated EBs (Fig.6 A-C). About 69% of AFP positive cells were red (Fig. 7 E). Remarkably, These AFP positive red cells were not found on the surface but rather scattered throughout the interior of the EBs. This data matches nicely with the *AFP* qRT-PCR results on red cells sorted at day 3.5, which showed them to have 6 fold increase in expression compared to green cells sorted at the same time (Fig. 5 B).

We then wanted to ask whether the AFP-positive cells went through a primitive endoderm stage as they developed. Therefore we examined our EBs for the appearance of GATA4, a protein which is needed for primitive endoderm formation and whose expression persists in the visceral and parietal endoderm (Cai et al., 2008; Nichols et al., 2009). GATA4 expression was studied in day 3 and 4 EBs (Fig.6 D-L). This shows that GATA4 arises, as expected, in green cells and that the first GATA4-positive cells are scattered throughout the EB. About 94% of GATA4 cells are green on day 3 (Fig.7 E). But if nearly all GATA4 cells are green at day 3 (Fig.6 D-F), then they cannot be the precursors to AFP cells, which are mostly red at 3 days. Conversely, by day 4 a number of GATA4-positive cells are red, indicating that they might have lost *Oct4* expression or day 3 red cells have acquired GATA4 (Fig.6 G-I). To exploit this possibility, we tamoxifen treated EBs at day 3 and fixed them 36h later. Upon doing so we found all GATA4 in green cells (Fig.6 J-L), indicating that the red GATA4 cells visible on day 4 labeling must have lost *Oct4* expression after upregulating GATA4, not the reverse. Therefore, the red AFP-positive cells cannot derive from the GATA4 positive ones,

although the minority of green AFP cells may do so. Collectively, our results indicate that the first cells to loss *Oct4* in our EBs are visceral endoderm and these endoderm cells do not pass through a primitive endoderm precursor. It is also safe to say both primitive endoderm and visceral endoderm cells are located in the interior of EBs.

