the FEBS Journal

The reprogramming factor nuclear receptor subfamily 5, group A, member 2 cannot replace octamer-binding transcription factor 4 function in the self-renewal of embryonic stem cells

Kyeng-Won Choi[1,*], Hye-rim Oh[1,*], Jaeyoung Lee[1], Bobae Lim[1], Yong-Mahn Han[2], Junseo Oh[3] and Jungho Kim[1]

1 Department of Life Science, Laboratory of Molecular and Cellular Biology, Sogang University, Seoul, Korea
2 Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon, Korea
3 Department of Medical Science, Graduate School, Korea University, Seoul, Korea

Keywords
embryonic stem cell; induced pluripotent stem cell; nuclear receptor subfamily 5, group A, member 2 (Nr5a2); octamer-binding transcription factor 4 (Oct-4); self-renewal

Correspondence
J. Kim, Department of Life Science, Laboratory of Molecular and Cellular Biology, Sogang University, Seoul 121-742, Korea
Fax: +82 2 716 2092
Tel: +82 2 705 8461
E-mail: jkim@sogang.ac.kr

*These authors contributed equally to this work.

(Received 27 March 2013, revised 15 November 2013, accepted 28 November 2013)

doi:10.1111/febs.12665

Although octamer-binding transcription factor 4 (Oct-4) is one of the most intensively studied factors in mammalian development, no cellular genes capable of replacing Oct-4 function in embryonic stem (ES) cells have been found. Recent data show that nuclear receptor subfamily 5, group A, member 2 (Nr5a2) is able to replace Oct-4 function in the reprogramming process; however, it is unclear whether Nr5a2 can replace Oct-4 function in ES cells. In this study, the ability of Nr5a2 to maintain self-renewal and pluripotency in ES cells was investigated. Nr5a2 localized to the nucleus in ES cells, similarly to Oct-4. However, expression of Nr5a2 failed to rescue the stem cell phenotype or to maintain the self-renewal ability of ES cells. Furthermore, as compared with Oct-4-expressing ES cells, Nr5a2-expressing ES cells showed a reduced number of cells in S-phase, did not expand normally, and did not remain in an undifferentiated state. Ectopic expression of Nr5a2 in ES cells was not able to activate transcription of ES cell-specific genes, and gene expression profiling demonstrated differences between Nr5a2-expressing and Oct-4-expressing ES cells. In addition, Nr5a2-expressing ES cells were not able to form teratomas in nude mice. Taken together, these results strongly suggest that the gene regulation properties of Nr5a2 and Oct-4 and their abilities to confer self-renewal and pluripotency of ES cells differ. The present study provides strong evidence that Nr5a2 cannot replace Oct-4 function in ES cells.

Introduction

Reprogramming of a somatic cell into a pluripotent cell represents a valuable resource for the development of clinical applications, such as patient-specific cell therapies, drug screening, and investigation of human disease models. An adult cell can be reprogrammed into a pluripotent state by nuclear transfer or by cell fusion [1], although these methods are hindered by ethical and technical limitations. Recently, a newly designed nuclear reprogramming method was achieved through retroviral transduction of fibroblasts with a

Abbreviations
BrdU, bromodeoxyuridine; Dox, doxycycline; EGFP, enhanced green fluorescent protein; ES, embryonic stem; Esrrb, estrogen-related receptor-β; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iPSC, induced pluripotent stem cell; miRNA, microRNA; Nr5a, nuclear receptor subfamily 5, group A; Nr5a2, nuclear receptor subfamily 5, group A, member 2; Oct-4, octamer-binding transcription factor 4; PI, propidium iodide.

set of transcription factors, such as octamer-binding transcription factor 4 (Oct-4), Sox2, Klf4, and c-Myc [2]. These factors are now called reprogramming factors or Yamanaka factors. This approach can be used to form induced pluripotent stem cells (iPSCs), which have properties similar to those of embryonic stem (ES) cells [1]. The generation of iPSCs with this or other modified approaches has now been reported from almost every mouse and human somatic cell type, by the use of various types of vector or small molecule [3]. Subsequent reports from many laboratories have provided a growing list of new combinations of factors for use in iPSC generation. The orphan nuclear receptor estrogen-related receptor-β (Esrrb) was reported to reprogram mouse embryonic fibroblasts to iPSCs without the need for the exogenous Klf4 transcription factor [4]. In addition, although Sox7 and Sox17, which are close family members of Sox2, cannot reprogram somatic cells in combination with Oct-4, Klf4, and c-Myc, rationally engineered Sox7 or Sox17 can replace Sox2 in the reprogramming of these cells [5,6]. Recently, it was reported that pluripotent stem cells can be derived from somatic cells with lineage specifier genes [7].

The *Oct-4* gene, also known as *Oct-3*, *Oct-3/4*, and POU domain, class 5, transcription factor 1 (*POU5F1*), encodes a nuclear protein that belongs to a family of transcription factors containing a POU DNA-binding domain [8–13]. Transcriptional regulation by Oct-4 is complex. In ES cells, the octamer sequence motif (5′-ATGCAAAT-3′) is active irrespective of its distance from the site of transcriptional initiation [8,14]. In differentiated cells, Oct-4 can transactivate its targets only when the octamer motif is proximal to the initiation site [9,15,16]. To be active from distal sites, it requires stem cell-specific bridging factors that link it to the transcription initiation site [17]. A number of factors, such as Sox2, HMG, E7, and E1A, are known to influence the ability of Oct-4 to act as an activator or a repressor [15,17–20]. Recently, physical associations of Oct-4 with EWS [21] and PKM2 [22] were documented, suggesting that EWS and PKM2 may also play a role in regulating Oct-4. However, in differentiated cells, it still remains to be determined whether Oct-4 binds to a site proximal to the transcription start site by genomics analysis. The expression of Oct-4 is normally restricted to pluripotent stem cells of pregastrulation embryos, including oocytes, early-cleavage-stage embryos, and the inner cell mass of the blastocyst, and is downregulated during differentiation [9,10,23,24]. In addition, *Oct-4* plays a predominant role in development. Homozygous germline mutations in *Oct-4* cause early lethality in mice, owing to the absence of an inner cell mass [25].

