Gene expression profiling reveals the heterogeneous transcriptional activity of Oct3/4 and its possible interaction with Gli2 in mouse embryonic stem cells

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A B S T R A C T

We examined the transcriptional activity of Oct3/4 (Pou5f1) in mouse embryonic stem cells (mESCs) maintained under standard culture conditions to gain a better understanding of self-renewal in mESCs. First, we built an expression vector in which the Oct3/4 promoter drives the monocistronic transcription of Venus and a puromycin-resistant gene via the foot-and-mouth disease virus self-cleaving peptide T2A. Then, a genetically-engineered mESC line with the stable integration of this vector was isolated and cultured in the presence or absence of puromycin. We identified approximately 4600 probes with statistically significant differential expression. The genes involved in nucleic acid synthesis were overrepresented in the probe set associated with mESCs maintained in the absence of puromycin. In contrast, the genes involved in cell differentiation were overrepresented in the probe set associated with mESCs maintained in the presence of puromycin. Therefore, it is suggested with these data that the transcriptional activity of Oct3/4 fluctuates in mESCs and that Oct3/4 plays an essential role in sustaining the basal transcriptional activities required for cell duplication in populations with equal differentiation potential. Heterogeneity in the transcriptional activity of Oct3/4 was dynamic. Interestingly, we found that genes involved in the hedgehog signaling pathway showed unique expression profiles in mESCs and validated this observation by RT-PCR analysis. The expression of Gli2, Ptch1, and Smo was consistently detected in other types of pluripotent stem cells examined in this study. Furthermore, the Gli2 protein was heterogeneously detected in mESC nuclei by immunofluorescence microscopy and this result correlated with the detection of the Oct3/4 protein. Finally, forced activation of Gli2 in mESCs increased their proliferation rate. Collectively, it is suggested with these results that Gli2 may play a novel role in the self-renewal of pluripotent stem cells.

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1. Introduction

Embryonic stem cells (ESC) are derived from preimplantation embryos and are capable of both long-term proliferation (self-renewal) and differentiation into cell types of all three germ layers (pluripotency). The self-renewal and pluripotency of ESCs are sustained by a combination of essential transcription factors [1] and the extracellular signals that drive the expression of these transcription factors [2]. Recent studies have observed that undifferentiated mouse ESC (mESC) cultures contain multiple cell populations showing fluctuating expression levels of genes associated with cellular pluripotency and cell differentiation [3–18]. Cellular pluripotency and cell differentiation genes are downregulated or expressed in approximately one-tenth of cells in steady state culture (for a review, see [19–22]). For example, when mESCs were sorted into Zscan4-positive and Zscan4-negative subpopulations based on expression levels, the subpopulations were able to regain Zscan4-negative and Zscan4-positive cells, respectively, when they were replated and cultured separately [11]. Interestingly, the constitutive knockdown of Zscan4 significantly decreased telomere length, whereas its constitutive expression increased the levels of telomeric sister chromatid exchange (T-SCE; [11]). Both telomere shortening and increased T-SCE rates lead to acceleration of the replicative senescence [23,24].
Therefore, the heterogeneous expression of Zscan4 is necessary for mESCs to self-renew indefinitely.

In studies with other genes [35,12–16,18], each subpopulation has exhibited unique differentiation potential. For example, Nanog-high mESCs are resistant to differentiation, whereas Nanog-low mESCs are prone to differentiation [3]. Consequently, the presence of subpopulations in mESC cultures typifies the plasticity of early embryonic cells and dynamically sustains their self-renewal and pluripotency. The autorepressive feedback of Nanog [25], extrinsic TGFβ signaling pathways, such as Nodal and BMP [26], and activity of the basic-helix-loop–helix transcription factor Tcf15 [16] are responsible for maintaining heterogeneous Nanog expression in mESC cultures. In addition, the stiffness of the culture dishes [27] and/or the uneven partition of the cytoplasm during cell division [28,29] may contribute to the variable expression of these genes in mESC cultures. However, the underlying molecular mechanism that is responsible for transcriptional heterogeneity in mESCs remains elusive.

In this study, we generated a genetically-engineered mESC line to examine its self-renewal. The mESC line contains an expression cassette with an Oct3/4 (Pou5f1) promoter that drives the monocistronic expression of Venus and a puromycin-resistant gene product. The mESCs were cultured under standard conditions with or without puromycin (puromycin-positive and puromycin-negative cultures, respectively) and subjected to Illumina expression microarray analysis. It is suggested with these data that mESCs exhibit fluctuations in Oct3/4 expression levels and that Oct3/4 plays an essential role in sustaining the basal transcriptional activities required for duplication of cells with equal differentiation potential. Surprisingly, we found that the genes involved in the hedgehog signaling pathway, i.e., Gli2 and Ptc1, showed unique expression profiles in mESCs. It is suggested with our results that Gli2 may play a novel role in the self-renewal of pluripotent stem cells.

2. Materials and methods

2.1. Vector construction

Standard molecular cloning techniques were used to build pOct2VAP in which the Oct3/4 promoter drives the expression of Venus [17,30] and pCAG_Gli2ERP from which the protein coding sequence of Gli2 was expressed as a fusion protein with the human estrogen receptor ERT2 [31,32]. A stepwise description of the vector construction is provided in the Supplementary Materials and methods.

2.2. Cell culture

Mouse embryonic stem cells (mESCs; OGR1 and W4) were cultured under standard conditions, as described previously [27,33,34]. Briefly, mESCs were plated on 0.1% gelatin-coated tissue culture dishes and cultured in Dulbecco’s modified Eagle’s medium (high glucose; Life Technologies, Carlsbad, CA) supplemented with 15% fetal bovine serum (FBS; Life Technologies, and Gemini Bio-Products, West Sacramento, CA), 0.1 mM non-essential amino acids (Life Technologies), 2 mM GlutaMax I (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich) and 1000 U/ml Leukemia inhibitory factor (IIF; EMD Millipore, Billerica, MA). To determine the best serum lot for mESC culture, several different serum lots from a few different companies were tested by plating mESCs at low density under 15 or 30% serum conditions. Although three serum lots contributed to the results presented in this manuscript due to the duration of the study, heterogeneity in the expression of the Oct3/4 reporter was consistently observed. To expand mESCs at 80% confluence, TrypLE Express (Life Technologies) and the same volume of the standard culture medium were sequentially added to the culture and a single cell suspension was prepared. The plating density was 1:5. Under these conditions, it took two days for mESCs to reach 80% confluence after plating. More than 80% of the mESCs exhibited an appearance of undifferentiated cells under these conditions. Mouse teratocarcinoma cell lines (F9 and P19 [35,36]), which were kindly provided by Dr. Minoru S. H. Ko, National Institute on Aging/NIH, were cultured under standard conditions for mESCs without LIF.

pOct2VAP (see Supplementary Materials and methods) was linearized with BspHII and delivered by electroporation into feeder-free W4 ESCs at passage 15 (10 µg DNA/1.0 × 107 cells/cuvette, 0.8 kV/cm, 12 pulses of 99 µs/pulse, BTX ECM200). After selection with 2 µg/ml puromycin (InvivoGen, San Diego, CA) for 11 days, the drug-resistant colonies, designated OW4, were collected (passage 1) and expanded. The OW4 cells were maintained under standard conditions and sorted at passage 18 based on fluorescence at 575 nm, as described previously [27].