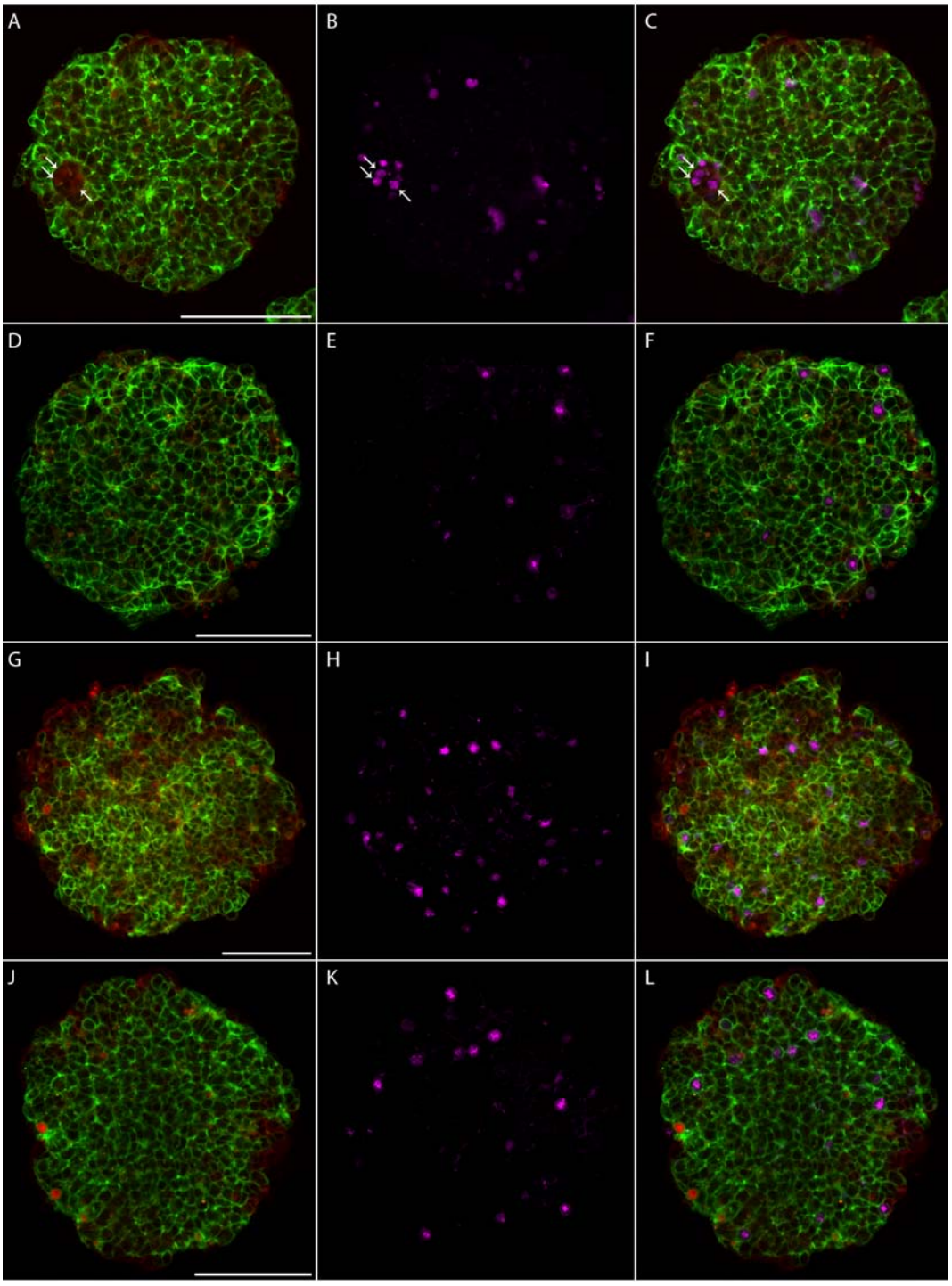


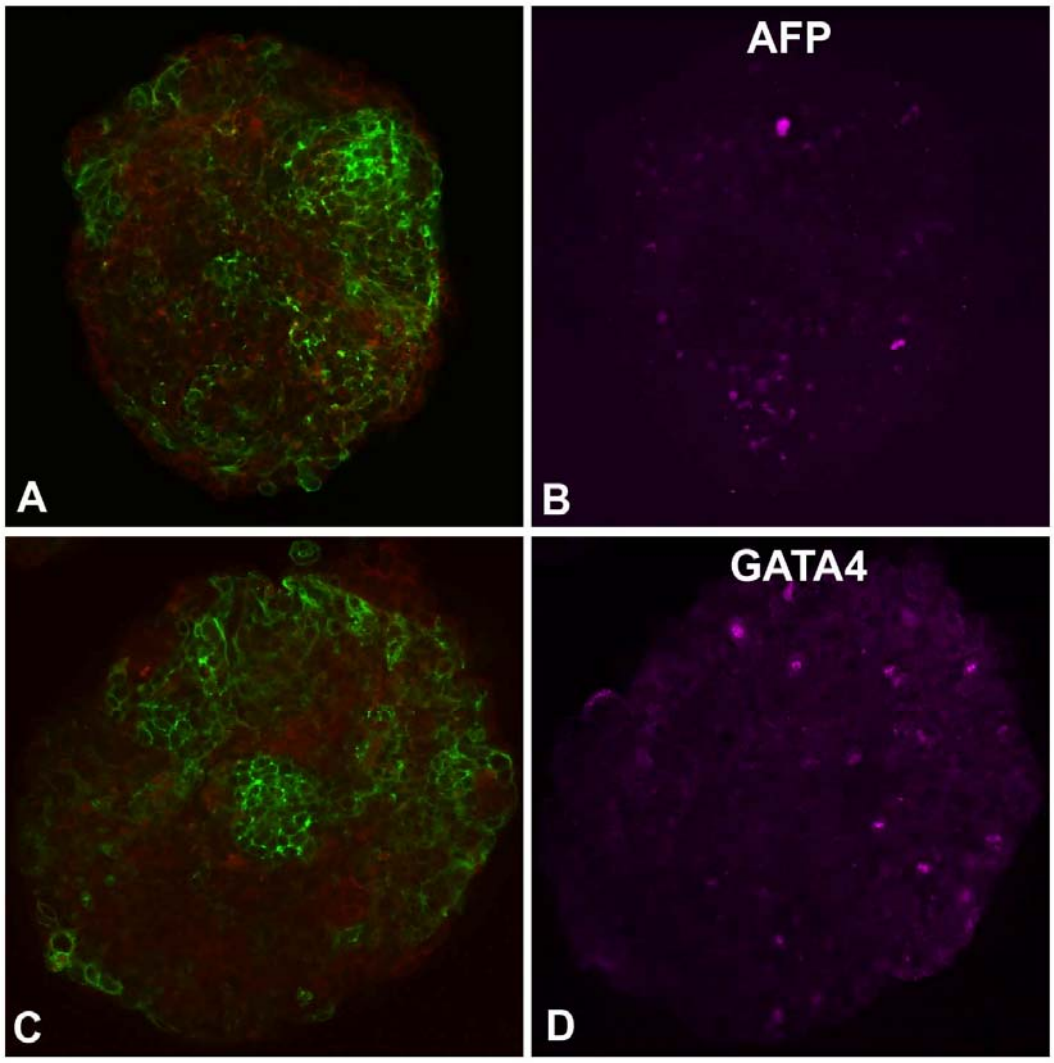
Figure 6. AFP marks the first cells to lose *Oct4* expression while GATA4 is found mostly in *Oct4*-expressing cells.

EBs were treated at the designated time points and left for 12h or 36h before fixation and immunostaining (magenta). (A) Red-green configuration of day 3 tamoxifen treated EBs.

(B) AFP staining. (C) Merged image showing AFP staining is mostly in red cells (arrows show a cluster). (D-F) The same for GATA4, which appears almost entirely in green cells. (G-I)The same for GATA4 with tamoxifen treatment at 4 days. Some GATA4 cells are now red. (J-L) GATA4 in in EBs treated on day 3 and fixed 36h later. The merged image shows most GATA4 staining in green cells, indicating that de novo expression occurs in cells that were *Oct4* positive at 3 days. Scale bars 100μm.

Formation of late primitive endoderm

After establishing that both primitive and visceral endoderm are formed early during EB development, we wanted to ask whether these cells go on to form an outer layer of endoderm in mature EBs, as often described in EB studies. In order to tackle this question we preformed a lineage tracing experiment of day 3 EBs. Thus, day 3 EBs were tamoxifen treated and left to develop in culture tell day 7.5 before being fixed and stained for AFP and GATA4. Surprisingly, both AFP and GATA4 were found to have a disperse appearance (Fig. 7 A-D). Therefore, demonstrating that our EBs did not form an outer layer of endoderm.