Oct-4 not only plays a pivotal role in mammalian development, but is also crucial for the derivation of iPSCs from somatic cells. Although Sox1 and Sox3, Klf2 and Klf5, and N-Myc and L-Myc, family members that are closely related to the Sox2, Klf4 and c-Myc reprogramming factors, are capable of replacing their counterparts, Oct-4 cannot be replaced by either of its two homologs, octamer-binding transcription factor-1 or octamer-binding transcription factor-6, to induce the development of iPSCs [26]. Recently, nuclear receptor subfamily 5, group A (NR5a), member 2 (Nr5a2) was shown to replace Oct-4 function in the reprogramming process [27]. Nr5a2 is also referred to as liver receptor homolog-1, and belongs to the NR5a subfamily (also known as the steroidogenic factor-like or Ftz-F1 subfamily). Four NR5a subfamily members have been identified: Nr5a1, Nr5a2, Nr5a3, and Nr5a4 [28]. The Nr5a2 protein is expressed mainly in endoderm-derived tissues and in the ovary [29]. *Nr5a2* knockout causes early embryonic lethality in mice, indicating its essential role in development [30]. In addition, Nr5a2 is involved in bile acid/cholesterol homeostasis and in the development of some human cancers [28,29]. Interestingly, a genome-wide binding analysis of Nr5a2 revealed that it colocalizes with the Oct-4–Sox2–Nanog cluster of transcription factors [31], suggesting that Nr5a2 may have an unknown, but significant, role(s) in the maintenance of ES cells.

Although Nr5a2 has been reported to replace Oct-4 in reprogramming of murine somatic cells to pluripotent cells, whether Nr5a2 can replace Oct-4 in the maintenance of self-renewal and pluripotency in ES cells has not been determined. To examine whether Nr5a2 can replace Oct-4 function in ES cells, we performed an ES cell-based complementation assay with ZHBTc4 ES cells. Like Oct-4, Nr5a2 localized to the nucleus in ZHBTc4 ES cells. However, expression of Nr5a2 failed to rescue the stem cell phenotype and to maintain the self-renewal ability of the ES cells. Moreover, as compared with Oct-4-expressing ES cells, Nr5a2-expressing ES cells showed a reduced number of cells in S-phase, and the cells neither expanded normally nor remained in an undifferentiated state. We also found that ectopically expressed Nr5a2 was not able to activate the transcription of ES cell-specific genes in ZHBTc4 ES cells, unlike Oct-4. In addition, gene expression profiling demonstrated differences between Nr5a2-expressing and Oct-4-expressing ZHBTc4 ES cells, and Nr5a2-expressing ZHBTc4 ES cells were unable to form teratomas in nude mice.

Taken together, these data imply that the gene regulation properties of Nr5a2 and Oct-4 and their abilities to confer self-renewal and pluripotency of ES cells differ. Thus, in contrast to the reprogramming process, the nuclear receptor Nr5a2 cannot replace Oct-4 function in ES cells.

Results

Establishment and characterization of Nr5a2-expressing ZHBTc4 ES cell lines

Although the *Nr5a2* gene encodes a transcription factor that is thought to replace Oct-4 in the reprogramming of somatic cells to pluripotent cells [27], whether Nr5a2 can substitute for Oct-4 in generating the self-renewal potential of ES cells is unknown. To investigate the ability of Nr5a2 to confer self-renewal ability on ES cells, we performed cell-based complementation assays [32] with ZHBTc4 ES cells. In ZHBTc4 cells, both endogenous alleles of *Oct-4* have been inactivated by gene targeting, and the cells instead harbor a tetracycline-regulated *Oct-4* gene [33]. In the absence of tetracycline, the *Oct-4* transgene is active, and ZHBTc4 cells behave as ES cells, but, in the presence of tetracycline, the *Oct-4* transgene is repressed [32]. In this system, the tetracycline analog doxycycline (Dox) can be used to prevent the expression of Oct-4 (Fig. S1).

To determine whether overexpression of Nr5a2 could replace the Oct-4-dependent self-renewal capacity of ES cells, an Nr5a2 cDNA under the control of the constitutive CAG expression unit was stably transfected into ZHBTc4 ES cells. Because it has been reported that an enhanced green fluorescent protein (EGFP)–Nr5a2 fusion protein is also functional as Nr5a2 [34], Nr5a2 was expressed as an EGFP fusion protein to monitor the expression of Nr5a2 in ES cells. In addition, a pCAG–IP expression vector lacking the Nr5a2 cDNA sequence (pCAG–IP/EGFP) was used as a negative control, and a pCAG–IP expression vector containing an Oct-4 cDNA sequence (pCAG–IP/Oct-4–EGFP) was used as a positive control. The structures of the expression vectors are shown schematically in Fig. 1A.

The amount of Nr5a2 protein in three different clones (cell lines #26, #41, and #73) was determined by western blot analysis (Fig. 1B). Whereas no Nr5a2–EGFP was detected in ZHBTc4 ES cells expressing EGFP or Oct-4–EGFP (Fig. 1B, lanes 1 and 2), Nr5a2–EGFP was expressed at similar levels in each of the three clonally derived cell lines used in this study (Fig. 1B, lanes 3–5). We also confirmed that EGFP, Oct-4–EGFP and Nr5a2–EGFP proteins were expressed at similar levels in all clonally derived ES cell lines used in the present study (Fig. 1B).

We tested whether ZHBTc4 ES cell lines expressing EGFP or Nr5a2 can be propagated as undifferentiated ES cell lines in the absence of Dox, a condition under which the *Oct-4* transgene is active. As shown in Fig. 1Ca–j, the self-renewal ability and stem cell phenotype of ZHBTc4 ES cells expressing EGFP or Nr5a2 was rescued when they were grown in the absence of Dox, indicating that they are not differentiated when they are propagated without Dox in the culture medium. As a positive control, ZHBTc4 ES cells expressing Oct-4 were cultured in the presence of Dox, because ZHBTc4 ES cells expressing Oct-4 can differentiate (owing to the superthreshold production of Oct-4) when they are propagated in the absence of Dox [32,33,35]. The colonies were also subjected to alkaline phosphatase staining. ZHBTc4 ES cells expressing EGFP or Nr5a2 had alkaline phosphatase activity when cultured in the absence of Dox (Fig. 1Ck–o), and the level of activity was comparable with that of ZHBTc4 ES cells expressing Oct-4 in the presence of Dox. These results indicate that the ZHBTc4 ES cells expressing EGFP or Nr5a2 used in this study are not differentiated when grown in the absence of tetracycline.

As the subcellular distribution of Nr5a2 in ES cells is unknown, we examined the localization of Nr5a2 by monitoring green fluorescence. In ZHBTc4 ES cells, Nr5a2–EGFP was clearly localized to the nucleus (Fig. 1Cc–e,h–j,m–o), whereas EGFP alone was present in both the nucleus and cytoplasm (Fig. 1Ca,f,k). Oct-4–EGFP localized to the nucleus (Fig. 1Cb,g,m), as previously reported [35]. These data indicate that Nr5a2 is a nuclear protein in ES cells.

