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P19 Cells Overexpressing Lhx1 Differentiate into the Definitive Endoderm by Recapitulating an Embryonic Developmental Pathway

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ABSTRACT

Background Epiblasts occur at the last pluripotent stage of embryonic development and are important in elucidating how the three germ layers are formed. However, little is known of the molecular mechanisms of their development. We have shown that LIM homeobox 1 (Lhx1) was involved in epiblast development in embryonic stem cells, especially meso- and endodermal differentiation. However, since epiblasts in embryoid bodies spontaneously develop into a further stage, it is difficult to study their development in this system.

Methods Mouse embryonal carcinoma P19 cells which have properties similar to those of epiblasts provided new avenues of investigation into the regulatory mechanism of epiblasts.

Results Overexpression of Lhx1 in P19 cells induced expression of organizer marker genes (Cer1, Gsc) and endoderm marker genes (Gata6, Foxa2, Sox17) but not extra-embryonic endoderm marker genes (Sox7 or Hnf4alpha).

Conclusion This study suggested that Lhx1 overexpression caused P19 cells to differentiate into an endodermal lineage. Thus, P19 cells and their derivatives can be a useful model system to study how the three germ layers are formed.

Key words cell differentiation; epiblast; germ layer; organizer; P19 cells

Early mammalian developmental processes such as organizer formation and primitive streak invagination, occur at epiblasts in blastocysts and lead to differentiation of cells in the three germ layers.[1] These developmental processes are extensively investigated in primitive vertebrate species. Little is known of the molecular mechanisms of epiblast development in mammals, primarily because it is difficult to directly observe or experimentally manipulate these cells that exist in the uterus. Embryonic stem (ES) cells in embryoid bodies (EBs) differentiated in vitro to epiblasts and then further to cells in the three germ layer lineages.[2, 3, 4] This differentiation does not happen in ES cells in a monolayer culture.

Comparison of gene expression profiles between ES cells cultured in EBs and those cultured in a monolayer, leads to identification of LIM homeobox 1 (Lhx1) as one of the key regulators of epiblast development (Hasegawa et al., submitted). Lhx1-null embryos displayed head truncation due to disruption in germ layer morphogenesis.[5] Since Lhx1 is required for the movement of the anterior visceral endoderm (AVE) at the onset of gastrulation, it is one of the gastrula organizers for specifying anterior parts of embryos along with A-P axis.[6, 7]

Mouse embryonal carcinoma (EC) P19 cells were derived from a teratocarcinoma formed by transplantation of E7.5 embryo into the testis.[8, 9] P19 cells exhibit typical flat compact colonies and express pluripotency-related genes such as Oct3/4 and Sox2, but not Rex1. Differentiation of P19 cells can be effectively induced by various drugs at non-toxic concentrations. Retinoic acid and DMSO induce their differentiation to neuronal cells[10] and cardiomyocytes,[11] respectively. Thus P19 cells are considered equivalent to epiblasts in blastocysts, and suitable as an experimental environment for studying Lhx1 function in epiblasts. Here we examined the role of Lhx1 on developmental processes of epiblasts using P19 cells as a model system.

MATERIALS AND METHODS

Cell culture and differentiation

P19 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical, Osaka, Japan), supplemented with 10% fetal bovine serum (FBS; Corning Life Sciences, Tewksbury, MA) and penicillin-streptomycin-L-glutamine solution (PSG;

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Abbreviations: ADE, anterior definitive endoderm; APS, anterior primitive streak; AVE, anterior visceral endoderm; DE, definitive endoderm; DMEM, Dulbecco's modified Eagle's medium; EB, embryoid body; EC, embryonal carcinoma; ES, embryonic stem; ExEn, extraembryonic endoderm; FBS, fetal bovine serum; HA, hemagglutinin; HRP, horseradish peroxidase; IgG, immunoglobulin G; KD, knockdown; Lhx1, LIM homeobox 1; MEF, mouse embryonic fibroblast; NEAA, MEM non-essential amino acid solution; O/E, overexpression; PBS, phosphate-buffered saline; PS, primitive streak; PSG, penicillin-streptomycin-L-glutamine solution; qPCR, quantative PCR; RT, reverse transcription; SBE, Smad binding element; TBS-T, Tris-buffered saline with Tween 20; TRC, the RNAi Consortium

