

Aggregation of embryonic stem cells induces Nanog repression and primitive endoderm differentiation

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Summary

When embryonic stem cells are allowed to aggregate, the outer layer of the aggregated spheres (referred to as embryoid bodies) differentiates into primitive endoderm. This initial specification of cell lineage facilitates further differentiation of the inner mass of the embryoid bodies. These processes are considered to recapitulate early embryonic development from the blastocyst stage to the egg-cylinder stage. Formation of the primitive endoderm layer in the embryoid bodies was induced solely by aggregation of embryonic stem cells, in the presence of leukemia inhibitory factor/STAT3 and serum/BMP4, which

were considered to be sufficient for embryonic stem cell self-renewal. Interestingly, cell aggregation by itself induced Nanog repression at the outer layer, which was essential for aggregation-induced primitive endoderm formation. These data illustrate aggregation-based cell-fate specification during early embryonic development, when downregulation of Nanog plays a crucial role.

Key words: Embryonic stem cell, Differentiation, Primitive endoderm, Aggregation, Nanog

Introduction

Murine embryonic stem (ES) cells are derived from the inner cell mass of blastocyst [day 3.5 (E3.5) preimplantation embryo] and maintained as undifferentiated stem cells on a feeder layer of mouse embryonic fibroblasts (MEFs). Undifferentiated ES cells can also be maintained without a feeder layer, if they are cultured on gelatin-coated dishes in a serum-containing medium supplemented with leukemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988). When LIF is removed from the medium *in vitro*, these ES cells start to differentiate into all three germ layers (endoderm, mesoderm and ectoderm) (Evans and Kaufman, 1981; Martin, 1981). In a typical experiment, ES cells are suspended in the medium without LIF on non-adherent culture plates or in hanging drops. The suspended ES cells spontaneously aggregate to form spheres, which are called embryoid bodies. Following the aggregation, the outer layer of the embryoid bodies is specified as extraembryonic primitive endoderm and further differentiates into visceral endoderm (Doetschman et al., 1985). Extracellular matrix, which is secreted from the primitive endoderm layer, then forms a thick basement membrane, separating the primitive endoderm from the inner mass of the embryoid bodies. Along the inside of the basement membrane, a primitive ectoderm layer is formed and cavitation occurs in the core of the embryoid bodies. This series of processes is considered to be an *in vitro* model recapitulating early embryonic development from the blastocyst stage to the egg cylinder stage (E3.5-E6.5) (Coucouvanis and Martin, 1995).

Although LIF is a key factor in maintaining ES cells in an undifferentiated state, the removal of LIF is not essential for initial specification of the primitive endoderm layer in

embryonic development. During gestation, LIF is secreted from the oviduct and upregulated during the blastocyst stage (E3.5). The extraembryonic endoderm layer is formed during this stage and in the presence of LIF (Shen and Leder, 1992). In agreement with this, previous studies have demonstrated that aggregated ES cells can give rise to primitive endoderm even in the presence of LIF (Murray and Edgar, 2001; Shen and Leder, 1992). However, the molecular mechanisms underlying this phenomenon are not fully understood.

Molecular mechanisms for self-renewal of pluripotent ES cells have been extensively studied using monolayer culture of murine ES cells. LIF was identified as a factor secreted by MEFs, and sufficient to maintain self-renewal of murine ES cells without the feeder layer in the presence of fetal calf serum (Smith et al., 1988; Williams et al., 1988). STAT3 activation was then shown to be the responsible downstream pathway of the LIF/gp130 signaling for ES-cell self-renewal (Niwa et al., 1998; Matsuda et al., 1999). More recently, supplementation with BMP4 inhibited differentiation of ES cells in a cooperative manner with LIF, and substituted for fetal calf serum (Ying et al., 2003). In addition to these external factors, a novel homeodomain transcription factor, Nanog, was recently discovered and shown to be an essential factor in maintaining pluripotency of ES cells (Chambers et al., 2003; Mitsui et al., 2003). Interestingly, knockout disruption of the *Nanog* gene resulted in differentiation of ES cells, primarily into the primitive endoderm lineage, suggesting that Nanog is a crucial factor that prevents ES cells from differentiating into primitive endoderm. With these as background, we investigated molecular events occurring during aggregation-mediated cell-fate determination of ES cells into primitive endoderm.