Different abilities of Nr5a2 and Oct-4 to confer self-renewal in ES cells

Oct-4 supports ES cell self-renewal, and pluripotent ES cells expressing Oct-4 can be maintained indefinitely [36]. Little is known regarding the function of Nr5a2 in ES cells; therefore, to investigate whether Nr5a2 can replace Oct-4 function in ES cells, we performed cell-based complementation assays with ZHBTc4 ES cells expressing Nr5a2. All cells were grown in the presence of Dox to repress expression of the tetracycline-regulated *Oct-4* gene carried in the ZHBTc4 cells, and thus to allow for monitoring of cell characteristics in cells transfected with Nr5a2–EGFP, Oct-4–EGFP, or EGFP alone. The growth properties of ZHBTc4 ES cells expressing Nr5a2–EGFP and cultured in the presence of Dox were mon-

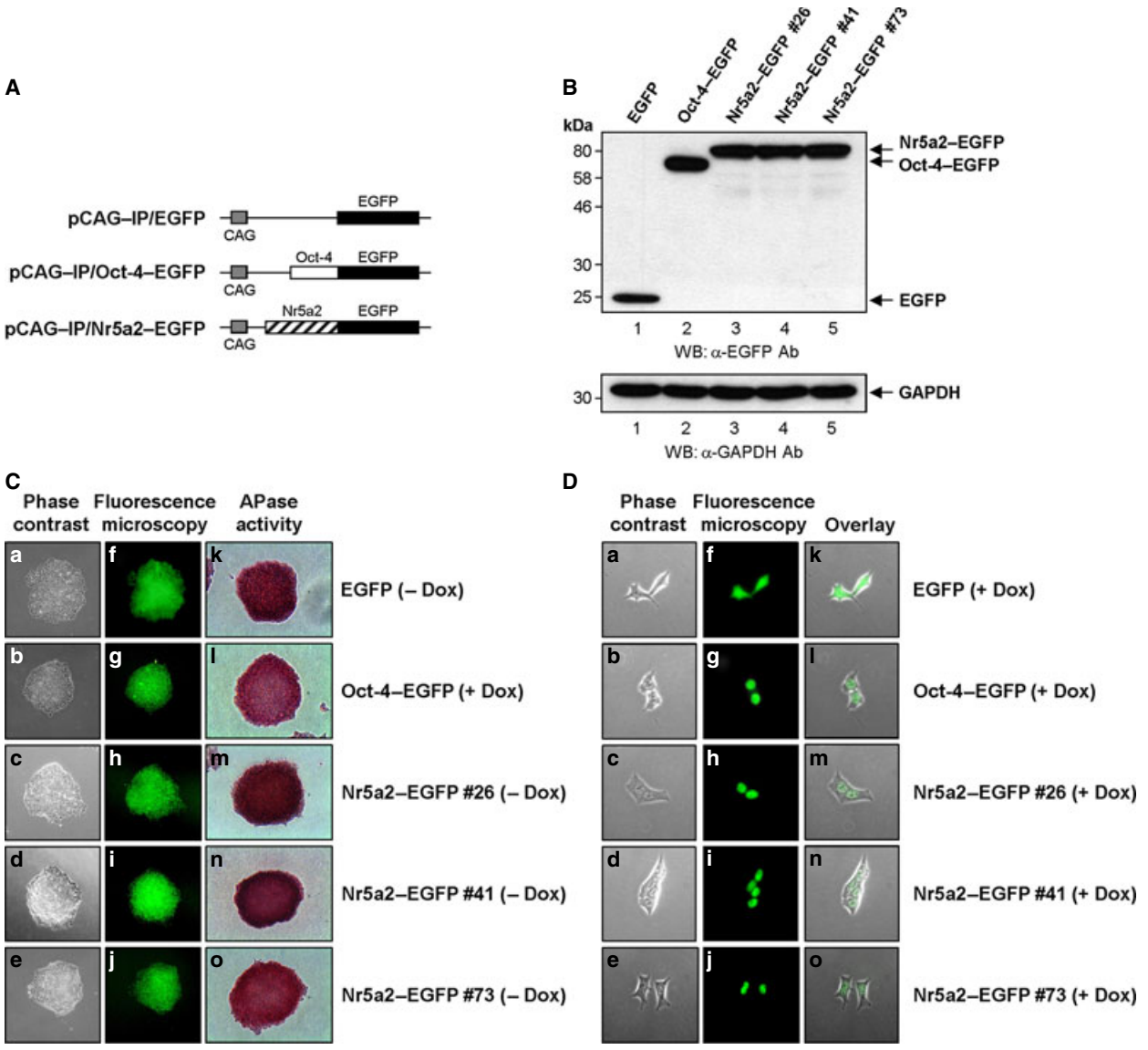


Fig. 1. Establishment of stable ES cell lines expressing Nr5a2, and the subcellular distribution of Nr5a2. (A) Schematic representation of the expression vectors used. Expression vectors pCAG–IP/Oct-4–EGFP or pCAG–IP/Nr5a2–EGFP express Oct-4 or Nr5a2, respectively, fused to EGFP. The pCAG–IP/EGFP expression vector was used as a control. (B) Immunoblot analysis of Nr5a2 expression in stably transfected ZHBTc4 ES cells. Total cell lysates (30 μg of protein) were fractionated by 10% SDS/PAGE and visualized by western blotting with antibody against EGFP (A11122; Invitrogen) or antibody against GAPDH (V-18; Santa Cruz Biotechnology). Lane 1: EGFP alone. Lane 2: Oct-4–EGFP. Lane 3: Nr5a2–EGFP (#26). Lane 4: Nr5a2–EGFP (#41). Lane 5: Nr5a2–EGFP (#73). (C) Characterization of ZHBTc4 ES cells expressing EGFP, Oct-4–EGFP, or Nr5a2. ZHBTc4 ES cells stably transfected with pCAG–IP/EGFP (labeled as EGFP), pCAG–IP/Oct-4–EGFP (labeled as Oct-4–EGFP), or pCAG–IP/Nr5a2–EGFP (labeled as Nr5a2–EGFP #26, #41, and #73) were cultured in the absence (for EGFP and Nr5a2–EGFP #26, #41, and #73) or presence (for Oct-4–EGFP) of Dox. Phase-contrast and fluorescence microscopy views are shown. Alkaline phosphatase (APase) activity was also assessed in ZHBTc4 ES cells expressing EGFP, Oct-4–EGFP, or Nr5a2–EGFP. (D) Subcellular localization of Nr5a2 in ES cells. ZHBTc4 ES cells (2.5×10^5) expressing EGFP (a, f, k), Oct-4–EGFP (b, g, l), Nr5a2–EGFP (#26) (c, h, m), Nr5a2–EGFP (#41) (d, i, n) or Nr5a2–EGFP (#73) (e, j, o) were plated on 0.2% gelatin-coated 60-mm dishes, and cultured in the presence of Dox (1 $\mu g \cdot mL^{-1}$). After 24 h, the cells were analyzed for EGFP by fluorescence microscopy. Ab, antibody; WB, western blot.