Linear pCAG_Gli2ERP digested with Scal (0.5, 3 and 4 µg) was nucleofected into OGR1 mESCs (at passages 21, 25 and 12, respectively) according to the manufacturer’s instruction (Lonza, Basel, Switzerland). Stable lines (referred to as Gli2ER hereafter) were isolated 10–14 days after selection with 2 µg/ml puromycin supplemented in the standard culture medium. Gli2ER clones were maintained and assayed in the presence of puromycin within 10 passages after isolation. After trypsinization, the same volume of a single cell suspension was plated into two sets of gelatin-coated wells of 24-well plates in the standard culture medium. Roughly 100–3000 cells were plated in this manner. One day after plating, one set of the wells were fed with the standard medium supplemented with 20 nM 4-hydroxytamoxifen (4OHT; T176, Sigma-Aldrich). We determined that 20 nM 4OHT was optimal: when a higher dose was applied to culture, OGR1 mESCs decreased the proliferation rate (data not shown). Four days after the 4OHT treatment, a single cell suspension was prepared using an electronic pipet (Biohit, Bohemia, NY) to reduce pipetting errors, although the final volume of the single cell suspension was measured using a pipetman (Eppendorf, Hauppauge, NY). The number of cells (larger than 8.4 µm and smaller than 33.6 µm in diameter) was counted using Scepter™ (Millipore). Results were statistically analyzed using one-tail Student’s t-test.

The dynamic of Oct3/4 reporter expression was examined using mESCs that express EGFP under the Oct3/4 promoter, namely OGR1 [27,33,34,37]. After a single cell suspension was diluted with the standard culture medium, EGFP expression levels in each OGR1 mESC were determined under an inverted microscope (Leica DM4000B) equipped with an epifluorescent lamp. Single OGR1 mESCs were individually plated in each well of a gelatin-coated 96-well plate (Sarstedt AG & Co., Nümbrecht, Germany) filled with the standard culture medium by the single cell manipulation method [38,39]. Five to seven days after plating, the plating efficiency and the morphology and EGFP expression of colonies developed from single OGR1 mESCs were measured. Images were processed using ImageJ and enhanced in the same way. For this set of experiments, OGR1 mESCs were used at passages 6–19.

2.3. Microarray hybridization and analysis

One day after a subclone of OVW4, namely A02 (at passage 5), was plated at 100 cells/cm², it was maintained under the presence or absence of puromycin (2 µg/ml; Invivogen) for 4 days. Total RNA was extracted from 3 separate dishes per condition and subjected to microarray analysis (MouseWG-6 v1.1 Expression BeadChips; Illumina Inc., San Diego, CA). More detailed descriptions of the microarray hybridization and data analysis are provided in the Supplementary Materials and methods.

After background correction and quantile normalization, the log2-transformed data were assessed for differential expression. We arbitrarily considered genes with a false discovery rate [40] less than 0.05 and a fold-change of 1.2 or greater differentially expressed. Then, a list of differentially expressed genes with an expression level of 200 or greater in the puromycin-positive OVW4 culture was generated. A
scatter plot and a heat map were constructed using Microsoft Excel and MultiExperiment Viewer [41,42], respectively. ConPath Navigator (http://conpath.dna-chip.co.jp/; DNA Chip Research Inc., Yokohama, Kanagawa, Japan) was used to compare this gene list with published data [42,43], and GOTOolBox (http://genome.crg.es/GOTOolBox/; [44]) was used to investigate its associated gene annotations.

2.4. Semi-quantitative (sq) and quantitative (q) RT-PCR

The 1.6μg of total RNA extracted from stem cells in each culture condition was used to synthesize the first cDNA strand, as described previously [27,45,46]. For gene expression analysis of undifferentiated and differentiated mESCs, OGR1 was plated at 100 cells/cm² and maintained in the presence or absence of LIF for 4 days under standard conditions with animal serum. PCR mixtures were prepared using Phusion DNA polymerase (New England Biolab, Ipswich, MA), according to the manufacturer’s instructions. The PCR conditions were as follows: initial denaturing at 98°C for 1 min followed by 25 cycles of denaturing at 98°C for 10 s, annealing at 65°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 7.5 min. The primer sets used for this study were described previously [3,27,33,34,45,47,48] or are listed in Supplementary Table 1. To quantify relative gene expression, images of ethidium bromide-stained PCR products in agarose gels were analyzed using ImageJ. An identical rectangular selection was used to measure the mean fluorescence intensity of each PCR product as well as a local background level near the product per image. After background subtraction, values were divided by the one of Sino as a reference to obtain relative gene expression levels. Quantitative (q) RT-PCR was carried out essentially as described previously [34], except that the One-Step qRT-PCR kit (Life Technologies) was used.

2.5. Immunofluorescence microscopy

After fixation with 4% paraformaldehyde (Sigma-Aldrich) in PBS at room temperature for 15 min, mESCs cultured on glass-bottom dishes (MatTek corporation, Ashland, MA) were washed with PBS and blocked with 0.1% Triton X-100 at room temperature for 10 min. After PBS washes, these mESCs were incubated with 10% Image-IT FX signal enhancer (Life Technologies) at room temperature for 30 min, followed by incubation with a mouse anti-human Oct4 monoclonal antibody (sc-5279, Santa Cruz Biotechnology, Santa Cruz, CA) and a rabbit anti-human Gli2 polyclonal antibody (ab26056, Abcam, Cambridge, MA) diluted with 10% Image-IT FX signal enhancer at a 1:200 ratio at 4°C overnight. After PBS washes, the samples were incubated with goat Alexa Fluor 488 anti-mouse IgG and goat Alexa Fluor 568 anti-rabbit IgG polyclonal antibodies (Life Technologies) diluted in 10% Image-IT in PBS at room temperature for 1 h. Cellular nuclei were stained with Hoechst 33258 (0.2 mg/ml; Sigma) after PBS washes. The immunostained mESCs were treated with ProLong Gold antifade reagent (Life Technologies) overnight. A confocal microscope LSM 700 was used. Image analysis was carried out using ImageJ. Mean fluorescence intensity values that are greater than 40 and 20 are considered positive for the expression of Oct3/4 and Gli2, respectively (see Supplementary Fig. 1).