Materials and Methods

ES-cell culture

Murine ES cells were maintained in an undifferentiated state on gelatin-coated dishes in Knock-out Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY) containing 10% knockout serum replacement (Gibco BRL), 1% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine, 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 25 mM HEPES (Gibco BRL), 300 µM monothioglycerol (Sigma, St Louis, MO) and 1000 unit ml⁻¹ recombinant mouse LIF (ESGRO; Chemicon, Temecula, CA). Aggregation of ES cells was induced by making hanging drops on the lids of Petri dishes (2000 cells per drop) using the same medium described above with or without supplementation with LIF (Hamazaki et al., 2001). To activate STAT3ER (fusion protein of STAT3 and estrogen receptor), 1 µM 4-hydroxytamoxifen (4HT) (Sigma) was added to the medium (Oka et al., 2002). In some experiments, 10 ng ml⁻¹ BMP4 (Sigma) was added to the medium.

Plasmid construction and transfection

Transgenic ES cells were created that contained a fusion gene between the promoter of the gene encoding α -fetoprotein (Afp) and the gene encoding green fluorescent protein (GFP) (*Afp-GFP*). A vector containing the fusion gene (pAfp-GFP) (Hamazaki and Terada, 2003) was cloned into pMP8NEB Δ LacZ vector using *NotI-MluI* site. The linearized vector was introduced into hypoxanthine guanine phosphoribosyl transferase (HPRT) deficient ES R1 cells (Misra et al., 2001) by electroporation. Hypoxanthine-aminopterin-thymidine (HAT)-resistant colonies were screened for cells with successful homologous recombination.

Inducible *Nanog*-overexpressing ES cells (*TRE-Nanog* ES cells) were created as follows. The open reading frame of *Nanog* cDNA was generated by polymerase chain reaction (PCR) using LA-Taq polymerase (TAKARA Bio, Otsu, Japan) from cDNA prepared from R1 ES cells. Primers used were; 5'-AAACGCGGATCCATGAGTGTGGGTCTTCCTGGT, and 5'-ACGCGTCGACTCATATTTACCTGGTGGAGTCA. The PCR fragment was ligated into the *Bam*HI-*Sal*I restriction site of the pTRE2hyg expression vector (Clontech, Palo Alto, CA), which contained a tetracycline-responsive element. This vector was introduced into tetracycline-regulated ES cells (Era and Witte, 2000) by Fugene 6 (Roche, Indianapolis, IN) and selected with hygromycin B (200 µg ml⁻¹) (Invitrogen, Carlsbad, CA). Tetracycline-dependent expression of *Nanog* was confirmed by the ability to maintain ES-cell morphology in the absence of LIF and to differentiate by addition of 1 µg ml⁻¹ doxycycline (Sigma).

RT-PCR

For reverse-transcription PCR (RT-PCR), total RNA was extracted by using RNA aqueous kit (Ambion, Austin, TX). cDNA was synthesized by using SuperScript II first-strand synthesis system with oligo(dT) (Gibco BRL). PCR was performed by using Taq DNA polymerase (Eppendorf, Westbury, NY). For each gene, the DNA primers were originated from different exons to ensure that the PCR product represents the specific mRNA species and not genomic DNA.

Immunofluorescence staining

ES cell aggregates were washed once with PBS, and fixed in 3.7% formamide/PBS for 15 minutes at room temperature. Fixed cells were embedded in Tissue-Tek OCT compound (Tissue-Tek; Sakura, Torrance, CA) and frozen in a dry-ice/isopentane bath. Cryostat section (7 µm) were treated with 0.5% Triton-X100 in PBS for 5 minutes and blocked with 5% bovine serum albumin in PBS for 1 hour at room temperature. Cells were further incubated with mouse monoclonal anti-mouse-Oct4 antibody (C-10) (Santa Cruz

Biotechnology, Santa Cruz, CA), goat polyclonal anti-mouse-osteopontin antibody (R&D Systems, Minneapolis, MN) or goat polyclonal anti-mouse-HNF4 α antibody (C-19) (Santa Cruz Biotechnology) for 2 hours at room temperature. After four washes with PBS, cells were incubated with anti-mouse-IgG or anti-goat-IgG antibodies conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, West Grove, PA). After four washes with PBS, cells were mounted by Vectashield containing 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA).