itored with an inverted phase-contrast microscope (Fig. 2A). Consistent with previous reports [32,33], ZHBTc4 ES cells transfected with Oct-4–EGFP and cultured in the presence of Dox formed colonies and grew like normal ES cells (Fig. 2Al,q). In contrast, expression of Nr5a2–EGFP failed to rescue the stem cell phenotype in the presence of Dox (Fig. 2Am,r for Nr5a2–EGFP in #26; Fig. 2An,s for Nr5a2–EGFP in

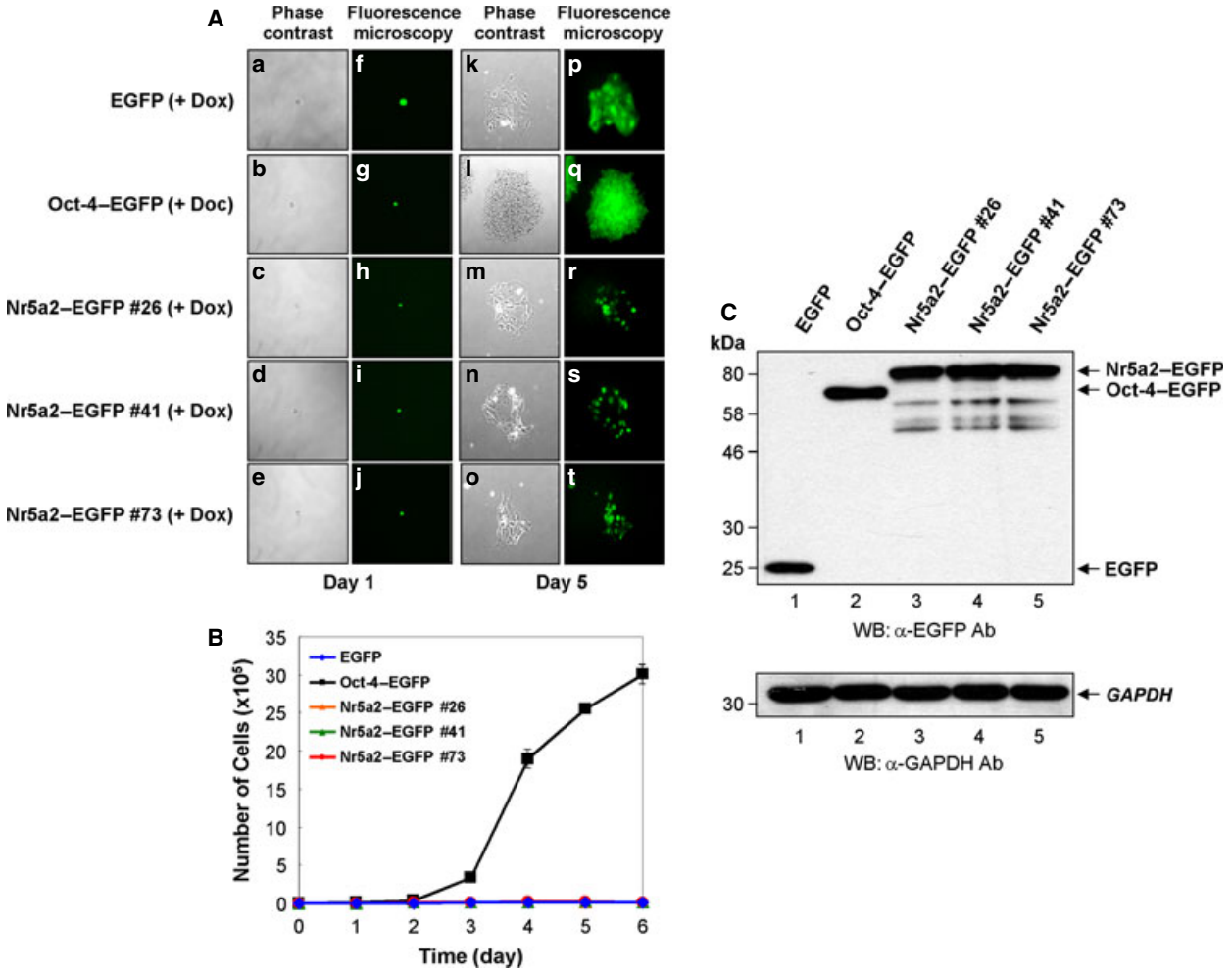


Fig. 2. Nr5a2 does not rescue self-renewal in ES cells. (A) Self-renewal potential of Nr5a2. ZHBTc4 ES cells (5×10^3) expressing EGFP (a, f, k, p), Oct-4–EGFP (b, g, l, q), Nr5a2–EGFP (#26) (c, h, m, r), Nr5a2–EGFP (#41) (d, i, n, s), or Nr5a2–EGFP (#73) (e, j, o, t) were plated onto 0.2% gelatin-coated 35-mm dishes, and cultured in the presence of 1 μg·mL^{-1} Dox for 6 days. Representative phase-contrast and fluorescence microscopy views are shown. (B) Growth characteristics of ZHBTc4 ES cells expressing Nr5a2 or Oct-4. Cells (5×10^3) were seeded onto 0.2% gelatin-coated 35-mm dishes, and counted at 1-day intervals with a hemocytometer for a total of 6 days. (C) Immunoblot analysis of the expression level of Nr5a2 in stably transfected ZHBTc4 ES cells at day 5. To compare Nr5a2, Oct-4 and EGFP protein levels at day 5, total cell lysates (30 μg of protein) were fractionated by 10% SDS/PAGE and visualized by western blotting with antibody against EGFP (A11122; Invitrogen) or antibody against GAPDH (V-18; Santa Cruz Biotechnology). Lane 1: EGFP alone. Lane 2: Oct-4–EGFP. Lane 3: Nr5a2–EGFP (#26). Lane 4: Nr5a2–EGFP (#41). Lane 5: Nr5a2–EGFP (#73). Ab, antibody; WB, western blot.

#41; and Fig. 2Ao,t for Nr5a2–EGFP in #73). These data indicate that the abilities of Oct-4 and Nr5a2 to confer self-renewal on ES cells differ. ZHBTc4 ES cells expressing EGFP alone also underwent differentiation in the presence of Dox (Fig. 2Ak,p). Similar results were obtained with a Flag-tagged Nr5a2 construct (Fig. S2).