Detection of the SSEA1 antigen [49] was carried out using the FITC-conjugated anti-SSEA1 antibody (sc-21702, Santa Cruz Biotechnology) under the standard fluorescence microscopy (Observer.Z1, Zeiss) essentially as described previously [34,27].

3. Results and discussion

3.1. Mouse embryonic stem cells showed fluctuating expression of Oct3/4

To examine the transcriptional activity of Oct3/4 in mESCs cultured under standard conditions with animal serum (referred to as standard conditions hereafter), the Oct3/4 promoter [50] was cloned and used to build a fluorescent reporter construct, namely pOctV2AP (Fig. 1A; see Supplementary Materials and methods). In this construct, the Oct3/4 promoter drives the expression of Venus, a variant of yellow fluorescent protein [30], which is co-expressed with a puromycin-resistant gene product via the self-cleaving peptide T2A [51]. After pOctV2AP was linearized and delivered to mESCs by electroporation, mESC clones that exhibited puromycin resistance were isolated. As a proof of principle, these mESC clones, referred to as OVW4, expressed Venus under standard culture conditions (Fig. 1B). Interestingly, Venus expression was not detected in all of the OVW4 mESCs under standard culture conditions (Fig. 1B), i.e., Venus exhibited a variable expression pattern across OVW4 mESCs. In contrast, Venus fluorescence was relatively uniform when OVW4 cells were cultured in the presence of puromycin (Fig. 1C). Thus, these results indicate that the transcriptional activity of Oct3/4 fluctuates in mESCs under standard culture conditions. Heterogeneity in the transcriptional activity of Oct3/4 is not under the influence of animal serum because we observed similar heterogeneous expression of the Oct3/4 reporter in mESCs maintained under chemically-defined serum-free conditions [33,52] (Supplementary Fig. 2). Flow cytometric analysis revealed that approximately one-fifth of the OVW4 cells maintained under standard conditions lacked Venus expression (data not shown). When OVW4 cells were introduced into host blastocysts, Venus was fluorescently detected in the testes of developing fetuses at embryonic day 17.5 (Fig. 1D), thus validating the promoter activity of the transgene.

Next, the expression of genes associated with cellular pluripotency (Oct3/4, Esg1, Fgf4, Nanog, Rex1 and Sox2) and cell differentiation (Rho6x, Rho9x and Tcf15) were examined in Venus-positive and Venus-negative OVW4 cells by semi-quantitative reverse transcriptase polymerase chain reaction (sqRT-PCR; Fig. 1E). Nanog, Rex1, Rho9x and Tcf15 are known to be heterogeneously expressed in mESCs [3–6,16,45]. Notably, Venus-negative OVW4 cells exhibited a marked downregulation of Fgf4, Rex1, Sox2, Rho6x and Tcf15, whereas Esg1, Nanog and Rho6x expression levels were roughly maintained in Venus-negative OVW4 cells (Fig. 1E). Therefore, multiple subpopulations of mESCs existed in the culture, as suggested previously [4].

We investigated whether or not the observed heterogeneity in the transcriptional activity of Oct3/4 is dynamic. Initially, single OVW4 mESCs were plated, but their recovery was poor due to W4’s high dependency on LIF [53]. Therefore, R1 mESCs that express EGFP under the Oct3/4 promoter (OGR1 [27,33,34,37]) were used. Single EGFP-high or low OGR1 mESCs were plated into each well of 96-well plates and maintained under standard conditions (Figs. 2A–C). Five to seven days after plating, 42.8% of EGFP-high and 36.6% of EGFP-low single OGR1 mESCs grew into individual colonies (p > 0.05). The appearance of each colony was arbitrarily categorized into the following three: a dome-like shape with bright EGFP fluorescence indicative of pluripotent mESCs, a flattened shape with moderate EGFP fluorescence indicative of reduced differentiation potential and a committed cell shape with reduced EGFP fluorescence indicative of cell differentiation (Figs. 2D–I). EGFP-low mESCs produced slightly more colonies with an appearance of committed cells (38.1%) than EGFP-high mESCs (34.3%) at the expense of dome-like colonies (28.6% in EGFP-low mESCs vs. 33.1% in EGFP-high mESCs; Fig. 2J). However, this result shows no statistical significance and both EGFP-high and EGFP-low mESCs formed colonies with heterogeneous EGFP expression. Thus, we conclude that heterogeneity in the transcriptional activity of Oct3/4 is dynamic.

3.2. Microarray analysis revealed global transcriptional heterogeneity in mouse embryonic stem cells

To evaluate the transcriptional heterogeneity in mESCs at a genomic scale, OVW4 cells were maintained under standard conditions in the presence or absence of puromycin, and subjected to expression microarray analysis (Fig. 3A). Hybridization experiments were carried out in
Fig. 1. Characterization of OVW4 mouse embryonic stem cells (mESCs). (A) A schematic representation of the expression cassette used to generate OVW4 mESCs. The Oct3/4 promoter drives the monocistronic transcription of the Venus fluorescent protein and a puromycin-resistant gene product (puromycin-N-acetyltransferase; Puror) via T2A (2A), pA, the bovine growth hormone polyadenylation signal. The bar denotes a length of 1 kb. (B–D) Bright (left) and dark (right) field images are shown. Bars, 50 μm in B and C and 100 μm in D. (B) A mESC line that harbors the Venus_T2A_Puror cassette driven by the Oct3/4 promoter (Oct3/4::Venus) was designated OVW4 and maintained under standard conditions (left). Venus showed fluctuating expression (right). (C) OVW4 was maintained under standard conditions supplemented with puromycin (left). Venus showed relatively homogeneous expression (right). (D) Fetal testes were isolated from a chimera at embryonic day 17.5, which was derived from injection of OVW4 into a host blastocyst (left). The expression of Venus was detected in seminiferous tubules (right). (E) Undifferentiated OVW4 was sorted according to Venus expression levels at 575 nm and subjected to first strand cDNA synthesis. The cDNA obtained from Venus-positive (+) or negative (−) OVW4 was used as a template for the subsequent polymerase chain reaction (PCR) with each gene-specific primer pair indicated on the right. Ef1α is a positive control, and RT− is a negative control.