AFP
A
B
C
GATA4
D

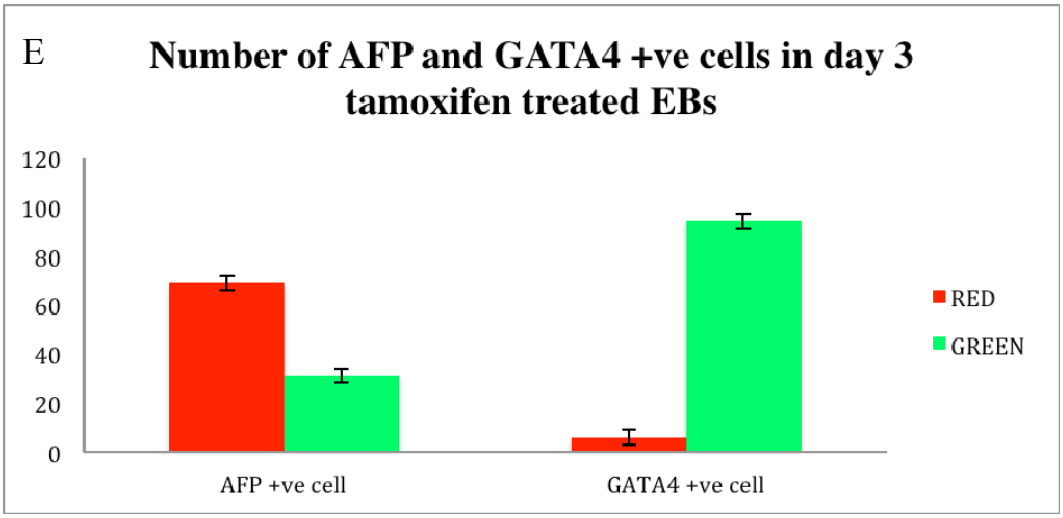

E
Number of AFP and GATA4 +ve cells in day 3 tamoxifen treated EBs
120
100
80
60
40
20
0
AFP +ve cell
GATA4 +ve cell
RED
GREEN

Figure 7. Mature EBs don not form an outer layer of primtive and visceral endoderm.

EBs were tamoxifen labeled at day 3 and allowed to develop to day 7.5 before fixation. In these specimens loss of *Oct4* has proceeded further than in Figs. 1, 3 and 4. (A,B) AFP immunostain. (C,D) GATA4 immunostain. There is no continuous exterior epithelium, and what there is does not display the endodermal markers. E) Quantitative graph of 3 independent samples showing the percentage of AFP and GATA4 in both red and green cells of day 3 Tamoxifen treated EBs fixed and stained 12h later.

Early cells to lose Oct4 fail to migrate to the outer surface of EBs.

To decisively show that early red cells, which are AFP-positive, do not migrate to the exterior of EBs as they develop in vitro and form a layer of endoderm, we sorted out red cells from day 3 tamoxifen treated EBs. We then added tamoxifen to iPS cells and mixed 500 of these cells with 500 sorted red cells and allowed the EBs to develop normally. As expected, red cells demonstrated a certain degree of aggregation within EBs, however failed to migrate to the surface of EBs and form a layer of endoderm (Fig. 8). Accordingly, we can safely state that early cells to lose *Oct4* do not migrate to the exterior and do not form an outer layer of primitive endoderm around mature EBs.