To determine whether overexpression of Nr5a2 could maintain ES cell growth kinetics, ZHBTc4 ES cells expressing Nr5a2–EGFP or Oct-4–EGFP were cultured in the presence of Dox. Cells (5×10^3) were plated in 35-mm dishes, and the cell numbers were counted daily. In agreement with our previous reports [37], self-renewal of ZHBTc4 ES cells was rescued by expression of Oct-4–EGFP when they were cultured in the presence of Dox (Fig. 2B). However, the growth properties of ZHBTc4 ES cells expressing Nr5a2–EGFP or EGFP were markedly different from those of cells expressing Oct-4. ZHBTc4 ES cells expressing Nr5a2–EGFP or EGFP did not maintain self-renewal capacity, indicating that Nr5a2 cannot replace Oct-4 in self-renewal of ES cells.

To check whether the expression levels of Nr5a2–EGFP, Oct-4–EGFP and EGFP proteins were similar in each ZHBTc4 ES cell line cultured in the presence of Dox, the amounts of EGFP, Oct-4-EGFP and Nr5a2-EGFP proteins were determined by western blot analysis at day 5 (Fig. 2C). Although all stable cell lines expressing Nr5a2 were grown in the presence of Dox to repress expression of the tetracycline-regulated *Oct-4* transgene in ZHBTc4 cells, the expression levels of Nr5a2–EGFP (Fig. 2C, lanes 3–5 for Nr5a2 in #26, #41, and #73, respectively), Oct-4–EGFP (lane 2), and EGFP proteins (lane 1) were similar in all clonally derived ZHBTc4 ES cell lines cultured for 5 days in the presence of Dox (Fig. 2C), indicating that the failure to rescue the stem cell phenotype with Nr5a2 is not attributable to downregulation of Nr5a2 protein levels in ZHBTc4 ES cells.

To investigate the cell cycle distribution, the ZHBTc4 ES cells expressing Nr5a2–EGFP were cultured in the presence of Dox, and the cell cycle distribution was examined. A two-dimensional fluorescence-activated cell sorting (FACS) assay in which cells were sorted for DNA content, measured by propidium iodide (PI) staining, as well as active replication, measured by bromodeoxyuridine (BrdU) incorporation, was used. As compared with Oct-4–EGFP-expressing ES cells cultured in the presence of Dox (Fig. 3B), Nr5a2–EGFP-expressing ES cell clones (#26, #41, and #73) cultured in the presence of Dox showed a reduced number of cells in S-phase (Fig. 3C–E). ZHBTc4 ES cells expressing Nr5a2–EGFP showed an ~ 60% decrease (from 76.2% to 31.0%) in the number of ES cells in S-phase of the cell cycle, as compared with ZHBTc4 ES cells expressing Oct-4–EGFP. The control ES cell lines (expressing EGFP alone, and cultured in the presence of Dox) showed an ~ 65% decrease (from 76.2% to 27.0%) in the number of ES cells in S-phase of the cell cycle, as compared with ZHBTc4 ES cells expressing Oct-4–EGFP. Taken together, these data suggest that

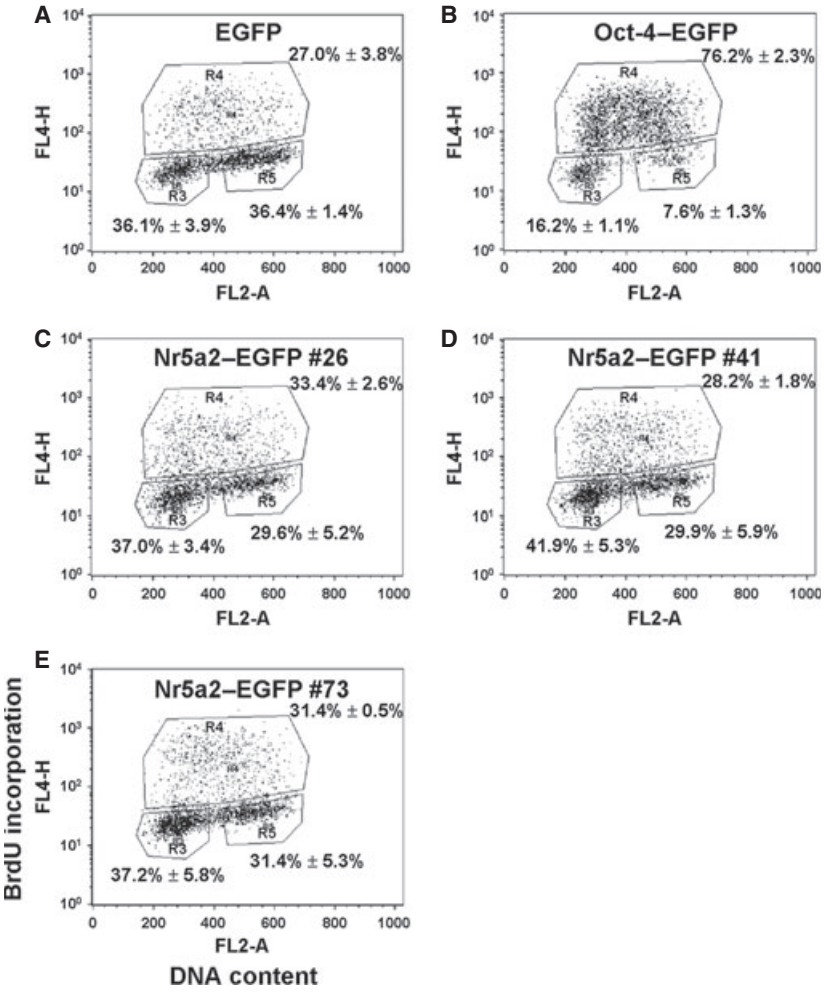


Fig. 3. Comparison of BrdU incorporation and DNA content in ZHBTc4 ES cells expressing EGFP (A), Oct-4–EGFP (B), Nr5a2-EGFP (#26) (C), Nr5a2–EGFP (#41) (D), and Nr5a2–EGFP (#73) (E). BrdU incorporation and DNA content of ZHBTc4 ES cells were measured by fluorescence with BrdU/PI staining. All cell lines were cultured in the presence of Dox. R3, R4 and R5 represent gating for G_0/G_1, S and G_2/M populations, respectively. Values are means ± standard deviations. FL4-H represents BrdU incorporation and FL2-A represents DNA content. Three independent experiments produced similar results. Ab, antibody; WB, western blot.

overexpression of Nr5a2 cannot substitute for Oct-4 in the self-renewal of ES cells.