Cell sorting

A single cell suspension was prepared from aggregates of ES cells by treating them with 0.05% trypsin (Gibco BRL). Dissociated cells were separated by fluorescence-activated cell sorting (FACS) using a Vantage machine (Becton Dickinson, Franklin Lakes, NJ). Data analysis was performed by using CellQuest Acquisition software (Becton Dickinson).

Whole-mount X-Gal staining

ES cell aggregates were fixed in solution containing 2% formaldehyde, 0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl₂ and 0.02% NP-40 for 10 minutes at room temperature. After washing twice with PBS containing 0.1% Tween-20 (PBST), the samples were stained in 5-bromo-4-chloro-3-indolyl-D-galactoside (X-Gal) mix [5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂, 10% dimethyl sulfoxide, 0.01% sodium deoxycholate, 0.02% NP-40, 1 mg ml⁻¹ X-gal] for 8-12 hours at 37°C and then rinsed in PBST. Post-stain fix was performed in 3.7% formaldehyde at 4°C overnight and followed by paraffin embedding. Sections (7 µm) were counterstained with Eosin Y.

Results

To monitor visceral endoderm differentiation during ES-cell differentiation, we previously constructed a vector that allows GFP production under the control of the *Afp* promoter (-259 to +24, *Afp-GFP*) (Hamazaki and Terada, 2003). In order to avoid the unexpected gene silencing that occurs occasionally with randomly integrated transgenes during ES-cell differentiation, we here knocked-in a single copy of the *Afp-GFP* transgene into the *HPRT* locus of wild-type R1 ES cells (*Afp-GFP* ES cell). The *HPRT* gene locus has been demonstrated to be excluded from the silencing effect of transgenes (Misra et al., 2001). *Afp-GFP* ES cells were maintained as a monolayer on gelatin-coated dishes in the presence of LIF and did not produce GFP as expected (Fig. 1A). Cell aggregation was induced by culturing ES cells in hanging drops in the same ES-maintenance medium with or without LIF. GFP expression was observed in the outer layer of aggregates within 2 days regardless of LIF addition.

Gene expression was then examined in embryoid bodies for genes related to the undifferentiated phenotype of ES cells and differentiation into various lineages (Fig. 1B). Genes indicating visceral endoderm differentiation, such as hepatocyte nuclear factor (HNF) families and *transthyretin* were upregulated even in the presence of LIF, consistent with the results shown in Fig. 1A. Differentiation into other lineages such as primitive ectoderm and mesoderm was also examined by expression of *FGF5* and *Wnt3*, respectively (Haub and Goldfarb, 1991; Liu et al., 1999). In contrast to endoderm markers, *FGF5* and *Wnt3* expression was induced in embryoid bodies only when LIF was withdrawn. *Rex1*, *BMP4* and *Nanog*, which are expressed in

undifferentiated ES cells, were rapidly downregulated upon withdrawal of LIF but sustained in the presence of LIF.

LIF is known to activate both the JAK/STAT and Ras/mitogen-activated-protein-kinase (MAPK) signal-transduction pathways in ES cells (Niwa et al., 1998; Ernst et al., 1996). Activation of STAT3 alone was shown to be sufficient to maintain pluripotency in the monolayer culture of ES cells (Matsuda et al., 1999). Ras/MAPK signal was proposed to induce differentiation rather than self-renewal of ES cells (Burdon et al., 1999). Because Ras/MAPK activation through LIF might affect endoderm differentiation in the context of cell aggregates, we tested whether cell-aggregation-induced