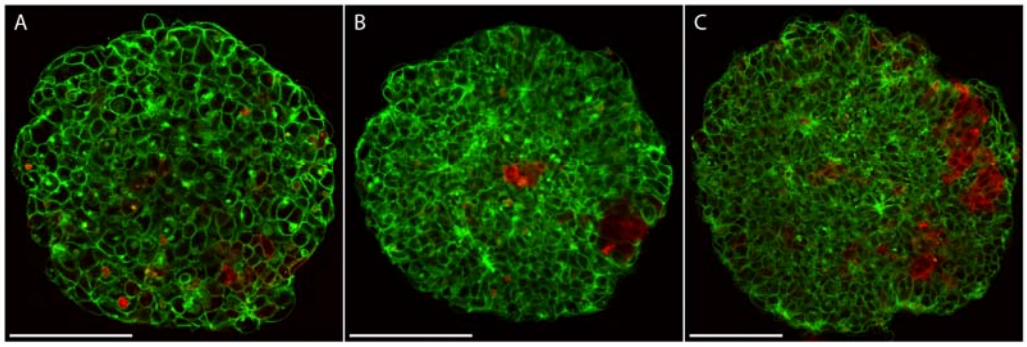


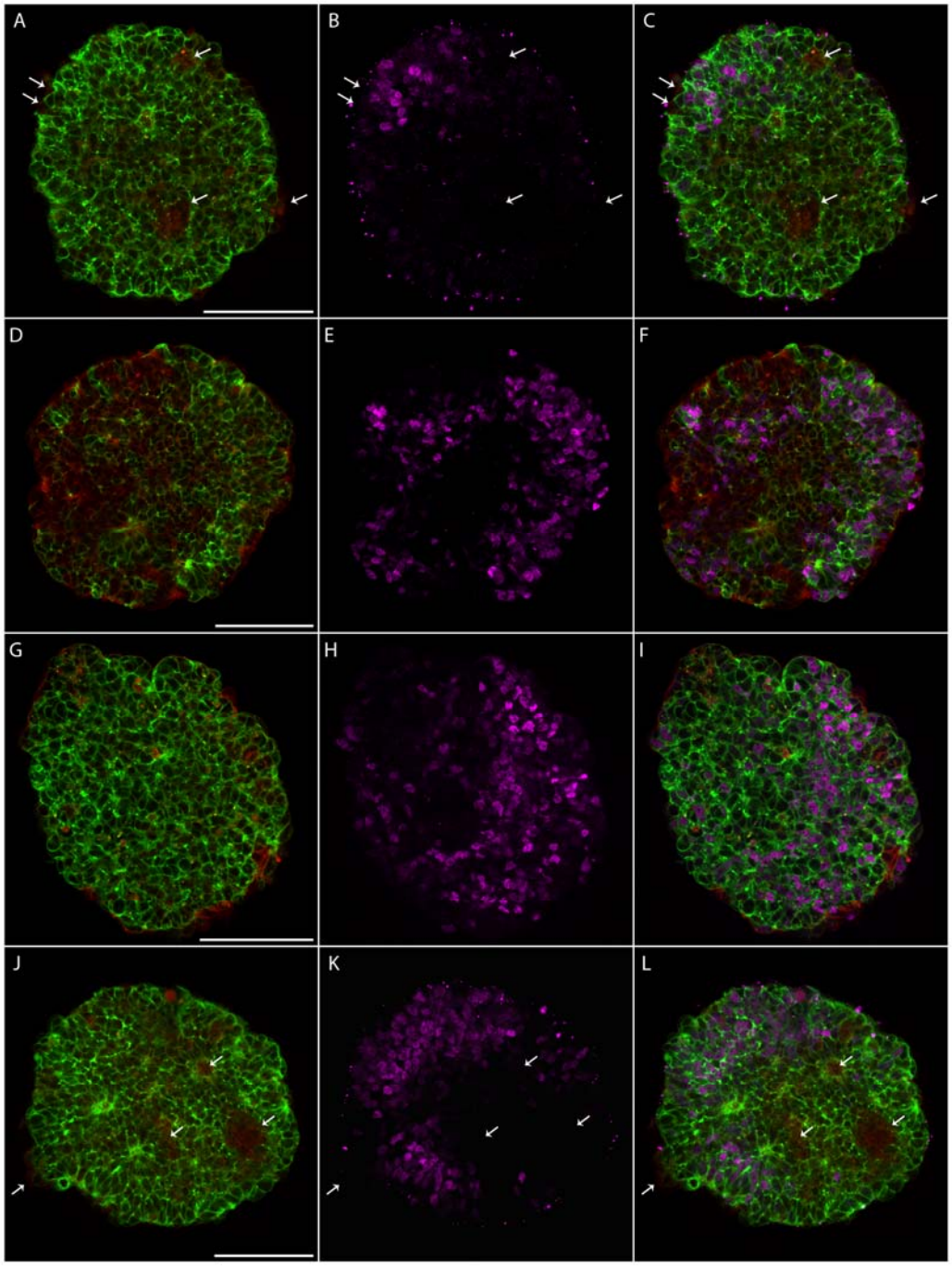
Figure 8. Early cells to lose *Oct4* do not form a layer of primitive endoderm in EBs.

EBs were given tamoxifen at day 3 and red cells were FACS sorted 12h later. 500 red FACS sorted cells were mixed with iPS cells pre-treated with tamoxifen while in iPS culture. A) Day 2 EB showing successful integration of red cells throughout the EBs. B) Day 3 EB showing a degree of red cell aggregation. C) Day 4 EB showing red patches throughout the EB, but no outer layer of primitive endoderm. Scale bars 100μm.

Formation of principal body structures

Primitive streak-like structures have been reported in EBs on occasion (Leahy et al., 1999; ten Berge et al., 2008). Here we examined the expression of BRACHYURY (BRA, also called T) and of FOXA2. In the normal mouse embryo, *T* is first expressed in the posterior epiblast, then in the nascent mesoderm of the primitive streak, and then in the node and the notochord of the late streak. Loss of function mutants show posterior defects (Wilkinson et al., 1990). *FoxA2* is first expressed in the anterior primitive streak, then in the node, the notochord, the floor plate of the neural tube, and the definitive endoderm (Ang et al., 1993; Sasaki and Hogan, 1993). Loss of function mutants show absence of the node and notochord (Ang and Rossant, 1994). It is also active in the anterior visceral endoderm, although not expressed at high level in this tissue (Kimura-Yoshida et al., 2007). Expression of the T and Foxa2 protein are both evident by day 3. Tamoxifen treated EBs at day 3 and fixed 12h later show a patch of T expression on one side of the EB, extending 5-6 cell diameters internally. There are also a number of scattered T-positive cells. Intriguingly, in these samples, T expression is almost entirely in green cells indicating that *Oct4* is still expressed and that early red cells are not part of the T patch (Fig.9 A-C). EBs treated with tamoxifen at day 4 and fixed 12h later show some red cells expressing T but the majority remains in green cells (Fig.9 D-F). When counted it can be shown that about 80% of T-positive cells are in green cells (Fig.9 U). We then questioned whether the red cells positive for T in day 4 treated EBs were either early day 3 cells acquiring T expression of day 3 green cells losing *Oct4.* Therefore, we tamoxifen treated EBs at day 3 and fixed them 36h later show. From (Fig.9 G-I) it can be