Different abilities of Nr5a2 and Oct-4 to maintain the undifferentiated state

The flat morphology of Dox-treated ZHBTc4 ES cells expressing Nr5a2–EGFP (Fig. 2A) suggested a failure to maintain the undifferentiated state of the ES cells. To verify this hypothesis, known molecular markers of undifferentiated ES cells were examined. To stain for alkaline phosphatase activity (a marker of pluripotent cells of embryonic origin) [38], 3×10^3 ZHBTc4 ES cells expressing Nr5a2–EGFP, Oct-4–EGFP or EGFP were seeded onto 35-mm dishes, and cultured for 5 days in medium containing 1 $\mu g \cdot mL^{-1}$ Dox; the colonies were then stained. ZHBTc4 ES cells expressing Oct-4–EGFP showed alkaline phosphatase activity (Fig. 4Ab,g). However, ZHBTc4 ES cells expressing EGFP (Fig. 4Aa,f) and clones expressing Nr5a2–EGFP (Fig. 4Ac–e,h–j) did not show alkaline phosphatase activity, revealing a failure to maintain the undifferentiated state.

We also examined other known molecular markers of undifferentiated ES cells, such as Sox2 [39] and SSEA-1 [40]. Consistent with the results for alkaline phosphatase activity, ZHBTc4 ES cells expressing Oct-4–EGFP were positive for Sox2 (Fig. 4Bb,g,l,q) and SSEA-1 (Fig. 4Cb,g,l,q). However, ZHBTc4 ES cells expressing EGFP alone (Fig. 4Ba,f,k,p for Sox2; Fig. 4Ca,f,k,p for SSEA-1) and clones expressing Nr5a2–EGFP (Fig. 4Bc–e,h–j,m–o,r–t for Sox2; Fig. 4Cc–e,h–j,m–o,r–t for SSEA-1) did not express either marker. Taken together, these results suggested that ES cells expressing Oct-4 proliferated normally and remained in an undifferentiated state, whereas ES cells expressing Nr5a2 did not.

Nr5a2 is unable to activate the transcription of Oct-4-responsive genes

ES cell-based complementation assays with ZHBTc4 ES cells showed that, unlike in the reprogramming process, Nr5a2 could not replace Oct-4 function in sustaining ES cell self-renewal (Figs 2 and 3). In addition, Nr5a2-expressing ZHBTc4 ES cells showed differentiated ES cell phenotypes (Fig. 4). All of these properties suggest that Nr5a2 is not a transcriptional activator of Oct-4-responsive genes. To address this question, transient transfection assays were performed with differentiated and undifferentiated cells. Reporter assays were performed with 293T cells cotransfected

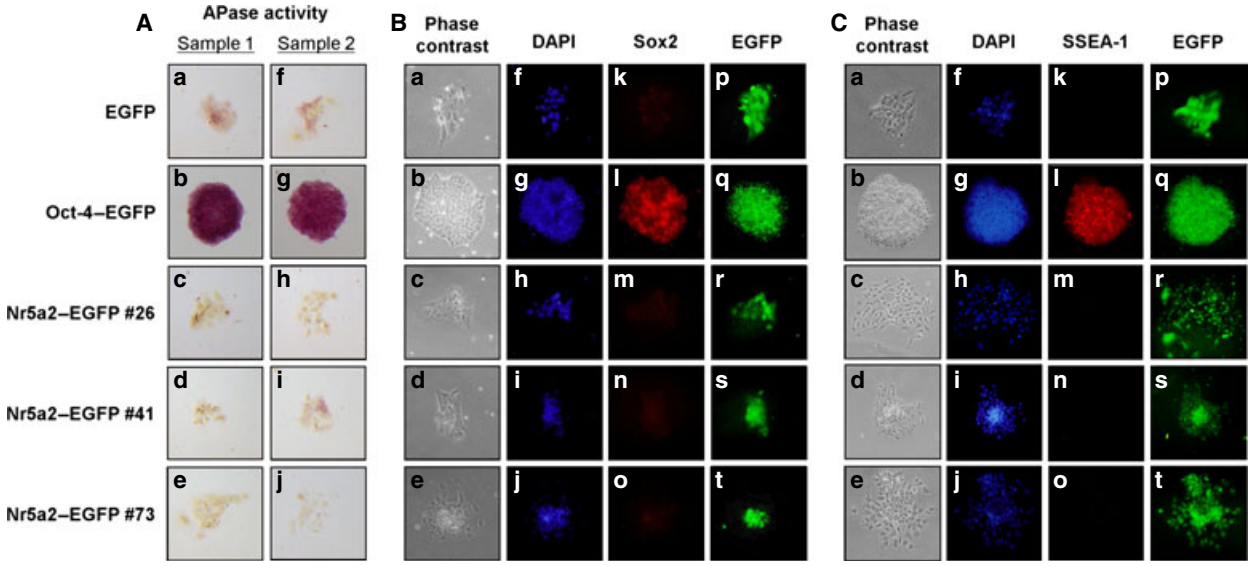


Fig. 4. Characterization of ZHBTc4 ES cells expressing Nr5a2. (A) Expression of alkaline phosphatase (APase). APase activity was assessed in ZHBTc4 ES cells expressing EGFP (a, f), Oct-4–EGFP (b, g), Nr5a2–EGFP (#26) (c, h), Nr5a2–EGFP (#41) (d, i), or Nr5a2–EGFP (#73) (e, j). (B) Expression of Sox2. The expression of Sox2 in ZHBTc4 ES cells expressing EGFP (a, f, k, p), Oct-4–EGFP (b, g, l, q), Nr5a2–EGFP (#26) (c, h, m, r), Nr5a2–EGFP (#41) (d, i, n, s) or Nr5a2–EGFP (#73) (e, j, o, t) was analyzed with an antibody against Sox2 (Y-17; Santa Cruz Biotechnology). Phase-contrast and fluorescence microscopy views for 4′,6-diamidino-2-phenylindole (DAPI), Sox2 and EFGP are shown. (C) Expression of SSEA-1. The expression of SSEA-1 in ZHBTc4 ES cells expressing EGFP (a, f, k, p), Oct-4–EGFP (b, g, l, q), Nr5a2–EGFP (#26) (c, h, m, r), Nr5a2–EGFP (#41) (d, i, n, s) or Nr5a2–EGFP (#73) (e, j, o, t) was analyzed with an antibody against SSEA-1 (480; Santa Cruz Biotechnology). Phase-contrast and fluorescence microscopy views for DAPI, SSEA-1 and EFGP are shown.