Table 1. Primer sequences in RT-PCR

Gene	Forward 5′-3′	Reverse 5′-3′
GAPDH	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA
Rex1	TTCACGGAGAGCTCGAAACT	CCATCCCCTTCAATAGCTCA
Pecam1	GCTTGGCAGCGAAACACTAA	TGCGATGGTGTATAACGTCA
Oct3/4	ATTCTCGAACCTGGCTAAGCT	ATGGTGGTCTGGCTGAACACCTTT
Sox2	GGCAGCTACAGCATGATGCAGGAGC	TGGAGTGGGAGGAAGAGGTA
Nanog	CACCCACCCATGCTAGTCTT	ACCCTCAAACTCCTGGTCCT
Fgf5	CTGTACTGCAGAGTGGGCATCGG	GACTTCTGCGAGGCTGCGACAGG
Otx2	CACTTCGGGTATGGACTTGC	CTCTCCCTTCGCTGTTTCC
Sox7	CTTCAGGGGACAAGAGTTCG	GCTTGCCTTGTTTCTTCCTG
Bmp2	GAGGCGAAGAAAAGCAACAG	GGGGAAGCAGCAACACTAGA
Ttr	TTCACAGCCAACGACTCTGG	AATGCTTCAGGGCATCTTCC
Hnf4alpha	CGAACAGATCCAGTTCATCAAG	ATGTGTTCTTGCATCAGGTGAG

Wako. Stock solution diluted 1:100). AB1 mouse ES cells were cultured on mouse embryonic fibroblasts (MEFs) in ES cell medium comprising DMEM supplemented with 20% FBS and PSG (Stock solution diluted 1:100) and MEM non-essential amino acid solution (NEAA; Wako. Stock solution diluted 1:100) and 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO) and 1000 U/mL ESGRO (Millipore, Milford, MA). MEFs were obtained from ICR-strain E13.5 fetuses and maintained in DMEM supplemented with 10% FBS and PSG (Stock solution diluted 1:100). MEFs were treated with mitomycin C and seeded as feeders. EB formation in ES cells was achieved by the hanging drop method. After ES cells were adjusted to 2.5×10^4 cells/mL, 20 μL aliquots were added to a 100-mm petri dish. The differentiation medium was comprised of DMEM, supplemented with 20% FBS and PSG (Stock solution diluted 1:100) and NEAA (Stock solution diluted 1:100) and 0.1 mM 2-mercaptoethanol, which was added 2 days later. ES cells were seeded onto 0.1% gelatin-coated dishes at a density of $1 \times 10^2/cm^2$ in the differentiation medium to form a monolayer.

Transfection

The pCAG IRES Puro vector DNA (2 μg) containing Lhx1 or Gata6 cDNA was complexed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and added to adherent P19 cells and ES cells in 24-well plates. The cells were trypsinized and re-seeded into 60-mm culture dishes 3 to 8 h after transfection. Puromycin (1.5 μg/mL) was added 24 h later to select transfected cells.

Luciferase analysis

DNA fragment, including the sequence immediately at 5′ end of the coding region, was PCR-amplified to construct the luciferase assay vector from ES cell gDNA using the following oligonucleotide primers: Cer1: F-5′-TATGGTACCATGGACCTCTGAAACAAGTAAAACATA-3′, R-5′-TATAAGCTTGCTTCCCAGAGACTGAGGTT-3′,[12] The Smad binding element (SBE) sequence was produced by artificial gene synthesis (IDT, Coralville, IA). Each fragment was inserted into pNluc1.3 (Promega, Madison, WI). Cells (1×10^4) were seeded in a 96-well plate and incubated with 0.15 μg reporter plasmid, 0.15 μg effector plasmid, 0.015 μg internal control plasmid pSEAP2-control vector (Clontech, Palo Alto, CA), and FuGENE HD (Promega) to transfect these plasmids as per the manufacturer's protocol. Luciferase assays were performed 24 h later using the Nano-Glo Luciferase Assay System (Promega) and the Great EscAPe SEAP Chemiluminescence Kit 2.0 (Clontech).

RNA preparation, RT-PCR, and RT-qPCR analysis

Total RNA was extracted from the cells using an RNeasy Mini Kit (Qiagen, Valencia, CA) The PrimeScript RT reagent Kit with gDNA Eraser 1 (Takara Bio, Shiga, Japan) was utilized to synthesize the cDNA. The PCR cycling conditions were as follows: one cycle of 94 ˚C for 2 min, 22 to 30 cycles of 98 ˚C for 10 s, 60 ˚C for 30 s and 72 ˚C for 30 s, followed by 4 ˚C. GAPDH was used as the invariant control. The primer sequences are described in Table 1. Reverse transcription (RT)-quantative PCR (qPCR) was performed with the appropriate ABI TaqMan Gene Expression Assays in a 10 μL mixture containing cDNA and specific probes and primers, and the EXPRESS qPCR Supermix with Premixed ROX (Invitrogen). Probes and primers were selected using web-based assay design software (Probefinder: https://qpcr.probefinder.com/organism.jsp). The PCR cycling conditions were as follows: one cycle of 95 ˚C for 20 s, 45 cycles of 95 ˚C for 1 s and 60 ˚C for 20 s. The results were expressed as the ratio between the gene of interest and the beta-actin reference gene. The primer sequences are described in Table 2.