noted that virtually all T is in green cells and not in red cells. This means that T expression arises in *Oct4*-positive cells, and that the *Oct4* expression is subsequently lost but at a slower pace. There is evidence that *Oct4* is needed for the initial expression of T (Zeineddine et al., 2006) and our result is consistent with this. The scenario with FOXA2 is somewhat different. As with T, a large patch of expression is found for EBs treated with tamoxifen at 3 days and fixed 12h later, almost entirely in green cells (Fig.9 J-L). However in EBs treated with tamoxifen at 4 days and fixed 12h later, the expression concentrates now mostly of red cells 78% (Fig.9 U) (Fig.9 M-Q). We then asked whether the FOXA2-posivite red cells observed in day 4 treated EBs are day 3 or day 4 red cells. Therefore, EBs were treated with tamoxifen at 3 days and allowed to develop for 36h before fixation and analyzed for FOXA2. These EBs show most of the FOXA2 positive cells are green (Fig.9 R-T). This indicates that the FOXA2 domain cells lost *Oct4* expression between 3 and 4 days of EB development, at about the same time as the expression of FOXA2. The presence of FOXA2 in some red cells in these specimens shows that it is to some extent upregulated in cells that have already lost *Oct4*, which might represent definitive endoderm precursors. Between them, the expression domains of T and FOXA2 make up most of the primary body axis so this study pinpoints the time of its determination in EBs. However it should be noted that the expression domains in our EBs are not very coherent. Although a clump of positive cells is apparent for both markers, there are also many scattered cells expressing them.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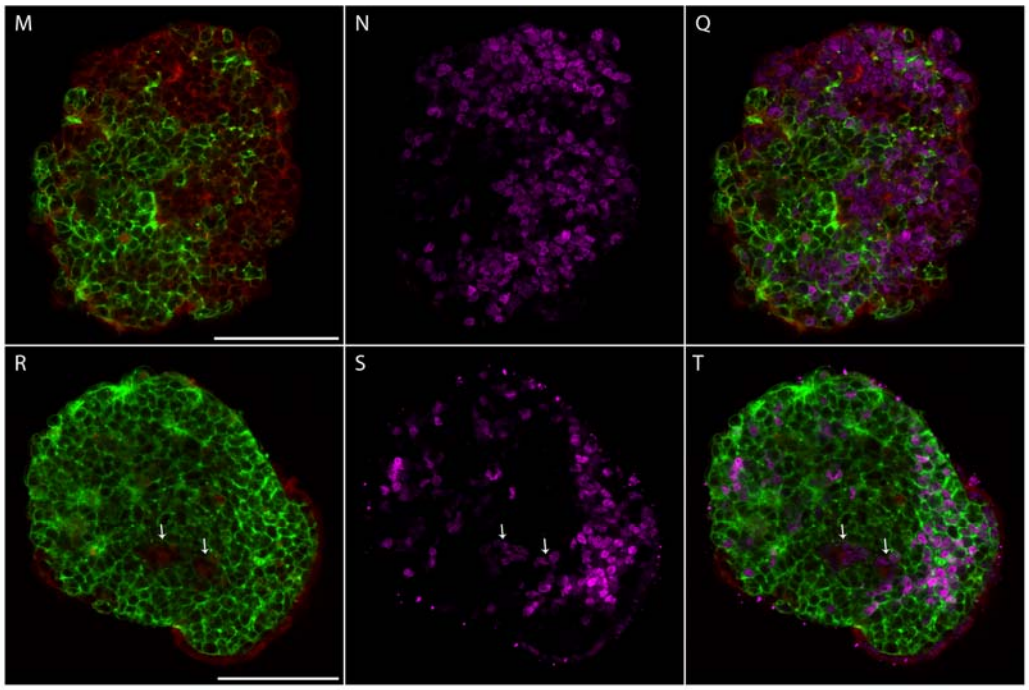
M
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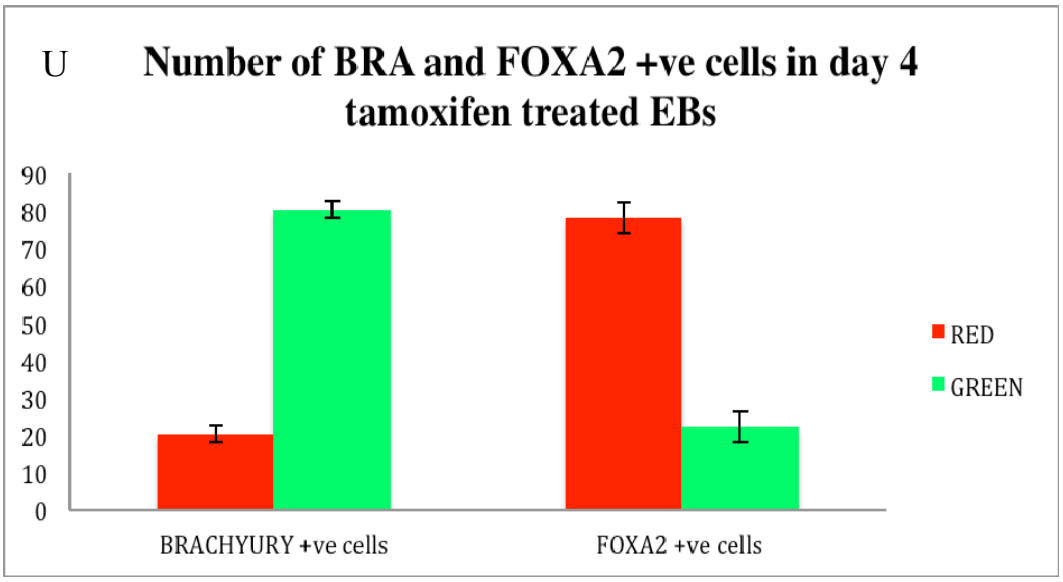

Figure 9. FOXA2 cells downregulate *Oct4* faster than T cells during EB development.