with either Oct-4 or Nr5a2 and a luciferase reporter plasmid with 10 consensus Oct-4-binding sites in the promoter (Fig. 5A). Cells cotransfected with Oct-4 showed up to 131-fold activation of the reporter (Fig. 5B), whereas Nr5a2 had no effect (Fig. 5B). We also performed an experiment with the known Nr5a2-regulated reporter pCYP11A1-Luc [41,42] as a positive control. Reporter assays were performed with 293T cells cotransfected with either Oct-4–EGFP or Nr5a2–EGFP, and a luciferase reporter plasmid carrying the murine *CYP11A1* promoter region [41,42] (Fig. S3). Cells cotransfected with Nr5a2–EGFP showed up to 45-fold activation of the reporter (Fig. S3, lanes 5–7), whereas Oct-4–EGFP had no effect (Fig. S3, lanes 2–4), indicating that the Nr5a2–EFGP used in this study is functional, as previously shown by others [34]. For undifferentiated cell assays, ZHBTc4 ES cells expressing Nr5a2–EGFP, Oct-4–EGFP or EGFP were transfected with the same reporter. Introduction of the pOct-4(10x)TATA–Luc reporter plasmid into ZHBTc4 ES cells expressing Oct-4–EGFP activated reporter gene transcription ~ 47-fold (Fig. 5C). Neither Nr5a2–EGFP nor EGFP alone activated the reporter in ZHBTc4 cells (Fig. 5C). Similar results were obtained with a reporter with an *Fgf-4* promoter/enhancer containing one Oct-4-binding site [43] (Fig. 5D). Hence, Nr5a2 cannot regulate Oct-4 target genes.

Distinct gene expression profiles of Nr5a2-expressing and Oct-4 expressing ZHBTc4 ES cells

To determine whether gene expression profiles in Nr5a2–EGFP-expressing or Oct-4–EGFP-expressing ZHBTc4 ES cells differed, a detailed, genome-wide expression analysis was performed with Nr5a2–EGFP-expressing and Oct-4–EGFP-expressing ES cells. The results showed 2.0-fold or greater changes in the level of gene expression between Nr5a2–EGFP-expressing and Oct-4-expressing ZHBTc4 ES cells. Scatter plot analysis showed that Nr5a2–EGFP-expressing ZHBTc4 ES cells and Oct-4-expressing ZHBTc4 ES cells could be distinguished by their gene expression signature (R^2 = 0.8001; Fig. 6A; Tables S1 and S2). The gene expression profiles of Nr5a2–EGFP-expressing ZHBTc4 ES cells and EGFP-expressing ZHBTc4 ES cells were also compared. Interestingly, although the phenotypic characteristics of Nr5a2–EGFP-expressing ZHBTc4 ES cells were quite similar to those of EGFP-expressing ZHBTc4 ES cells (Figs 2–4), the scatter plot analysis demonstrated that Nr5a2-expressing ZHBTc4 ES cells and EGFP-expressing ZHBTc4 ES cells could also be distinguished by their gene expression signatures (R^2 = 0.8288; Fig. 6B; Tables S3 and S4).

To investigate further the features of Nr5a2–EGFP-expressing ZHBTc4 ES cells, the expression levels of known ES cell-specific genes, such as *Nanog* [44,45], *Sox2* [39], *Rex-1* [46], and *Fgf-4* [47], were examined by real-time PCR. As shown in Fig. 6C, expression of all four genes was detected in Dox-treated Oct-4–EGFP-expressing ZHBTc4 ES cells. However, these genes were downregulated in Dox-treated Nr5a2–EGFP-expressing and EGFP-expressing ZHBTc4 ES cells. Dox-treated EGFP-expressing ZHBTc4 ES cells served as a negative control.

The expression of ES cell-specific microRNA (miRNA) RNAs, such as the Pri-miR-290 cluster and Pri-miR-302 cluster [48], were also examined. As shown in Fig. 6D,E, expression of the Pri-miR-290 cluster and Pri-miR-302 cluster was detected in Oct-4–EGFP-expressing ZHBTc4 ES cells, but not in Nr5a2–EGFP-expressing or EGFP-expressing ZHBTc4 ES cells. These properties all suggest that Oct-4-expressing ZHBTc4 ES cells proliferated normally and remained in an undifferentiated state, whereas Nr5a2-expressing ZHBTc4 ES cells did not.

Nr5a2-expressing ZHBTc4 ES cells do not form teratomas in nude mice

Finally, the ability of Nr5a2–EGFP-expressing ZHBTc4 ES cells to induce teratomas in nude mice was determined. Nude mice were given Dox in their drinking water for 2 weeks prior to injection of Nr5a2–EGFP-expressing ES cells, and the drug treatment was continued thereafter. As shown in Fig. 7, all six mice receiving Oct-4–EGFP-expressing ZHBTc4 ES cells developed large teratomas within a relatively short latent period. However, mice injected with Nr5a2–EGFP-expressing ZHBTc4 ES cells (#26) did not form teratomas, showing that Nr5a2 does not maintain stem cell pluripotency as efficiently as Oct-4. Similar results were obtained with the two other independent clones of Nr5a2-expressing ZHBTc4 ES cells (#41 and #73). ZHBTc4 ES cells expressing EGFP alone were used as a negative control. These results demonstrate that expression of Nr5a2 is not sufficient to maintain pluripotency of ES cells.

Discussion

Oct-4 plays a pivotal role in ES cell self-renewal and in the derivation of iPSCs from somatic cells. Despite a recent study demonstrating that the nuclear receptor Nr5a2 can replace Oct-4 in the reprogramming of somatic cells to iPSCs [27], little is known regarding Nr5a2 function in ES cell self-renewal. In this study,