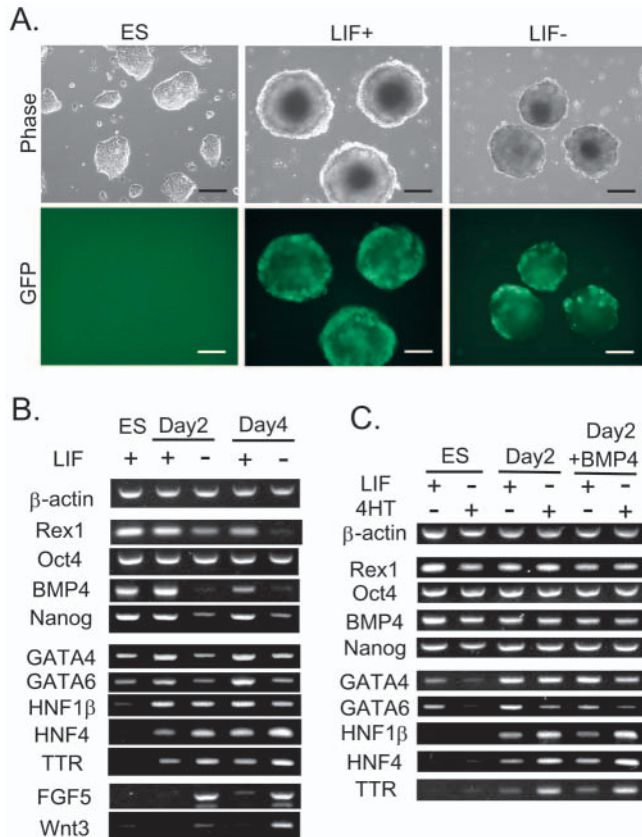


Fig. 1. (A) Visualization of visceral endoderm differentiation in ES cell aggregates by using GFP expression under the control of the promoter of the *Afp* gene (which encodes α -fetoprotein). GFP expression was not detected when *Afp-GFP* ES cells were cultured as monolayer in the presence of LIF (ES, left). GFP-positive cells appeared on the outer layer of aggregates within 4 days regardless of the presence of LIF in the medium (LIF- or LIF+, middle and right). (top) Phase-contrast image under a light microscope; (bottom) pictures under a fluorescence microscope. Bars, 200 μ m. (B) Expression of ES-cell-specific genes and differentiation markers upon ES-cell aggregation. Total RNA was isolated from undifferentiated ES cells (ES) and ES-cell aggregates (Day2, Day4). RNA was subjected to RT-PCR analysis and the PCR products were separated on a 2% agarose gel and visualized by ethidium-bromide staining. *TTR*, *transthyretin*. (C) The LIF/STAT3 pathway and BMP4 stimulation did not inhibit visceral endoderm differentiation upon ES-cell aggregation. Total RNA was isolated from *STAT3ER* ES cells, which were maintained as monolayer cells in the medium containing either LIF or 4HT (ES) and the cells, which were cultured as aggregates for 2 days in the same medium with or without BMP4 as indicated.

endoderm differentiation was indeed independent of the STAT3 activity. To activate STAT3 directly, we used ES cells containing a vector expressing a fusion protein of STAT3 and the ligand-binding domain of the estrogen receptor (*STAT3ER* ES cells) (Matsuda et al., 1999; Oka et al., 2002). In these cells,