EBs were treated with tamoxifen at the designated time points and left for 12h or 36h before fixation and immunostaining (magenta). A,C) Red-green configuration and T immunostaining of an EB treated at 3 days and fixed 12h later. T cells are mostly green. Arrows show selected red cells. (D,F) The same at 4 days. T cells are still mostly green. (G,I) T in EBs treated on day 3 and fixed 36h later. T is overwhelmingly in green cells. (J,L) Red-green configuration and FOXA2 immunostaining of an EB treated at 3 days and fixed 12h later. FOXA2 cells are mostly green. Arrows indicate red patches lacking FOXA2. (M,Q) The same at 4 days, now the FOXA2 cells are mostly red. (R,T) FOXA2 in EBs treated on day 3 and fixed 36h later. FOXA2 is mostly in green cells, but its presence in red cells indicates that it can be upregulated in cells that have already lost *Oct4*. (U) A graph showing the percent of both T and FOXA2 positive cells in red or green cells in day 3 tamoxifen treated EBs fixed 12h later. Scale bars 100μm.

The formation of cardiomyocytes in developing EBs

As an example of a terminally differentiated structure arising early in the development of the normal embryo, we chose the heart. This can be visualized by expression of the cardiac variant of troponin T (cTnT), a subunit of troponin, which connects tropomyosin to actin in the contractile apparatus of cardiac muscle cells. By 7-8 days of EB development, patches of cTnT- positive cells are clearly visible. Therefore,

we wanted to trace when the cardiomyocyte precursors appeared during the development of our EBs. To do so, we carried-out a number of lineage tracing experiments from day 3, 4, and 5 tell day 8, when beating patches are visible. In EBs treated with tamoxifen at day 3 and fixed at day 8, the clumps of cardiac tissue are all green (Fig.10 A-C). This is consistent with the continued expression of *Oct4* in the prospective principal body structures of this stage, visualized with T and FOXA2. This further indicates that cardiac progenitors are formed after day 3. Interestingly, In EBs treated with tamoxifen at day 4 and fixed at 8 days, the cardiac tissue is now composed of both red and green cells (Fig.10 D-F). Conversely, in EBs treated with tamoxifen at day 5 and fixed at 8 days, the cardiac tissue is all red (Fig.10 G-I) Collectively, our results shows that cardiac precursors are formed from cells that downregulate *Oct4* between 3 and 5 days during EB development, a little later than the rest of the FOXA2/T-positive cell population. It can also be noted that some cardiac precursors retain *Oct4* expression more than others, however if this points to the formation of 2 distinct populations of cardiomyocytes such as primary and secondary heart field tissue remains to be explored.