Table 2. Primer sequences in RT-qPCR

Gene	Forward 5′-3′	Reverse 5′-3′
Beta-actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
Gsc	GAGACGAAGTACCCAGACGTG	GCGGTTCTTAAACCAGACCTC
Cer1	GACTGTGCCCTTCAACCAG	AGCAGTGGGAGCAGAAGC
Gata6	GGTCTCTACAGCAAGATGAATGG	TGGCACAGGACAGTCCAAG
Foxa2	AAATAATGTAAGAGTCTGGTGTACCG	CCATGTCCAGAATGGGATGT
Sox17	CACAACGCAGAGCTAAGCAA	CGCTTCTCTGCCAAGGTC
Fgf5	AAAACCTGGTGCACCCTAGA	CATCACATTCCCGAATTAAGC
Otx2	AAATCAACTTGCCAGAATCCA	GGCCTCACTTTGTTCTGACC
Gata4	GGAAGACACCCCAATCTCG	CATGGCCCCACAATTGAC
Lhx1	CAGGAGACTGGCCTCAACAT	GTTTCATCCTTCGCTCCTTG

Immunofluorescence

The cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Furthermore, they were rinsed in phosphate-buffered saline [PBS (–), Wako] and incubated with 0.1% Triton X-100 in PBS for 15 min, then blocked in 5% skim milk in Tris-buffered saline with Tween 20 (TBS-T) for 2 h. Primary antibody was applied using 0.1% bovine serum albumin in TBS-T overnight at 4 °C. The cells were washed and secondary antibody was applied for 1 h at room temperature, followed by counterstaining with DAPI (Molecular Probes, Sunnyvale, CA). The primary antibodies used were anti-Lhx1 (Millipore), anti-hemagglutinin (HA) (Wako), anti-Oct3/4 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Pecam1 (anti-CD31; BD Biosciences, San Diego, CA). The secondary antibodies used were Alexa Fluor 546 anti-mouse immunoglobulin G (IgG), 488 anti-rabbit IgG and 568 anti-rat IgG (Molecular Probes).

Western blotting

Cells were lysed in sample buffer (62.5 mM Tris-HCl, 2% sodium dodecyl sulfate, 25% glycerol, 0.01% bromophenol blue) and boiled for 10 min at 95 °C. The samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a membrane (Millipore). The membranes were blocked with 5% skim milk for 2 h and then incubated with primary antibody for 1 h at room temperature. After washing the membranes, the blots were incubated with horseradish peroxidase (HRP)-coupled secondary antibody and developed using ECL-Plus regents (Thermo Scientific, Rockford, Il). The primary antibodies used were anti-HA (Wako), anti-actin (CP01; Calbiochem, San Diego, CA). The secondary antibody was the ECL Mouse IgG HRP-linked whole antibody (GE Healthcare, Piscataway, NJ). Western blotting for Nodal-Smad signaling was conducted with the Phospho-Smad Antibody Sampler Kit (Cell Signaling Technology, Danvers, MA).

Lentiviral transfection to generate P19 Lhx1 knockdown cells

We used the RNAi consortium (TRC) Lentiviral Mouse Lhx1 shRNA (TRCN0000070525 and TRCN0000070527; GE Healthcare) and the TRC Lentiviral pLKO.1 Empty Vector Control (GE Healthcare). The lentivirus was produced in HEK293T cells using the Trans-Lentiviral shRNA Packaging Kit with the Calcium Phosphate Transfection Regent (Thermo Scientific). The cell culture medium containing the virus particles was collected and filtered through a 0.45 μm filter 72 h after transfection. The viral particles were further concentrated using PEG-it Virus Precipitation Solution (SBI, Mountain View, CA). The pellets were resuspended in DMEM containing 25 mM HEPES. P19 cells were seeded at a density of 5.0×10^4 to 1.0×10^5 cells/well in a 24-well plate and infected with lentivirus to express either Empty or Lhx1 shRNA. The cells were trypsinized 24 h after infection and re-seeded into 100-mm culture dishes. Puromycin (1.5 μg/mL) was added to select the infected cells 72 h after infection.

Alkaline phosphatase staining

The cells were washed with PBS twice, and 3.6% formaldehyde was added for 15 min at room temperature. The cells were washed in PBS twice again and incubated with PBS and 1.5 M Tris-HCl and naphthol AS-BI phosphate (Sigma) and Fast Red Violet LB salt (Sigma) for 5 to 10 min at room temperature. After incubation, the cells were washed with PBS.

RESULTS

P19 cells express low levels of Lhx1 and have epiblastic properties

P19 cells showed the flat compact colonies (Fig. 1A). P19 cells expressed pluripotent cell markers Oct3/4 and Sox2 and Nanog like ES cells, but not ICM markers Rex1 and Pecam1. Importantly, they expressed high levels of an epiblast marker Fgf5 (Fig. 1B). Immunostaining showed