Fig. 2. Heterogeneous expression of the Oct3/4 reporter is dynamic. (A–I) Phase contrast (left) and fluorescence (right) images of mouse embryonic stem cells (mESCs) that express EGFP under the Oct3/4 promoter, namely OGR1, are shown. The EGFP fluorescence indicates the transcriptional activity of Oct3/4. Bars, 50 μm. (A & B) Single EGFP-high (A) and EGFP-low (B) OGR1 mESCs are shown. (C) An EGFP-high mESC (arrowhead) was plated in a well of a 96-well plate. No other cell exists in the same well. (D–I) Colonies with a dome-like shape (D & E), a flattened shape (F & G) and a committed cell shape (H & I) were formed from single EGFP-high (D, F & H) or EGFP-low (E, G & I) OGR1 mESCs. Note that colonies shown in E and G were formed on the plastic plate showing “grooves” on the opposite side of the bottom due to the manufacturing-associated issue. (J) The frequency of the appearance of a dome-like, flattened or committed cell shape was measured 5–7 days after plating single EGFP-high and EGFP-low mESCs. The total numbers of single cells plated are 181 for EGFP-high and 21 for EGFP-low mESCs from five replications. The Chi-square test showed no statistical significance.

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triplicate for each condition. A total of 4606 probes were selected as
differentially expressed based on their statistical con
didence (see Section 2.3 in Materials and methods). In the puromycin-positive
OVW4 culture, 2717 probes were overexpressed, whereas 1889 probes
were underexpressed (the gene list is available in Supplementary
Table 2). The variation in gene expression levels between puromycin-
positive and puromycin-negative conditions was evident in a scatter
plot (Fig. 3B). Among the variably expressed genes,
Spink3, which en-
codes a serine peptidase inhibitor, showed the largest fold induction
in OVW4 cells grown in the presence of puromycin. In addition to
Spink3, expression levels of Enox1, Gbx2 and Myo1f were upregulated
when OVW4 cells were maintained in the presence of puromycin
(Fig. 3B). These four genes were consistently downregulated when the
Oct3/4 expression level was knocked-down [54]. Genes associated
with the self-renewal and pluripotency of mESCs, such as
Dppa2, Dppa4, Eras, Foxa15, MybI2, Sox2 and Utf1, were enriched in OVW4
cells that were cultured in the presence of puromycin, whereas genes
associated with cell differentiation, such as Acta1, Acta2, Etv1, Hes1,
Htg2, Ihbp4, Lefty2, Msx1, Notch3, Pitx2 and T, were under-represented.
Collectively, these results indicate that a standard culture of mESCs
contains a cell population with reduced Oct3/4 transcriptional activity,
which leads to the generation of subpopulations with expression pro-iles that are similar to differentiated cells.

Next, we investigated the gene enrichment and functional annota-
tions associated with this gene list. The enriched probe sets in
puromycin-positive OVW4 cultures contained 2368 unique gene sym-
bols, which yielded 1264 functionally annotated genes (53%), whereas
enriched probes in puromycin-negative OVW4 cultures contained
1689 unique gene symbols, which included 1034 annotated genes
(61%). Uncharacterized genes were enriched in puromycin-positive
OVW4 cultures, which may be indicative of a naive state of undifferen-
tiated cells [55]. Similar results were obtained when mESC gene expres-
sion profiles were compared with lineage-committed trophoblast stem
cells [47]. By arbitrarily selecting probes with greater than 2-fold
expression level differences between the two conditions, the gene lists
were reduced to 286 and 365 annotated genes in puromycin-positive
and puromycin-negative OVW4 cultures, respectively (Tables 1 and
2). Interestingly, the gene list for the puromycin-positive OVW4 cul-
tures contained genes involved in nucleic acid metabolism (Table 1),
whereas genes involved in embryonic development, cholesterol

Fig. 3. Global transcriptional heterogeneity in OVW4. (A) A schematic representation of the experimental design. OVW4 mESCs were maintained under standard conditions with or without puromycin, and subjected to Illumina expression microarray analysis. (B) A scatter plot of the 4606 probes exhibiting differential expression between OVW4 maintained in the presence or absence of puromycin (referred to as puro+ or puro−, respectively). Differential expression was defined by arbitrarily determined cut-off values (see Section 2.3 in Materials and methods). For each probe, the log2-transformed average expression level of hybridizations for puro+ (collected in triplicate) was plotted on the x-axis and the log2-transformed ratio of expression levels between puro+ and puro− (“puro+/puro−”) was plotted on the y-axis. Probes representing indicated genes are marked on the plot. (C) Two representative pathways that exhibited significant enrichment of genes highly expressed in puro+ (left, general transcription factors and RNA polymerases) or puro− (right, cholesterol synthesis) are shown. The color of each box represents the normalized levels of fold-differences in gene expression between puro+ and puro−, as indicated in the legend.

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metabolism, and cytoskeletons were noted on the puromycin–negative gene list (Table 2). Similar results were independently obtained using ConPath Navigator (Fig. 3 C; http://conpath.dna-chip.co.jp/). ConPath Navigator is a search tool for genes relative to biological signaling pathways built by GenMAPP [56]. Taken together, it is suggested with these data that Oct3/4 plays an essential role in maintaining basal cellular transcriptional activities while suppressing the expression of genes involved in cell differentiation. This function of Oct3/4 is indicative of a gene that is responsible for regulating the duplication of cells with equal differentiation potential, i.e., self-renewal.

Our expression microarray analysis identified about 2300 annotated genes that potentially exhibit heterogeneous expression under the influence of Oct3/4 in mESCs. This analysis was done using OV4W4 cultured in the presence or absence of puromycin under conditions with LIF and animal serum without feeder layers. These conditions might have enriched a population of cells that express Oct3/4 at a very high level (e.g., [57]) and contributed to magnifying the gene list. Interestingly, single-gene expression analysis showed that nine genes associated with cellular pluripotency including Nanog and Oct3/4 exhibited variable expression similar to Gapdh [58]. In this analysis, mESCs were maintained under conditions with LIF, animal serum and feeder layers. Under these conditions, fluctuating expression of the nine genes might have been less pronounced than in conditions without feeder layers. However, these two studies clearly demonstrated that gene expression in mESCs is quite variable, so that bulk analysis of gene expression is not suited for understanding the mechanism of cellular pluripotency.