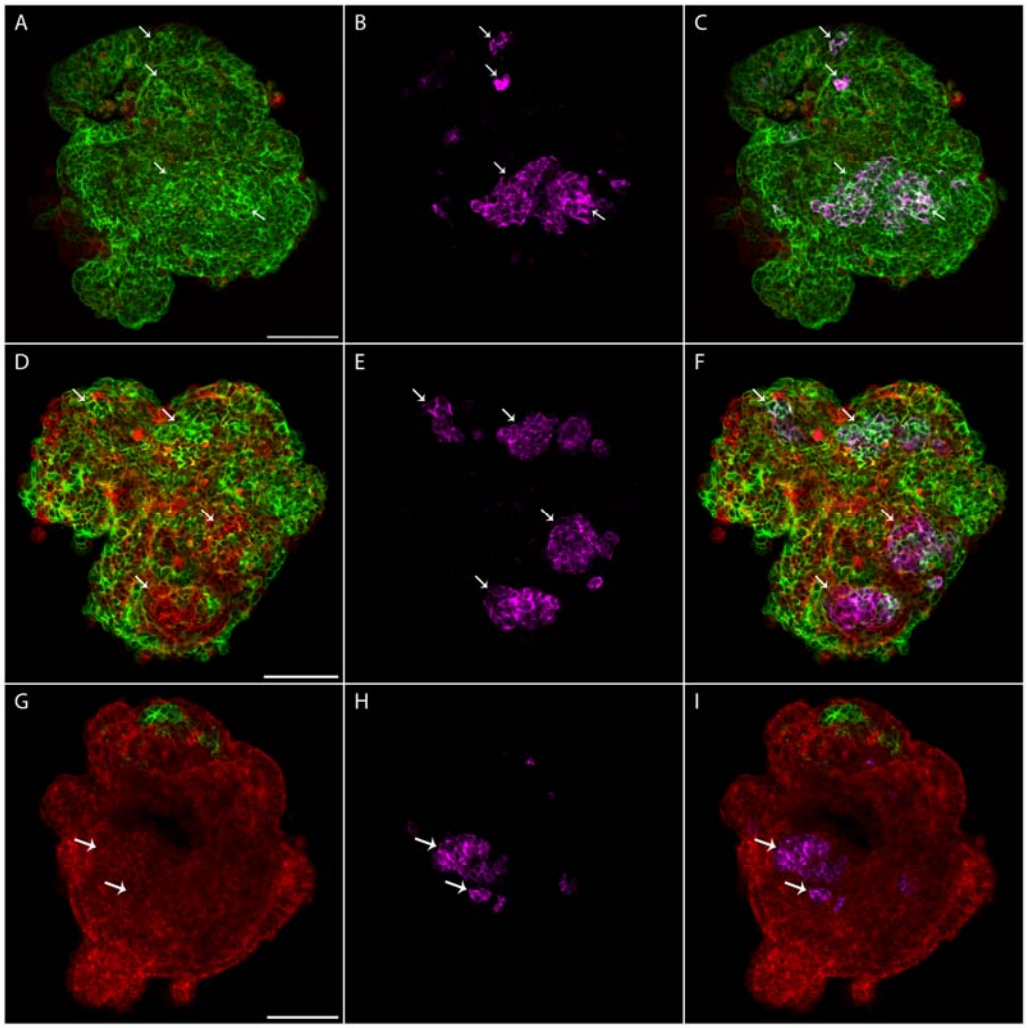

Figure 10. Cardiac precursors loses *Oct4* between day 3 and 5 during EB development.

Embryoid bodies were treated with tamoxifen at day 3,4, and 5, and allowed to develop in culture until day 8 before being fixed and stained for cardiac troponin (magenta). A,C) All cardiomyocytes rising by day 8 are in green cells when tamoxifen is added at day 3. D,F) In day 4 treated EBs, cardiomyocytes arise in both green and red cells. G,I) In day 5 treated EBs, all cardiomyocytes arise in red cells. Scale bars 100μm.

Discussion

Despite the widespread use of embryoid bodies as a first step in the differentiation of pluripotent stem cells (Keller, 1995; Murry and Keller, 2008) our knowledge of what actually occurs during EB development is quite limited. They are usually described as being "like embryos" but without any detail of what this means. Early work that described EBs were done on EC cells and later on ES cells. The nature of pluripotency between these 2 types of cells is quite different. The tumor EC cells gain their potency by duplication in *Nanog*, a pluripotency master regulator, thus they are in a severe unstable state (Chambers, 2004). However, the state is different in ES cells, as they are looked in an early stage during normal development, therefore they are considered to be more stable (Wray et al., 2010). In addition, many recent studies of murine EB structure have examined them with the only goal of deriving certain cell types at the end of a protracted culture period extending for 10-20 days. In this paper we have analyzed the development of EBs over a 7 day culture period which includes the expected time of determination of extraembryonic structures and the primary body axis. We have done this both by examining the location of cells bearing certain markers of early developing cell types and also by using the *Oct4-MerCreMer x mTmG* system, which enables us to permanently label cells that are expressing *Oct4* at a particular stage of development (Greder et al., 2012; Muzumdar et al., 2007). We have used a standard size of EB, 500 cells at aggregation, as the size is known to cause differences in subsequent behavior (Bauwens et al., 2008); and a standard shape, the sphere, achieved using the spin EB method of formation (Ng et al., 2005).

Murine ES or iPS cells do not correspond in transcriptional profile to either the inner cell mass (ICM) or the epiblast of the normal embryo but are generally considered to resemble the ICM in developmental properties (Brons et al., 2007; Sato et al., 2003; Tesar et al., 2007). If they are considered approximately equivalent to the cells of a 3 day mouse ICM, which has lost the ability to form trophectoderm, then the number of days of EB development should correspond to the same number plus 3 days of mouse embryo development.

Our results enable us to identify the time of loss of *Oct4* expression in the future primary body axis as between 3-4 days of EB development. This should be compared to between 6-7 days of normal embryo development. In normal development the primitive streak appears about E6.5 and the head fold about E7.5 (Kaufman and Bard, 1999). The loss of *Oct4* over this period in the embryo is similar to its loss from the T and FOXA2 positive cells in our EBs (Kehler et al., 2004; Scholer et al., 1990b; Yeom et al., 1996). The time of *Oct4* loss that we observe for cardiomyocytes precursor, at 3-5 days of EB development, is a little later than that of the main axial structures, as might be expected. Although OCT4 is usually considered as a pluripotency factor, there is evidence that elevated levels are associated with, or even necessary for, determination of cells to become mesoderm or more specifically to become cardiac tissue (Niwa et al., 2000b; Zeineddine et al., 2006). So there may be an increase of *Oct4* expression in the early stages of development of these structures.

Overall then the tempo of development in our EBs and the timing of *Oct4* loss is quite similar to that of normal development of a mouse embryo. However there are various

respects in which the EBs diverge sharply from normal development. One is the formation of the primitive endoderm, and its derivative, the visceral endoderm. The primitive endoderm appears as a layer on the blastocoelic surface of the ICM in the late blastocyst, about 4.5 days. Although there was initially speculation that it arose as a result of an environmental difference between the superficial and deep cells of the ICM, it is now known that its formation is stochastic, with cells that become primitive endoderm developing spontaneously and sorting out to form the superficial layer (Chazaud et al., 2006; Nichols et al., 2009). In our EBs we find that the first GATA4-positive cells do arise as single scattered cells in the interior. They arise in green cells, consistent with the persistence of *Oct4* expression at the time of primitive endoderm formation. However, they do not seem ever to become a superficial layer. We believe that the observance of an outer endoderm layer forming in the first few days of EB development (Abe et al., 1996; Doetschman et al., 1985; Leahy et al., 1999) may be due to the presence of significant numbers of cells in the original cultures that had already lost *Oct4* and become primitive endoderm. Due to the method of preparation, our cultures contain virtually none of these, but the very few that are present do spontaneously sort to the exterior of the aggregates (Fig.4).