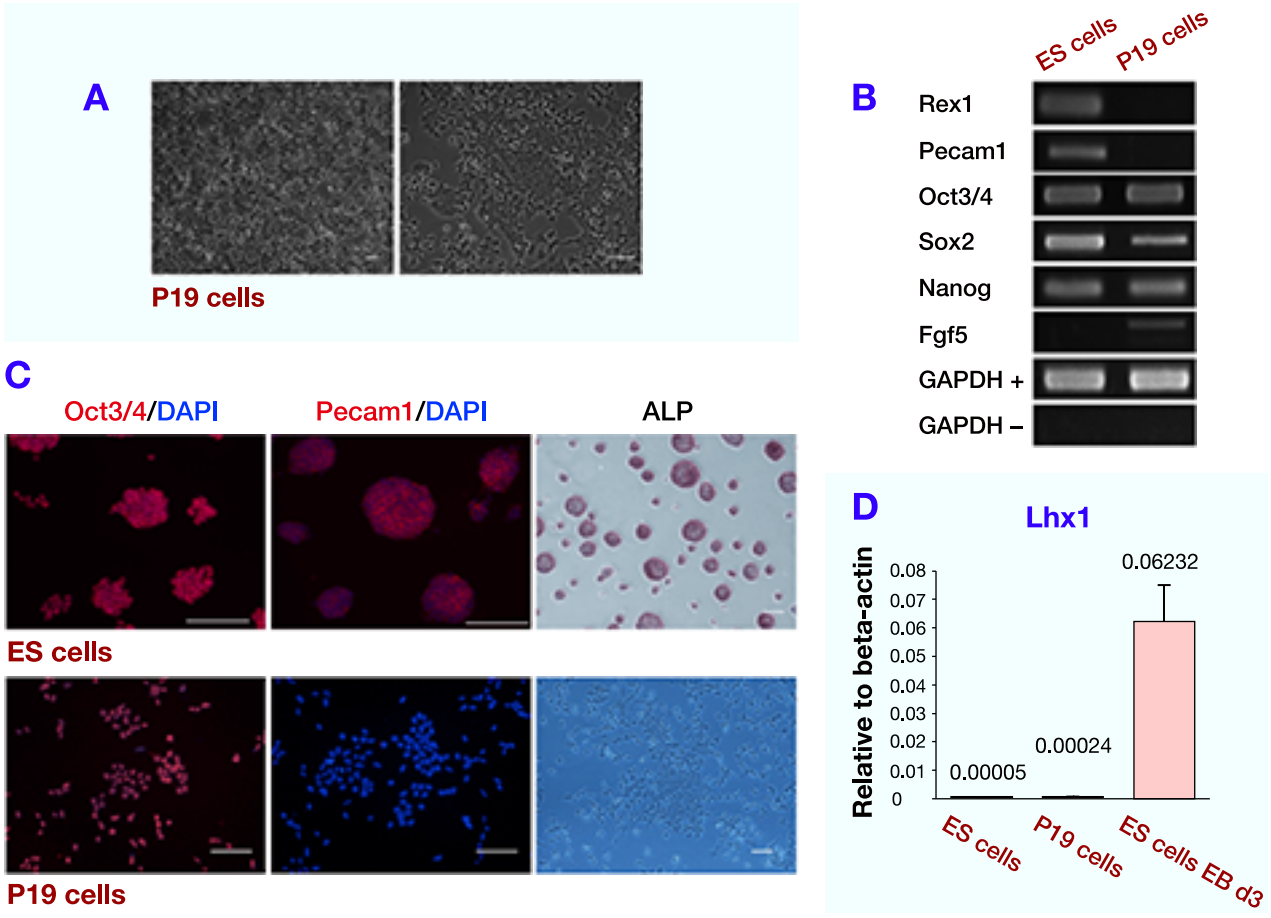

Fig. 1. The characteristics of embryonal carcinoma P19 cells.
A: Typical P19 cells morphology. Bar = 100 µm.
B: RT-PCR to determine the properties of P19 cells.
C: Immunofluorescence and alkaline phosphatase staining of ES cells and P19 cells. Bar = 100 µm.
D: Lhx1 mRNA expression in ES cells and the EBs on day 3 and P19 cells was evaluated by RT-qPCR. Relative to beta-actin.
ALP, alkaline phosphatase staining; EB, embryoid body; qPCR, quantitative PCR; RT, reverse transcription.

uniform expression of Oct3/4 but no expression of Pecam1, and a much lower level of alkaline phosphatase than in ES cells (Fig. 1C). P19 cells expressed much less Lhx1 than EBs on day 3 (as a positive control), whereas undifferentiated ES cells did not express Lhx1 (Fig. 1D). Taken together, these results reveal that P19 cells possess characteristics similar to those of epiblasts and slightly express Lhx1.

Lhx1 induces expression of organizer and endoderm marker genes

To investigate the effect of Lhx1 during epiblast cell differentiation, P19 cell lines overexpressing Lhx1 were established in which Lhx1 was tagged with the influenza HA epitope at the C-terminus. After selection with puromycin, more than thirty colonies morphologically different from wild-type were picked up. The highest level of exogenous Lhx1 expression was detected in cell lines #4 and #5 (Fig. 2A). Therefore, this #5 line [Lhx1 overexpression (O/E) P19 cells] was used in the current study. After several passages with drug selection, Lhx1 O/E P19 cells maintained high and uniform expression of exogenous Lhx1 (Fig. 2B).