### 3.3. Undifferentiated pluripotent stem cells expressed genes involved in the hedgehog signaling pathway

Our expression microarray data clearly demonstrate that standard cultures of mESCs consist of heterogeneous populations. Next, we used ConPath Navigator to perform a cross-platform comparison of our current data with published datasets that reported gene expression profiles of mESCs in the course of Oct3/4 downregulation [42,43]. These published datasets were generated using bulk preparations of mESCs maintained under standard conditions. On the other hand, our dataset took into account the heterogeneous transcriptional activity of Oct3/4 in mESCs maintained under similar conditions. Therefore, we aimed at investigating whether our dataset is consistent with or offers alternative interpretations of the published datasets. Because not all of the probes tested in our dataset were necessarily examined in the published datasets, we used two datasets for comparison. Interestingly, we found unique expression patterns in genes that are involved in the hedgehog (Hh) signaling pathway. For example, the transcription factor Gli2 and the transmembrane receptor Ptch1 were consistently highly expressed in mESCs that displayed high transcriptional activity of Oct3/4 (i.e., OV4W4 cells that were cultured in the presence of puromycin, “puro+”; Table 3), whereas the transcription repressor Gli3 was highly expressed in OV4W4 cells that were cultured in the absence of puromycin (“puro−”; Table 3). The expression levels of Gli2, Gli3 and Ptch1 were comparable to that of Sox2, a co-factor of Oct3/4 (Table 3). The forced downregulation of Oct3/4 transcription [43] induced downregulation and up-regulation of Gli2 and Gli3, respectively (Fig. 4A). Thus, the positive correlation between Gli2 and Oct3/4 expression, and the negative correlation between Gli3 and Oct3/4 expression were independently validated. The Ptch1 expression pattern observed in the current dataset was unexpected because, according to Walker et al. [42], Ptch1 expression markedly increased a few days after cell differentiation was induced (by the withdrawal of LIF from the culture or the addition of retinoic acid to the culture), but returned back to its basal level within 5 days [42]. Another study independently reported a similar expression profile of Ptch1 [59]. However, Ptch1 expression became further down-regulated when mESCs were differentiated in the absence of LIF but the presence of retinoic acid for 20 days [60]. Ptch1 may be influenced by both LIF and differentiation-inducing signals.

These results were unexpected because the Hh signaling pathway governs cell type specification and embryonic patterning [61]. In

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**Table 1**
The functional annotations of genes overrepresented in puromycin-positive OV4W4 cultures.

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**Table 2**
The functional annotations of genes overrepresented in puromycin-negative OV4W4 cultures.

<table>
<thead>
<tr>
<th>Level</th>
<th>Gene Ontology ID</th>
<th>Term</th>
<th># in reference</th>
<th>Frequency in the reference</th>
<th># in the gene list</th>
<th>Frequency in the gene list</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0002520</td>
<td>Developmental process</td>
<td>2915</td>
<td>0.1889</td>
<td>112</td>
<td>0.3681</td>
<td>0.00004047</td>
</tr>
<tr>
<td>3</td>
<td>0007725</td>
<td>Multicellular organisinal development</td>
<td>2476</td>
<td>0.1689</td>
<td>96</td>
<td>0.263</td>
<td>0.00259056</td>
</tr>
<tr>
<td>4, 5</td>
<td>0009792</td>
<td>Embryonic development ending in birth or egg laying</td>
<td>421</td>
<td>0.0287</td>
<td>28</td>
<td>0.0767</td>
<td>0.00001938</td>
</tr>
<tr>
<td>2</td>
<td>0010926</td>
<td>Anatomical structure formation</td>
<td>775</td>
<td>0.0529</td>
<td>43</td>
<td>0.1178</td>
<td>0.00007994</td>
</tr>
<tr>
<td>4, 5, 6</td>
<td>0004728</td>
<td>Nucleosome organization</td>
<td>96</td>
<td>0.0065</td>
<td>12</td>
<td>0.0329</td>
<td>0.0072877</td>
</tr>
<tr>
<td>5, 6, 7</td>
<td>0016125</td>
<td>Sterol metabolic process</td>
<td>78</td>
<td>0.0053</td>
<td>12</td>
<td>0.0329</td>
<td>0.0007878</td>
</tr>
<tr>
<td>3, 4, 5</td>
<td>0016102</td>
<td>Vesicle-mediated transport</td>
<td>419</td>
<td>0.0286</td>
<td>28</td>
<td>0.0767</td>
<td>0.0028278</td>
</tr>
</tbody>
</table>

---

* Terms belonging to the same parental term (level 2) are grouped. A total of 15 terms showed statistical significance (see “d” below). Only the representative terms are shown. At least 4 different parental terms (level 2) are expected, although 2 of them did not show statistical significance.

* Gene symbols in Mouse Genome Informatics were used as a reference.

* A total of 613 genes exhibiting a 2-fold increase in their expression levels at FDR < 0.05 contain 640 non-redundant gene symbols, of which 286 symbols are functionally annotated.

