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

Yanzhen Li [a,b], Jenny Drnevich [c], Tatiana Akraiko [c], Mark Band [c], Dong Li [b,d], Fei Wang [b,d], Ryo Matoba [e], Tetsuya S. Tanaka [a,b,f,g,*]

[a] Department of Animal Sciences, University of Illinois at Urbana–Champaign, Urbana, IL 61801, USA
[b] Institute for Genomic Biology, University of Illinois at Urbana–Champaign, Urbana, IL 61801, USA
[c] The W.M. Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana–Champaign, Urbana, IL 61801, USA
[d] Department of Cell and Developmental Biology, University of Illinois at Urbana–Champaign, Urbana, IL 61801, USA
[e] DNA Chip Research Inc., Yokohama, Kanagawa 230-0045, Japan
[f] Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA
[g] Department of Chemical and Biomolecular Engineering, University of Notre Dame, Notre Dame, IN 46556, USA

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ABSTRACT

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. The cultures were subsequently subjected to Illumina expression microarray analysis. 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 presence of puromycin. In contrast, the genes involved in cell differentiation were overrepresented in the probe set associated with mESCs maintained in the absence 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 (ESCs) 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].

Abbreviations: Hh, hedgehog; mESCs, mouse embryonic stem cells; sqRT-PCR, semi-quantitative reverse transcriptase polymerase chain reaction.

* Corresponding author at: 49 Galvin Life Sciences, Notre Dame, IN 46556, USA.
E-mail address: Tetsuya.Tanaka.9@nd.edu (T.S. Tanaka).

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Therefore, the heterogeneous expression of *Zscan4* is necessary for mESCs to self-renew indefinitely.

In studies with other genes [3,5,12–16,18], 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 *Ptch1*, 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 pOctV2AP in which the *Oct3/4* promoter drives the expression of Venus [17,30] and pCAG_Gli2ERP from which the protein coding sequence of *Gli2* is 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 (LIF; 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 a 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.

pOctV2AP (see Supplementary Materials and methods) was linearized with BspHI and delivered by electroporation into feeder-free W4 ESCs at passage 15 (10 μg DNA/1.0×10^7 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 OVW4, were collected (passage 1) and expanded. The OVW4 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 ScaI (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 optimum: 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 DMI4000B) 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/cm^2, 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

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