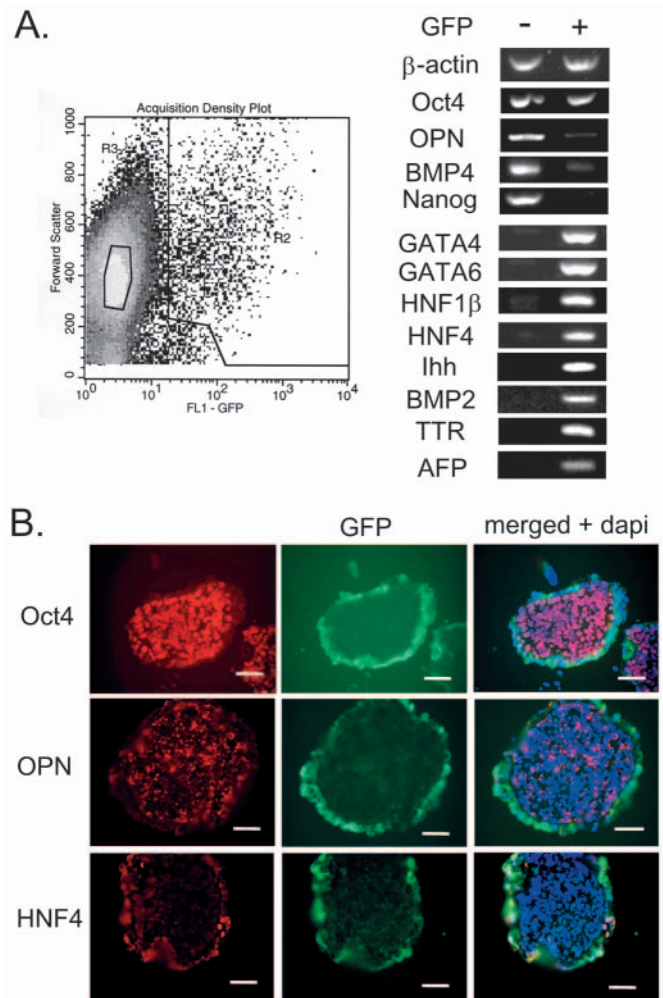


Fig. 2. (A) Separation of GFP-positive visceral endoderm cells from ES-cell aggregates. *Afp-GFP* ES cells were aggregated for 4 days in the presence of LIF and subjected to FACS. The cells in R2 region were collected as GFP-positive cells and those in R3 as GFP-negative cells. The y axis indicates forwards scatter and the x axis indicates GFP expression (left). Total RNA was isolated from both GFP-positive and GFP-negative cells, and the RNA was subjected to RT-PCR analysis. PCR products were separated on a 2% agarose gel and visualized by ethidium-bromide staining (right). *OPN*, *osteopontin*; *Ihh*, *Indian hedgehog*; *TTR*, *transthyretin*; *AFP*, α -*fetoprotein*. (B) Oct4 and osteopontin (OPN) expression were downregulated in the outer layer of ES-cell aggregates. After 4 days of aggregation of *Afp-GFP* ES cells in the presence of LIF, the aggregates were fixed with 3.7% formaldehyde and subjected to frozen section. The sections were incubated initially with anti-Oct4 antibody (1:100 dilution), anti-osteopontin antibody (1:500 dilution) or anti-HNF4 α antibody (1:100 dilution), and subsequently with fluorescein-isothiocyanate-conjugated anti-mouse-IgG (1:200 dilution; for Oct4) or anti-goat-IgG (1:200 dilution; for osteopontin and HNF4 α) (left, red). Endogenous GFP expression derived from *Afp-GFP* ES cells was detected in the corresponding section (middle, green). Cells were co-stained with 4,6-diamidino-2-phenylindole to demonstrate nuclei (right, blue). Bars, 50 μ m.

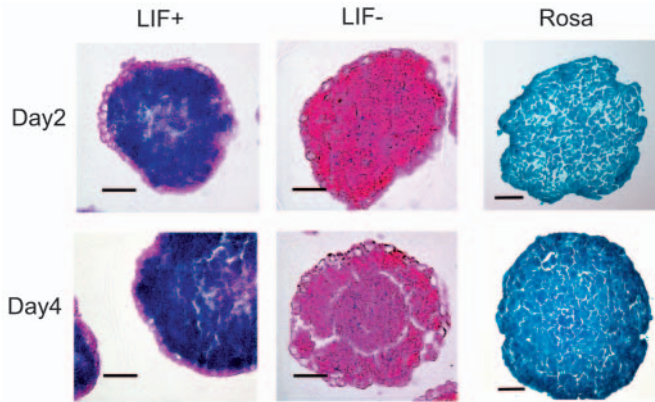


Fig. 3. Downregulation of *Nanog* expression in ES cells upon aggregation. Whole-mount X-Gal staining of *Nanog* β geo ES cells that were aggregated for the indicated number of days in the presence of LIF (LIF+) or the absence of LIF (LIF-). Rosa26 ES cells were stained as a positive control for whole-mount X-gal staining (Rosa). The paraffin sections were counterstained with eosin Y (pink). Bars, 50 μ m.

a synthetic ligand of estrogen, 4-hydroxytamoxifen (4HT) is able to maintain the *STAT3ER* ES cells in an undifferentiated state in the absence of LIF. By using these ES cells, we examined visceral endoderm gene expression upon aggregation in the presence of 4HT (Fig. 1C). Even when *STAT3* was continuously activated by 4HT, the differentiation toward endoderm proceeded upon aggregation.