* p-Values were calculated using the hypergeometric test (default setting) and the Bonferroni correction. GO annotations that have p-values less than 0.01 are considered statistically significant.
Table 3
The differential expression of genes involved in the Hh signaling pathway in OVW4a.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Refseq ID</th>
<th>Entrez ID</th>
<th>FDR</th>
<th>Puro+</th>
<th>Puro-</th>
<th>Log2 (Puro+/Puro-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Genes showing statistically significant differential expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gli2</td>
<td>NM_001081125.1</td>
<td>14633</td>
<td>0.00</td>
<td>1238</td>
<td>839</td>
<td>0.6</td>
</tr>
<tr>
<td>Gli3</td>
<td>NM_008130.2</td>
<td>14634</td>
<td>0.00</td>
<td>1052</td>
<td>1743</td>
<td>−0.7</td>
</tr>
<tr>
<td>Igf2</td>
<td>NM_010514.3</td>
<td>16002</td>
<td>0.00</td>
<td>1036</td>
<td>4279</td>
<td>−2.0</td>
</tr>
<tr>
<td>Pchg1</td>
<td>NM_008957.2</td>
<td>19206</td>
<td>0.00</td>
<td>1107</td>
<td>494</td>
<td>1.2</td>
</tr>
<tr>
<td>Rb23</td>
<td>NM_008999.4</td>
<td>19335</td>
<td>0.01</td>
<td>231</td>
<td>193</td>
<td>0.3</td>
</tr>
<tr>
<td>Sap18</td>
<td>NM_009119.3</td>
<td>20220</td>
<td>0.03</td>
<td>268</td>
<td>227</td>
<td>0.2</td>
</tr>
<tr>
<td>Smo</td>
<td>NM_176966.4</td>
<td>319757</td>
<td>0.00</td>
<td>226</td>
<td>394</td>
<td>−0.8</td>
</tr>
<tr>
<td>Sufu</td>
<td>NM_011443.3</td>
<td>20674</td>
<td>0.00</td>
<td>1016</td>
<td>577</td>
<td>0.8</td>
</tr>
<tr>
<td>(ii) Genes that did not show statistical significance or had expression levels less than 200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdk1</td>
<td>NM_007659.3</td>
<td>12534</td>
<td>&lt;0.05</td>
<td>3679</td>
<td>4071</td>
<td></td>
</tr>
<tr>
<td>Crebbp</td>
<td>NM_001025432.1</td>
<td>12914</td>
<td>&lt;0.05</td>
<td>455</td>
<td>540</td>
<td></td>
</tr>
<tr>
<td>Dhh</td>
<td>NM_007857.4</td>
<td>13363</td>
<td>&lt;0.05</td>
<td>&lt;200</td>
<td>&lt;200</td>
<td></td>
</tr>
<tr>
<td>Dyrk1a</td>
<td>NM_007890.2</td>
<td>13548</td>
<td>&lt;0.05</td>
<td>&lt;200</td>
<td>&lt;200</td>
<td></td>
</tr>
<tr>
<td>Pch2</td>
<td>NM_008958.2</td>
<td>19207</td>
<td>&lt;0.05</td>
<td>&lt;200</td>
<td>&lt;200</td>
<td></td>
</tr>
<tr>
<td>Sin3a</td>
<td>NM_011378.2</td>
<td>20466</td>
<td>&lt;0.05</td>
<td>2581</td>
<td>2593</td>
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<tr>
<td>Ski</td>
<td>NM_011385.2</td>
<td>20481</td>
<td>&lt;0.05</td>
<td>784</td>
<td>692</td>
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<tr>
<td>Shh36</td>
<td>NM_175031.3</td>
<td>269209</td>
<td>&lt;0.05</td>
<td>&lt;200</td>
<td>&lt;200</td>
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</tr>
<tr>
<td>Sufu</td>
<td>NM_015752.2</td>
<td>24069</td>
<td>&lt;0.05</td>
<td>413</td>
<td>426</td>
<td></td>
</tr>
</tbody>
</table>

* The expression microarray did not contain probes for the following genes involved in the Hh signaling pathway: Gli1, Ihh and Shh.

b Sox2 is not directly involved in the Hh signaling pathway, but it has been listed for comparison.

Fig. 4. The expression of genes involved in the hedgehog pathway of mESCs. (A) A heat map of genes (rows) involved in cellular pluripotency and the hedgehog pathway based on present (OVW4 puro+/−) and published (ZHBTc4, LIF+, LIF−, RA + [41,42]) datasets (columns). ConPath Navigator was used to compare data obtained from multiple platforms. In the published datasets, mESC differentiation was induced by either the forced downregulation of Oct3/4 transgenes using Oct3/4-null mESCs (ZHBTc4 [42]), or the withdrawal of LIF (LIF−) for 5 days and the addition of all-trans retinoic acid (RA+) under the LIF− condition for 2 days using OGR1 mESCs ([41]). The triangles indicate the level of EGFP expression in OGR1. The color scale indicates relative gene expression levels. Missing values are shaded in gray. (B) A schematic representation of the Sonic hedgehog (Shh) pathway. Without the growth factor Shh, the Shh receptor Patched (Pch) represses Smoothened (Smo). When Shh binds to Pch, Shh represses Pch, allowing activation of Smo. Subsequently, Smo can activate protein activity of transcription factors Gli1, Gli2 and Gli3. (C) The expression of genes shown in B was examined in undifferentiated (LIF+), left) and differentiated (LIF−, middle) OGR1 mESCs. Phase contrast (top) and fluorescence (bottom) images of the representative mESC colonies are shown. Bars, 100 μm. For LIF− conditions, mESCs were cultured in the absence of LIF for 4 days. Semi-quantitative RT-PCR analysis was performed using cDNAs derived from OGR1 mESCs cultured under LIF+ or LIF− conditions as templates with each gene specific primer set indicated on the right. Ef1α is a positive control. The size of the DNA markers is shown on the left. These results were independently validated by quantitative RT-PCR (Supplementary Fig. 3).
vertebrates, three orthologs of Drosophila Hh, i.e., Desert hedgehog (Dhh), Indian hedgehog (Ihh) and Sonic hedgehog (Shh), have unique roles in embryonic development [62–67]. Shh is the most broadly expressed ortholog and is involved in embryogenesis, organogenesis and the maintenance of adult stem cells [68]. Without the Shh ligand, the Shh receptor, Patched (Ptc), represses another membrane-bound receptor, Smoothened (Smoo). In mammals, two Ptc isoforms, Ptc1 and 2, exist, although Ptc1 is mainly involved in the Hh signaling pathway [69]. When Shh binds to Ptc, Shh represses Ptc, allowing the activation of Smoo. Subsequently, Smoo can activate the protein transcription of transcription factors Gli1, Gli2 and Gli3. Gli3 is the main modulator of the Shh signal [70–72]. Gli3 mostly acts as a transcriptional repressor [73].

In accordance with this knowledge, genes involved in the Hh signaling pathway, except for Gli2 and Ptc1, (Cdk1, Crebpb, Dhh, Dyrk1a, Gas1, Gli1, Gli3, Igf2, Ihh, Ptc2, Rab3b, Sap18, Shh, Sin3a, Ski, Smo, Sotr3c6 and Sufu, according to ConPath Navigator) were either constitutively expressed or downregulated in puromycin-positive OVV4 cultures (“pure +”). Furthermore, many of these genes were upregulated when a forced downregulation of Oct3/4 was induced (Crebpb, Dyrk1a, Dhh, Sap18, Gli3, Sufu and Cdk1; Fig. 4A) or when LIF was withdrawn from the culture (Gas1, Ski, Igf2, Smo, Ptc1 and Ptc2; Fig. 4A). In contrast to these results, our expression microarray analysis showed that Gli2 and Ptc1 were consistently highly expressed in mESCs with high transcriptional activity of Oct3/4.