Lhx1 O/E P19 cells showed increased expression of organizer markers Cer1 and Gsc (Fig. 2Cb), and persistent expression of Oct3/4 and Nanog (data not shown). The gastrula organizer is divided into some centers: the anterior primitive streak (APS) and the node and the AVE. Since these structures are barely distinguishable from each other at the molecular level, we could not determine which one was induced from Lhx1 O/E P19 cells. Interestingly, the epiblast marker Fgf5 was upregulated by Lhx1 approximately 10-fold, whereas another epiblast marker Otx2 was downregulated (Fig. 2Ca). It is notable that Cer1 mRNA expression increased 50-fold in Lhx1 O/E P19 cells. To verify whether this induction of Cer1 was due to transcriptional activation by Lhx1, we measured Cer1 promoter activity by reporter assay. Cer1

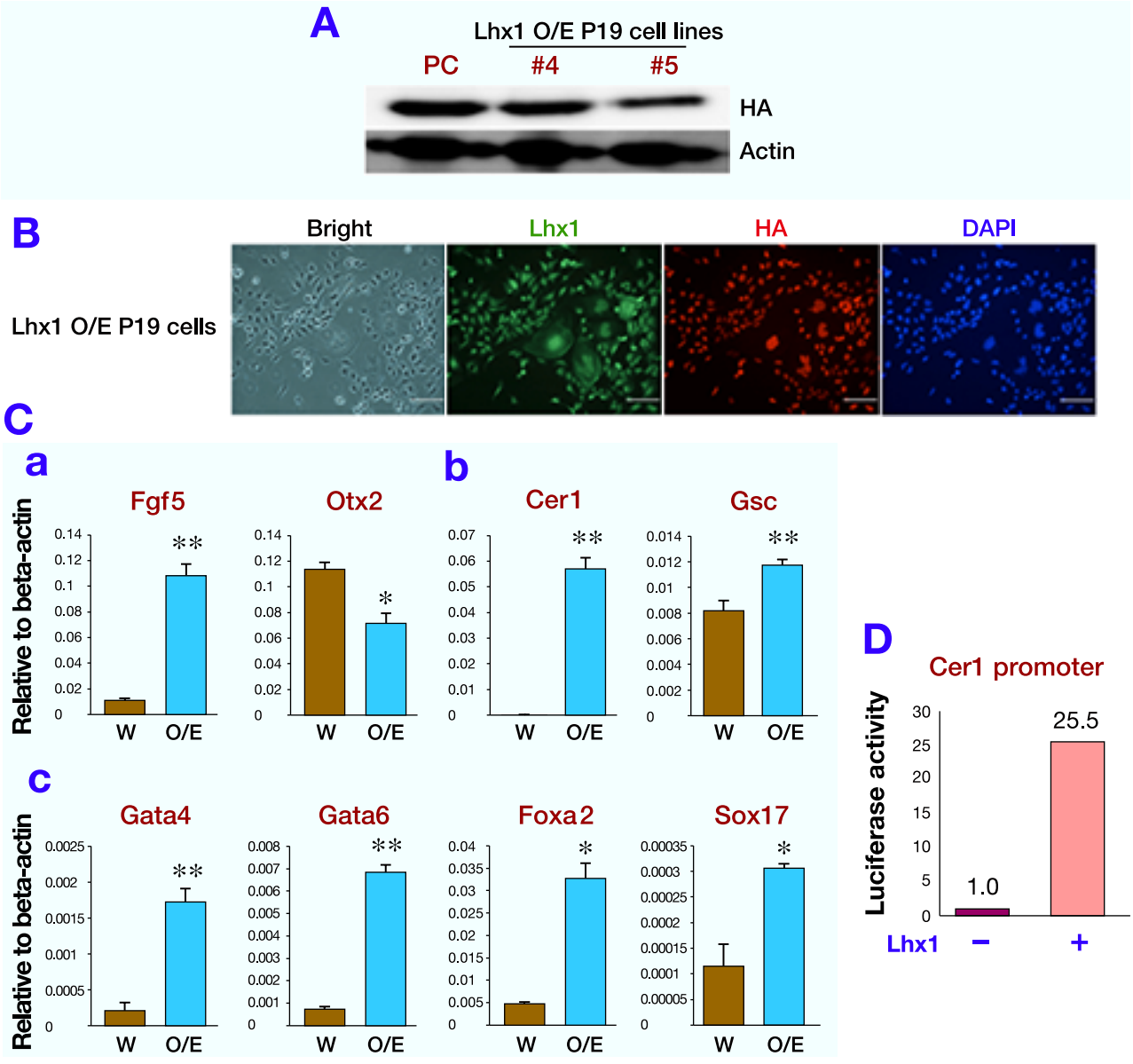

Fig. 2. The establishment of P19 cell lines overexpressing Lhx1.
A: Western blotting of P19 cell lines overexpressing Lhx1 #4 and #5.
B: Immunofluorescence staining of Lhx1 O/E P19 cells. Bar = 100 µm.
C: Gene expression levels determined by RT-qPCR are indicated. (**a**) epiblast marker, (**b**) organizer marker, (**c**) endoderm marker. $*P < 0.05$, $**P < 0.01$, versus W. Relative to beta-actin.
O/E, overexpression (Lhx1 O/E P19 cells); PC, positive control; W, wild type P19 cells.

promoter activity was increased 20-fold in Lhx1 O/E P19 cells (Fig. 2D), suggesting that organizer induction was a direct consequence of Lhx1 O/E.