BMP4 was recently reported to be a cofactor that is required to suppress differentiation of ES cells in the presence of LIF and that can substitute for the requirement for fetal calf serum for ES-cell maintenance (Ying et al., 2003). We tested the effect of additional BMP4 on visceral endoderm differentiation using *STAT3ER* ES cells. As shown in Fig. 1C, supplementation with BMP4 did not prevent ES-cell differentiation into visceral endoderm. In summary, the soluble factors, which have been known to be essential factors for maintaining ES cells, LIF/STAT3 and BMP4, failed to block initiation of visceral endoderm differentiation upon aggregation.

To define further the molecular events occurring in embryoid bodies, particularly in the presence of LIF, we sorted the outer endoderm layer cells and other inner cells by taking advantage of *Afp-GFP* expression (Fig. 2A). The cell aggregates were dissociated using trypsin and subjected to FACS. Based on the forward-scatter and side-scatter analysis, the GFP-negative cells were shown to be relatively homogeneous compared with the GFP-positive cells, which exhibited larger cells and coarser nuclear configuration. RT-PCR analysis for those isolated cell populations demonstrated that visceral endoderm marker genes including *GATA4/6*, HNF families, *transferrin* and *Afp* were exclusively expressed in the GFP-positive population. Furthermore, we could also detect gene expression of *BMP2* and *Ihh*, which are known to be secreted from visceral endoderm and are crucial for mesoderm differentiation of epiblasts (Dyer et al., 2001; Maye et al., 2000). ES-specific genes including *Nanog* (with exception of *Oct-4*) were downregulated in this GFP-positive population. Although *Oct-4* gene expression was detectable by RT-PCR, the downstream target gene of *Oct-4* (which encodes osteopontin) was

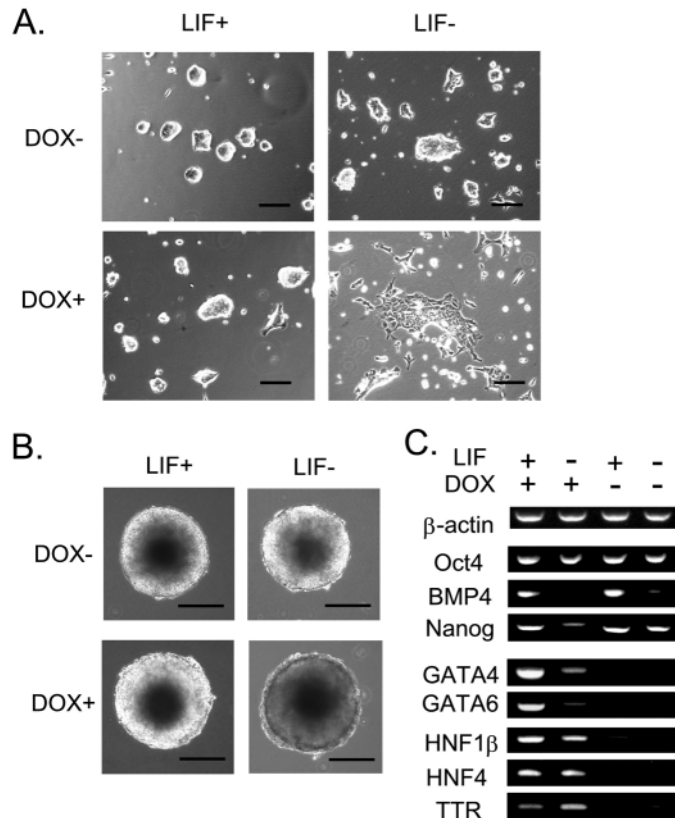


Fig. 4. (A) *TRE-Nanog* ES cells were plated on gelatin-coated plate and cultivated for 3 days in the presence or absence of LIF and doxycycline (DOX, 1 μ g ml⁻¹). In the absence of DOX, *Nanog* transgene was overexpressed in the cells (tet-off system). *Nanog*-overexpressed ES cells maintained undifferentiated morphology regardless of the presence of LIF. Bars, 500 μ m. (B) Morphology of *TRE-Nanog* ES-cell aggregates. *TRE-Nanog* ES cells were subjected to aggregation culture in the presence or absence of LIF and DOX. The phase-contrast images were captured under a light microscope after 4 days of aggregation. Bars, 200 μ m. (C) Overexpression of *Nanog* inhibited visceral endoderm gene expression in the aggregates. Isolated total RNA from each condition in B was subjected to RT-PCR analysis. PCR products were separated on a 2% agarose gel and visualized by ethidium-bromide staining.