To further validate our expression microarray analysis, we examined the expression of selected genes in the Hh signaling pathway (Fig. 4B) in undifferentiated and differentiated mESCs by qRT-PCR (Fig. 4C). For this analysis, we used a bulk preparation of OGR1 mESCs [27,33,34,37,42] (see Fig. 2) that were maintained under culture conditions with animal serum in the presence or absence of LIF for 4 days. Although significant downregulation of EGFP fluorescence was observed in OGR1 maintained without LIF for 4 days (Fig. 4C “LIF-” on the left vs. “LIF+” on the middle), EGFP fluorescence did not become fully undetectable under these conditions.

We found that undifferentiated mESCs expressed Gli1, Gli2, Smo and Ptc1 (Fig. 4C right). However, the endogenous expression of Shh was not detected, which is consistent with the fact that Gli2 can be expressed in the absence of a Shh signal [71,72]. The expression of Gli1, Gli2 and Ptc1 was downregulated 4 days after differentiation of mESCs were induced, whereas the expression of Gli3 and Smo was upregulated (Fig. 4C, see Supplementary Fig. 3 for quantitative results). Because the increased transcription of Gli1, Gli2 and Ptc1 serves as an indicator of Hh pathway activity [69,74,75], it is suggested with these results that Shh signaling was stimulated in undifferentiated mESCs, but not in mESCs cultured in the absence of LIF for 4 days. Interestingly, when mESCs were maintained under chemically-defined serum-free (CDSF) conditions [33,52], we observed downregulation of Gli1, Gli2 and Ptc1 (data not shown) in these mESCs. Therefore, animal serum may provide a stimulus to activate the Hh-mediated signal. However, when recombinant Shh was supplemented in a CDSF medium, it failed to provide any significant impact on the growth of mESCs (data not shown). Similarly, endogenous activation of the Hh signal was detected in undifferentiated human ESCs, although exogenously supplied Shh played no significant role in their self-renewal and pluripotency [76]. The self-renewal of human ESCs is dependent on the TGFβ and BMP signals [77,78], which are known to interact with the Shh signal [79,80]. Therefore, it is suggested with these observations that the Hh-mediated signaling may originate from TGFβ and/or BMP-like activity in animal serum.

Next, we investigated whether the genes involved in the Hh pathway were expressed in other mouse pluripotent stem cells. The mouse teratocarcinoma cell lines F9 (derived from the testis [35]) and P19 (derived from postimplantation embryos [36]) consistently expressed Gli2, Smo and Ptc1, although the expression level of Gli2 was lower in P19 than in F9 and Gli1 expression was undetectable in P19 (Fig. 5). We could not detect endogenous expression of Shh in F9 or P19 (Fig. 5). Because Gli1 encodes a transcription factor and, interestingly, the expression of Gli2 was consistently detected in all of the pluripotent stem cells examined, it is suggested with these results that Gli2 may play a novel role in the self-renewal of pluripotent stem cells.

3.4. Gli2 exhibited heterogeneous expression in undifferentiated mouse embryonic stem cells

It is suggested with our expression microarray data that Gli2-positive and Gli2-negative cells may both exist in mESCs maintained under standard conditions. Confocal microscopy confirmed that the Gli2 protein was localized in the nuclei and exhibited a patchy staining pattern (Fig. 6A), and that 89% of Gli2-positive mESCs were undifferentiated and Oct3/4-positive (Fig. 6B). In addition, 50% of mESCs with reduced expression levels of the Oct3/4 protein also exhibited low expression levels of the Gli2 protein (arrowheads in Fig. 6A). Therefore, Gli2 expression patterns are highly correlated with Oct3/4 expression patterns in undifferentiated mESCs. Collectively, we suggest that Gli2 may be involved in the network of transcription factors that sustain mESCs self-renewal and pluripotency. However, Gli2 is expected to play a modulatory role instead of an essential role in mESCs because Gli2-deficient embryos exhibited defects in body plans but not in cell differentiation per se [81].

3.5. Forced activation of Gli2 significantly enhanced the proliferation rate of mouse embryonic stem cells

To gain an insight into the role that Gli2 plays in mESCs, the protein-coding sequence of the Gli2 cDNA was subcloned into a novel expression vector (Fig. 7A) and stably expressed in OGR1 mESCs maintained under standard conditions. This vector allows Gli2 to be expressed as a fusion protein with the human estrogen receptor ERT2 [31,32]. When 4-hydroxytamoxifen (4OHT) is supplemented in the culture, Gli2 fused with ERT2 will be activated and localized to nuclei. In addition, this vector assures us a strong expression level of the transgene by the CAG promoter [82] combined with a transcriptional enhancer [45,83] and a transcriptional enhancer [84]. Furthermore, DsRedT3 [85,86] is linked with the immediate downstream of the transgene by the self-cleaving peptide T2A [51] in this vector (Fig. 7A). Therefore, DsRedT3 fluorescence helps us monitor expression levels of Gli2 in real-time (Fig. 7B).

Nine clones that express Gli2 fused with ERT2 (referred to as Gli2ER hereafter) were used for assays. They exhibited varying levels of DsRedT3 fluorescence, whereas quantitative (q) RT-PCR indicated that, on average the expression level of Gli2 increased 244 (±48.24 s.e.m., n = 7) folds in these Gli2ER clones. Parental OGR1 mESCs were used as a control. When 4OHT was added to standard cultures in the presence of LIF and animal serum for 4 days, Gli2ER clones did not exhibit any appearance of cell differentiation (Fig. 7B) and expressed the SSEA1 antigen [49] (Fig. 7C). In addition, heterogeneity in the transcriptional activity of Oct3/4 was observed in Gli2ER clones treated with 4OHT (Fig. 7B). This was not due to the forced activation of Gli2ER because most of the Gli2ER cells maintained the expression of both EGF and DsRedT3 (Fig. 7B). This result indicates that Gli2 does not regulate heterogeneous transcriptional activity of Oct3/4. However, Gli2ER clones significantly increased their proliferation rate when maintained in the presence of 4OHT for 4 days (1.59 ± 0.230 s.e.m-fold increase in the nine Gli2ER clones vs. 1.08 ± 0.0601 s.e.m-fold increase in OGR1, p < 0.025, Fig. 7D). When five Gli2ER clones that exhibited bright DsRedT3 fluorescence (260 ± 71.03 s.e.m-fold increase in the Gli2 level on average) were selected and independently assayed twice or three times, we consistently observed a similar increase in their proliferation rate (1.47 ± 0.157 s.e.m-fold increase, n = 11, p < 0.025). Finally, using five randomly selected Gli2ER clones that were maintained in the presence or absence of 4OHT for 4 days, expression levels of Oct3/4, Eras and Gsk3β were examined by qRT-PCR (the reason why these genes were chosen is described below). As expected from the results...
presented in Figs. 7B and C, the expression of Oct3/4 was stable under these conditions (Fig. 7E). However, the expression level of Eras in Gli2ER clones under 4OHT+ conditions decreased to about 40% of the Eras level in Gli2ER clones under 4OHT-conditions (Fig. 7E). On the other hand, Gsk3β showed a dramatic increase in Gli2ER clones under 4OHT+ conditions (Fig. 7E). Perhaps a positive feedback loop exists