The morphology of Lhx1 O/E P19 cells in normal culture condition medium was similar to that of endoderm-like cells (Fig. 2B). This morphological change in Lhx1 O/E P19 cells to endodermal cells was confirmed by RT-qPCR as they expressed pan-endodermal markers Gata4 and Gata6 and Foxa2 (Fig. 2Cc). Taken together, Lhx1 O/E lead P19 cells to differentiate into endodermal cells.

Lhx1 activates Nodal signaling to induced differentiation of P19 cells into the definitive endoderm

The morphological change of Lhx1 O/E P19 cells was investigated in more detail. Early embryonic endodermal cells are divided into two categories: definitive endoderm (DE) and extraembryonic endoderm (ExEn). Marker expression was examined by RT-PCR to determine which endodermal lineage was induced by Lhx1. The primitive endodermal marker Sox7 and visceral endoderm markers Bmp2 and Ttr and Hnf4alpha were

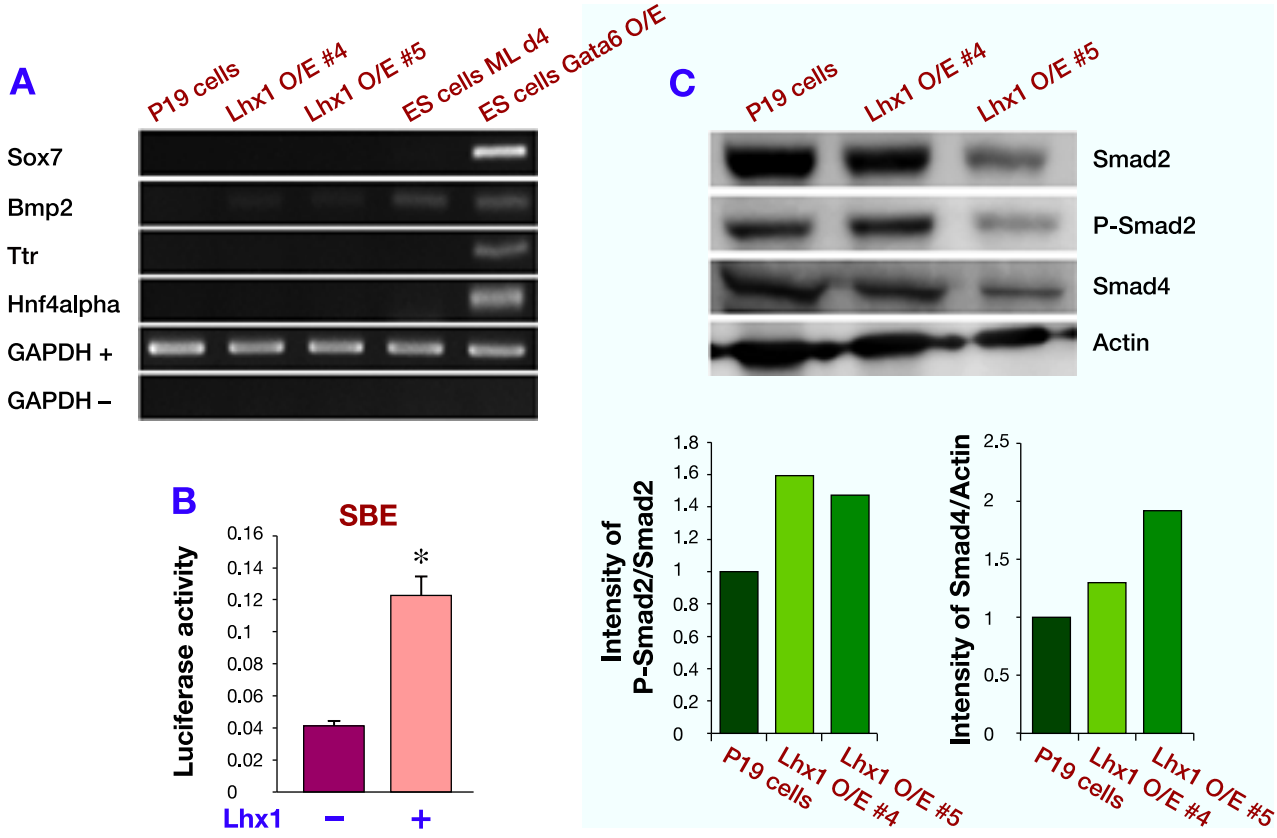

Fig. 3. The investigation of definitive endoderm via Nodal signaling in P19 cell lines overexpressing Lhx1.