downregulated in the GFP-positive cells. Therefore, we further investigated protein expression of Oct-4 by immunofluorescence staining and revealed that Oct-4 expression was remarkably reduced in the outer layer of aggregates, implying post-transcriptional suppression (Fig. 2B) (Xu et al., 2004). By contrast, HNF4 was detected primarily in the GFP-positive outer layer of the aggregates.

Among several essential molecules for maintaining pluripotency of ES cells, we were particularly interested in *Nanog* as a potential negative regulator of primitive endoderm differentiation. The gene-targeting study revealed that *Nanog*-deficient blastocysts failed to develop inner cell mass and predominantly differentiated into extraembryonic endoderm lineage (Chambers et al., 2003; Mitsui et al., 2003). Because the isolated GFP-positive endoderm cells demonstrated significant reduction of *Nanog* expression, we further confirmed that downregulation of *Nanog* in the outer layer cells by using *Nanog* heterozygous-null ES cells, in which *Nanog*

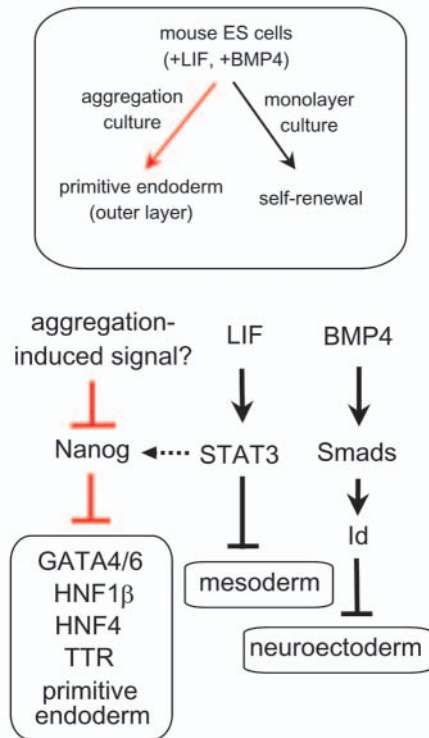


Fig. 5. Primitive endoderm specification of mouse ES cells can be induced solely by cellular aggregation regardless of the presence of external factors (LIF and BMP4), which are sufficient to maintain self-renewal of ES cells in monolayer culture (top). ES-cell aggregation induces repression of the *Nanog* gene in the outer layer independent of the LIF/STAT3 and BMP4/Smads pathways (Cavaleri and Schöler, 2003; Ying et al., 2003). The repression of *Nanog* subsequently leads to ES cells differentiating into the primitive endoderm (bottom).

homeodomain locus was targeted by IRES β -geo (internal ribosomal entry site, β -galactosidase/neomycin-resistance gene) cassette (Fig. 3) (Mitsui et al., 2003). In these ES cells, β -galactosidase expression represents endogenous *Nanog* expression. Consistent with the FACS data above, *Nanog* expression was downregulated only in the outer layer of the aggregates in the presence of LIF within 2 days. X-Gal staining was sustained in the inner mass of aggregates over 4 days. By contrast, X-gal staining eventually disappeared in whole aggregates within 2 days in the absence of LIF.

We then examined whether induced overexpression of *Nanog* could inhibit the visceral endoderm differentiation upon ES-cell aggregation. We constructed a vector in which *Nanog* expression was driven by tetracycline-responsive promoter and introduced this into tetracycline-regulated ES cells (tet-off system) (Era and Witte, 2000). We cloned a stable ES-cell line (*TRE-Nanog* ES cells) that could be maintained in an undifferentiated state in the absence of doxycycline and differentiate in the presence of doxycycline (Fig. 4A). By using the *TRE-Nanog* ES cells, we examined visceral endoderm differentiation upon aggregation in the presence of LIF. Interestingly, the aggregates remained as smooth spheres lacking a distinguished outer layer when *Nanog* was constitutively expressed. There were no obvious changes in cell growth or apoptosis in these aggregates (Fig. 4B). When

gene expression was examined in these aggregates, overexpression of *Nanog* (absence of doxycycline) completely inhibited the production of endoderm markers such as GATA4/6, HNF families and transthyretin (Fig. 4C). *Nanog* overexpression inhibited the differentiation even without LIF. This result demonstrated that *Nanog* downregulation was indispensable for visceral endoderm differentiation of ES cells upon aggregation.