Surprisingly, we find that the first cells to lose *Oct4*, which are presumably visceral endoderm due to the AFP staining, arise at a stage when virtually all the GATA4-positive cells are green. This indicates that the initial AFP-positive population cannot be descended from the GATA4 population, and may arise directly from the pluripotent cells.

Another respect in which EBs are not like embryos is that their internal structure is much more disorganized. In general our results are quite comparable to those of (Leahy et al., 1999), perhaps the most comprehensive previous study of EB structure using early developmental markers. They saw patches of *Nodal* and *Bra* from about 4 days of EB development and of *Nkx2.5*, a cardiac marker, from about 6 days. The resolution of their ^{35}S-in situs is inferior to that of modern techniques but the overall results look similar. There is a more recent report indicating a discrete principal body axis, based on the use of a Wnt-beta-catenin reporter system (ten Berge et al., 2008). While these studies have laid stress on the discreteness of the gene expression patches observed, in reality it is more of a diffuse cloud, surrounded by scattered single cells expressing the relevant markers, as is clearly apparent in our Fig.8. We do see discrete masses of cardiac tissue by day 7-8 (Fig.9), although these are always multiple patches without the morphology of a heart. So our conclusion is that, although EBs do follow a similar time course of development to normal embryos, the spatial organization is much more disorganized, and they are more like mosaics of many mini-regions of embryo rather than integrated body plans.

One reason for this difference is probably that the scale of events is different. The normal ICM comprises about 20-40 cells, and the epiblast at the time of implantation about 20-25 cells (Hogan et al., 1994).The growth rate is extremely rapid, with the epiblast reaching about 15,000 cells by headfold/early somite stage (Hogan et al., 1994). By contrast our EBs start out as aggregated spheres of 500 cells and grow about 10 fold over 7 days. Studies on the behavior of EBs of different controlled sizes have indicated that this is a very important variable affecting development (Bauwens et al., 2008). Moreover,

our EBs are suspended in medium, which is a relatively homogeneous environment, whereas the embryo has a highly asymmetrical environment. In the embryo the epiblast is in close contact with signaling centers in the anterior visceral endoderm, emitting inhibitors of Wnt and Nodal (Perea-Gomez et al., 2002; Thomas and Beddington, 1996; Varlet et al., 1997), and the posterior visceral endoderm, emitting Wnt3 (Rivera-Pérez and Magnuson, 2005). In general developmental systems display a lot of symmetry-breaking processes in which small perturbations bias delicately poised cell populations to develop down particular pathways, often in a mutually autocatalytic manner. If such processes can generate a single predictable embryonic axis in a small group of cells surrounded by specific signaling centers, it is not surprising that a large mass of cells in a uniform environment will generate many small regions of pattern in a disorganized manner.

We expect that the system we describe here will allow for many new kinds of study on embryoid bodies. These will include the effects of aggregate size, the effects of different culture media and substrates, and the effects of specific differentiation-inducing treatments of the type that are currently used to make cells for therapeutic transplantation. Moreover it can also be used to study the development and organization of teratomas in vivo, as administration of tamoxifen to the hosts will label green those cells in the teratoma that are currently expressing *Oct4*. The system also provides means to study the biology of pluripotent cells exiting from pluripotency as *Oct4* expression can be detected.

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Appedix 1

PRIMER NAME	PRIMERS
Kdr (Flk1)	5' TCTTGTTCTCGGTGATGTACAC 3' 3'AGCCTCCACTGTTTATGTCTATG 5'
Oct4	5'CTCGAAGCGACAGATGGTG 3' 3' AGAAGGAGCTAGAACAGTTTGC 5'
GAPDH	5' GTGGAGTCATACTGGAACATGTAG 3' 3' AATGGTGAAGGTCGGTGTG 5'
Cardiac troponin	5' CATCAAAGTCCACTCTCTCTCC 3' 3' CTGATGAAGAAGCCAAAGATGC 5'
Foxa2	5' GTATGTGTTCATGCCATTCATCC 3' 3' GCCAGCGAGTTAAAGTATGC 5'
Brachyury	5' AACTCTCACGATGTGAATCCG 3' 3' GCAAAGTCAAACTCACCAACA 5'
Nanog	5' AGGCAGGTCTTCAGAGGAA 3' 3' TGAGGAAGCATCGAATTCTGG 5'
Fgf5	5' CGGACGCATAGGTATTATAGCTG 3' 3' GGATTGTAGGAATACGAGGAGTT 3'
Gata4	5' GTGGTAGTCTGGCAGTTGG 3' 3' CTCTATCACAAGATGAACGGCAT 5'
Gata6	5' CTCACCCTCAGCATTTCTACG 3' 3' TCATCAAGCCACAGAAGCG 5'
Cdx2	5' TCTTGATTTTCCTCTCCTTGGC 3' 3' TCACTTTAGTCGATACATCACCATC 5'
Sox17	5' GGGGAAATAGGAAGGCTGAA 3' 3' GAACCTCCAGTAAGCCAGAT 5'
AFP	5' CATGCTCATTTAACATGCTTCCT 3' 3' CTGCTCAGTACGACAAGGTC 5'

Table 1. List of pre-designed primers from IDT for genes expression analysis.