A: RT-PCR analysis of extra-embryonic endoderm marker genes. ES cells ML day 4 are shown as a positive control of early primitive endoderm. Gata6 O/E ES cells are a strong positive control of extra-embryonic endoderm.

B: SBE activity levels determined by the luciferase assay. *$P < 0.05$, versus Lhx1 (–).

C: Western blotting of Smad family in Lhx1 overexpression P19 cell lines (upper panel). Lower panels show P-Smad2/Smad2 (left) and Smad4/Actin (right) staining intensity.

Lhx1 O/E #4, Lhx1 overexpression P19 cell line #4; Lhx1 O/E #5, Lhx1 overexpression P19 cell line #5; ML, monolayer; SBE, smad binding element.

not detected in Lhx1 O/E P19 cells (Fig. 3A). The lack of ExEn marker expression indicated that Lhx1 induced DE differentiation. This gene expression pattern was consistent with the results of RT-qPCR, which demonstrated increased expression of DE markers Cer1 and Gsc and Sox17 in Lhx1 O/E P19 cells (Fig. 2Cb and Cc). Since DE differentiation is triggered by Nodal signaling, we hypothesized that Lhx1 facilitated endodermal differentiation by activating Nodal signaling. This signaling pathway is activated by binding of Smad transcriptional activator to SBE. Luciferase reporter assay in P19 cells showed transcriptional activation through SBE 24 h after Lhx1 O/E (Fig. 3B). However, only a slight increase in Smad2 phosphorylation and Smad4 was observed in Lhx1 O/E P19 cells (Fig. 3C), which may reflect induction of Foxh1 expression in these cells (data not shown). These results suggest that Lhx1 activates Nodal signaling to induce differentiation of P19 cells into DE.

Lhx1 is not required for the maintenance of P19 cells

Two types of Lhx1 shRNA (#25, #27) were introduced into P19 cells to determine whether Lhx1 was necessary for maintenance and differentiation of P19 cells. Two Lhx1 knockdown (KD) P19 cell lines KD-#25 and KD-#27 showed reduced expression levels of Lhx1, respectively (Fig. 4A). No substantial changes in morphology or self-renewing activity were detected in the two KD P19 cell lines compared to those in P19 cells (data not shown). The KD-#25 and KD-#27 P19 cells exhibited gene expression profiles similar to that of wild-type cells (Fig. 4B). These KD cells did not differentiate spontaneously in normal culture media. These results suggest that Lhx1 is not involved in the maintenance of P19 cells, which is consistent with the low expression level of Lhx1 in these cells.

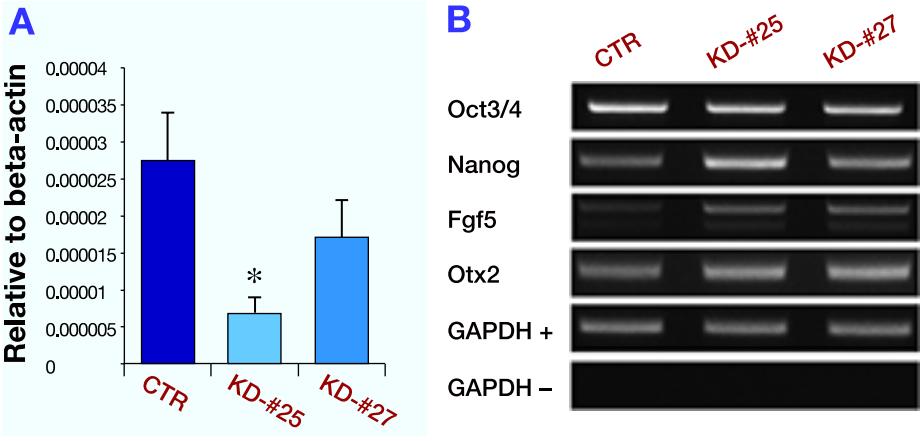

Fig. 4. The establishment of Lhx1 knockdown P19 cell lines.
A: Lhx1 expression levels (relative to beta-actin), as determined by RT-qPCR, are shown for the Lhx1 KD P19 cell lines. *$P < 0.05$ versus CTR.
B: RT-PCR of pluripotent and epiblast marker genes in the Lhx1 KD P19 cell lines.
CTR, control (P19 cells infected Empty vector); KD, knockdown.

DISCUSSION

Here we showed that Lhx1 O/E induced differentiation of P19 cells into DE. DE arises at gastrulation from epiblasts in the APS, which later gives rise to a specialized structure called the node, the organizer for posterior parts of embryos. DE specification is regulated by Nodal signaling and induction of the Sox17 transcriptional factor in primitive streak (PS) stage embryos. Lhx1 O/E P19 cells expressed Sox17 and their Nodal signaling pathway was activated with pan-endodermal markers Gata4 and Gata6 and Foxa2. Together with DE marker expression, the expression of the organizer related genes Cer1 and Gsc remained detectable in Lhx1 O/E P19 cells. Thus the differentiation of DE in Lhx1 O/E P19 cells recapitulated an embryonic developmental pathway for DE formation.