Fig. 5. The expression of genes involved in the Hh pathway in pluripotent stem cells. Semi-quantitative RT-PCR analysis was performed using cDNAs derived from pluripotent stem cells indicated on the right with each gene specific primer set indicated along the top. Ef1α is a positive control and RT− is the negative control. The size of the DNA markers is shown on the left. (A) Mouse ESCs (OGR1) and teratocarcinoma cell lines (F9 and P19) were cultured in a standard medium with animal serum. (B) Relative expression levels of genes indicated on the bottom were examined by quantifying mean fluorescence intensities of the PCR products shown in A using ImageJ. Smo was used as a reference.

Fig. 6. Oct3/4 and Gli2 are heterogeneously expressed in undifferentiated mESCs. (A) Confocal microscopy was used to examine the localization of Oct3/4 proteins (green; first row, second image), nuclei (blue; first row, second image) and Gli2 proteins (red; first row, third image) in undifferentiated mESCs (first row, first image). Sections were taken every 0.4μm on the z-axis. Twenty-one sections were stacked and projected for all images. Arrowheads indicate the mESC nuclei with reduced Oct3/4 and Gli2 expression levels. Arrows indicate the mESC nuclei that express Oct3/4, but display reduced expression of Gli2. An asterisk indicates the mESC nuclei with reduced expression of Oct3/4, but expression of Gli2. As a negative control, only secondary antibodies were used ("2° only", second row, second and third images). The nuclear staining of three cells in the bottom left corner of the second row, second image is not visible because these nuclei are out of focus. Bars, 10μm. (B) The bar chart indicates the frequency (%) of mESC nuclei that show the presence (+) or absence (−) of Oct3/4 and Gli2 expression.

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between Gsk3β and Gli2 because Gli2 is one of the Gsk3β substrates and targeted on proteasome-mediated processing [87].

### 3.6. The possible role that Gli2 plays in mouse embryonic stem cells

Gli1 was initially identified due to its amplification in human glioblastomas [88]. Two homologues of Gli1, Gli2 and Gli3, were also expressed in human glioblastomas [89]. The overexpression of Gli2 in the skin of transgenic mice induced the development of basal cell carcinomas, which are the most common skin tumors in Caucasians [90]. In addition, the overexpression of Gli2 in a human prostate epithelial cell line resulted in growth acceleration and cell cycle progression [91]. Therefore, it is possible to speculate that Gli2 may be involved in the tumor-like growth of ESCs.

The tumor-like growth of mESCs is dependent on the activity of Eras via the activation of Akt1 [92]. Because Akt1 inactivates Gsk3β [93–95] and Gsk3β inhibits c-Myc (Myc) [93,96], Eras may indirectly activate c-Myc, which subsequently drives the self-renewal of mESCs [97]. However, human Eras is not expressed in human ESCs [98,99]. Therefore, we hypothesize that other genetic factors drive the tumor-like growth of both mouse and human ESCs. Interestingly, our data showed that Gli2ER clones significantly increased their proliferation rate (Fig. 7D) while they decreased the expression level of Eras (Fig. 7E) under 4OHT+ conditions. Although it needs to be addressed whether the interaction between Gli2 and Eras is direct and whether a feedback loop exist between Eras and Gli3β or not, we suggest with our data that Gli2 may be involved in the tumor-like growth of ESCs.

We recently demonstrated that mESCs cultured under chemically defined serum-free conditions downregulated Eras and c-Myc and failed to grow into teratomas [33]. Interestingly, mESCs maintained under serum-free conditions restored their tumor-like growth without the upregulation of Eras and c-Myc when the culture was supplemented with a Gsk3β inhibitor [33]. Therefore, this result strongly supports our hypothesis that other genetic factors are involved in promoting the tumor-like growth of ESCs. Because the serum-free culture provides a unique platform to screen genetic factors responsible for teratoma development in mESCs, further study using this culture system may identify the role of Gli2 in the tumor-like growth of mESCs.

### 4. Conclusions

A standard culture of mESCs contains a cell population with reduced Oct3/4 transcriptional activity, which leads to the generation of subpopulations with expression profiles that are similar to differentiated cells. Heterogeneity in the transcriptional activity of Oct3/4 was dynamic. Oct3/4 plays an essential role in maintaining basal cellular transcriptional activities while suppressing the expression of genes involved in cell differentiation. The expression of Gli2, Ptc1 and Smo was consistently detected in pluripotent stem cells examined in this study. Gli2 expression patterns are highly correlated with Oct3/4 expression patterns in undifferentiated mESCs. Forced activation of Gli2 in mESCs increased their proliferation rate. It is suggested with our results that Gli2 may play a novel role in the self-renewal of pluripotent stem cells.
Description of additional data files

“Li_et_al_SupInfo.docx” provides a list of primer sets used in this study (Supplementary Table 1), supplementary figures 2, 3, and 4, and supplementary Table 2. Supplementary data related to this article can be found online at: http://dx.doi.org/10.1016/j.ygeno.2013.09.004.

Competing interests

All authors declare no competing interest.

Authors' contributions


Acknowledgments


Y. Li, T.S. Tanaka, Yokohama-Tamaki, Nanog, Short-term serum-free culture reveals that inhibition of GSK3β induces the tumor-like growth of mouse embryonic stem cells, PLoS ONE 6 (2011) e21355.


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