Discussion

In accordance with previous reports (Murray and Edgar, 2001; Shen and Leder, 1992), we have demonstrated that primitive endoderm specification occurs in the outer layer of ES-cell aggregates regardless of the presence of LIF (Fig. 5). Furthermore, continuous activation of STAT3, which is a crucial downstream mediator of LIF signaling, could not prevent primitive endoderm formation upon aggregation. By contrast, expression of primitive ectoderm and mesoderm markers were induced in ES-cell aggregates only when LIF was withdrawn (Fig. 1), suggesting that the LIF/STAT3 pathway probably plays a pivotal role in preventing ES cells from differentiating into these lineages.

BMP4 has recently been identified as another essential factor for ES-cell self-renewal in monolayer culture (Ying et al., 2003). It works as a cofactor to suppress the differentiation of ES cells, especially into neuroectoderm lineage, by activating the Smads/Id pathway in the presence of LIF. In aggregation culture, the addition of BMP4 to the culture medium did not alter the differentiation preference of ES cells towards the primitive endoderm, regardless of the presence of LIF. Thus, even the combination of both LIF and BMP4 was insufficient to inhibit primitive-endoderm differentiation upon ES-cell aggregation. It should be noted here that BMP4 is known rather to induce visceral endoderm differentiation in the absence of LIF (Cocouvanis and Martin, 1999; Turksen and Troy, 2001).

We further investigated here how *Nanog* was involved in primitive-endoderm specification upon aggregation. The present study clearly demonstrated that cell aggregation by itself induced *Nanog* repression at the outer layer. Furthermore, by using the inducible *Nanog* overexpression system, we proved that this *Nanog* downregulation was essential for subsequent ES-cell differentiation into primitive endoderm upon aggregation. These cell-aggregation-based changes in vitro should represent the process of cell-lineage specification in the inner cell mass of blastocysts. Importantly, *Nanog* repression occurred solely by cell aggregation, independent of the LIF/STAT3 or BMP4 pathways. The data strongly suggested that there should be a novel regulatory mechanism by which cell aggregation repressed *Nanog* expression (Fig. 5).

Aggregation itself would generate polarity that would separate a single outer layer cells and other inner cells. Obviously, the inner cells stayed as pluripotent stem cells in the presence of LIF/STAT3. Indeed, in Fig. 2, when the inner (GFP-negative) cells were re-aggregated, they again formed the GFP-positive outer layer (data not shown). The outer layer cells, by contrast, will have membrane polarization. E-Cadherin has been specifically implicated in the segregation of extraembryonic cells during preimplantation mouse development, at both trophectoderm segregation in compaction and endoderm segregation in the inner cell mass (Damsky et

al., 1993). Of interest, the outer layer of the embryoid bodies forms a tight junction, whereas the monolayer culture of ES cells express a very low level of claudins (Kubota et al., 2001) (data not shown). Polar distribution of E-cadherin might lead to signals such as activation of Cdc42/Rac and atypical protein kinases C as well as membrane sequestration of β -catenin (Etienne-Manneville and Hall, 2002).

A recent report has also claimed that forced expression of an active Ras mutant in ES cells caused downregulation of *Nanog* expression and led to an endoderm phenotype (Yoshida-Koide et al., 2004). Aggregation-based polarity might activate the Ras pathway, leading to repression of the *Nanog* gene. Of interest, ES cells with a deletion of *Grb2*, an upstream activator of the *Sos1/Ras* pathway, are known to fail to generate primitive endoderm (Cheng et al., 1998). Such possibilities are now under investigation. Cell-fate specification following aggregation is a process repeatedly seen during embryogenesis. The aggregation-based in vitro ES-cell differentiation into primitive endoderm would serve as a useful model to study molecular mechanisms underlying this universal process in development.

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