Genes uniquely upregulated in Lhx1 O/E P19 cells included anterior endodermal markers such as Cer1 and Gsc and Foxa2 and Sox17. Lhx1–/– cells in the endodermal layer of the null-mutant embryo did not express Sox17 and Foxa2 and Shh and Cer1 appropriately.[13] These results suggested that anterior endodermal marker genes could be the downstream targets of Lhx1 as indicated by our Cer1 promoter assay. There are two distinct endodermal lineages, AVE and anterior definitive endoderm (ADE) in the distal parts of PS stage embryos.[14,15] AVE is extra-embryonic tissue, but has quite a unique capacity as a gastrula organizer for specifying anterior parts of embryos including the head region. AVE showed a gene expression profile similar to ADE as well as the node. Therefore Lhx1 O/E P19 cells could contain AVE in addition to DE. Basically, the identity of anterior parts of cells was defined based on the allocation in an embryo at a specified stage and showed very similar expression profiles to marker genes. Mouse Lhx1 is expressed in both the AVE and in primitive streak-derived tissues including the node and the mesodermal wings and the anterior mesendoderm. Considering their morphology and marker expression in Lhx1 O/E P19 cells, the various Lhx1 expressing cells at anterior parts of PS stage embryos were possible candidates as induced tissue by Lhx1 O/E. At present, we could not specify the whole signature of Lhx1 O/E P19 cells.

Lhx1 deficiency leads to abnormal morphogenesis of the visceral endoderm and primitive streak and nascent mesoderm and the mesendoderm derivatives of the gastrula organizer in the gastrula embryo, which result in head truncation. Amot acts downstream to Lhx1, and a loss of Amot activity impairs the movement of AVE from the anterior region of embryo to the extraembryonic sites.[16] This disruption impeded anterior migration of DE in Lhx1-deficient mice. Thus Lhx1 is indirectly implicated in the production of definitive endoderm and a primary function of Lhx1 in early embryos is to enable proper cell movement during gastrulation. In this study, most Lhx1 O/E P19 cells showed endodermal morphology with DE and/or AVE marker expression such as Cer1 and Foxa2 and Gsc and Sox17, suggesting that Lhx1 O/E caused P19 cells to differentiate directly into DE and/or AVE from mesendodermal cells like APS. In addition to the regulation of morphogenetic movement and cell behavior in gastrulation, Lhx1 could regulate the fate of epiblasts or anterior primitive streak.

In accordance with very low expression of Lhx1 in P19 cells, Lhx1 KD did not affect the state of P19 cells, indicating that Lhx1 was not involved in the pluripotency of epiblasts. However, Lhx1 overexpression induced Fgf5 expression in P19 cells. Although Fgf5 is a marker for epiblasts,[17] neither pluripotent stem cell markers like Oct3/4, Sox2 and Nanog nor the population of stem like cells was increased. Therefore increased expression of Fgf5 was not directly involved in the maintenance or propagation of epiblasts. Recently ADE cells were generated and isolated from differentiating ES cell cultures.[18] The production of ADE in culture required FGF signaling in addition to activin and BMP signaling.

Although Fgf5 does not promote the inductive processes of ADE, the active induction of Fgf5 in Lhx1 O/E P19 cells might suggest an unknown function of Fgf5 for ADE and/or AVE formation.

Organizers form intricate regulatory networks for the patterning of the anterior-posterior and dorsal-ventral axes in developing gastrula. These processes are regulated by secreted factors from the organizer that induce the TGF-beta pathway and inhibit the BMP and WNT pathway, which is accomplished by secreting proteins such as Nodal related genes and Dkk1 and Noggin/Chordin. Cer1, another marker for the organizer, is an inhibitor of all three signaling pathways. Since Lhx1 O/E P19 cells expressed Cer1 and Dkk1 and Noggin (data not shown) with activated nodal signaling, all essential factors for organizer activities might be provided in Lhx1 O/E P19 cells. Moreover, the fundamental transcriptional factors for organizer function such as Gsc and Foxa2 and Lhx1 itself were all expressed in Lhx1 O/E P19 cells. Taken together, Lhx1 O/E P19 cells might show organizer function in vitro so that Lhx1 O/E P19 cells could be a very good cell source for organizer factors, which were essential for gastrulation, subsequent specification and / or differentiation of organizer tissues and their induced anterior tissues. Since Lhx1 O/E P19 cells could serve as a model for AVE and/or ADE, P19 cells and their Lhx1 expressing derivatives give us a useful model system for studying the underlying mechanisms of head formation and three germ layer cell specification.

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The authors declare no conflict of interest.

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