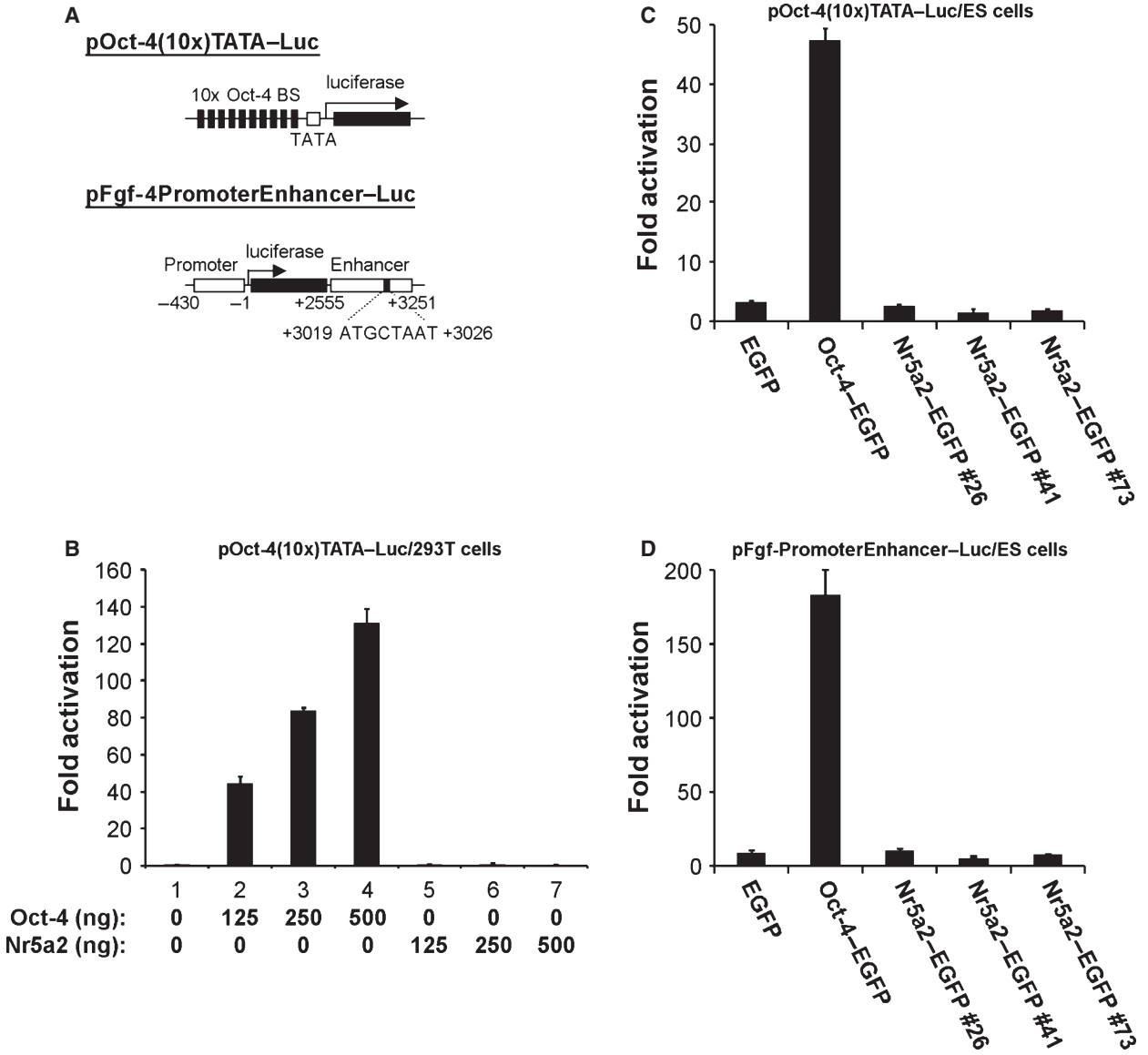
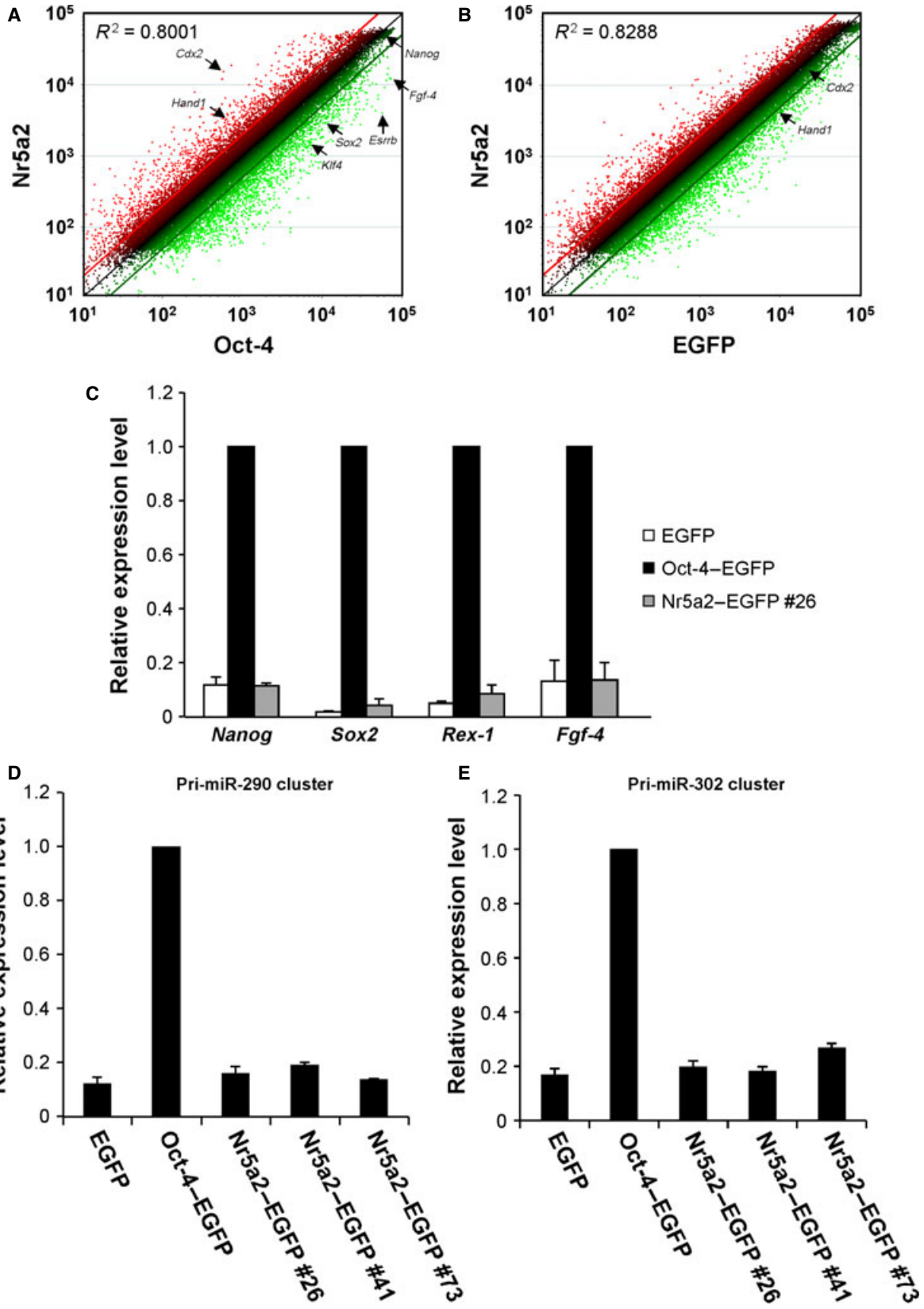


Fig. 5. Different transactivation properties of Nr5a2 and Oct-4. (A) Schematic representation of the pOct-4(10x)TATA–Luc and pFgf-4Enhancer–Luc reporter plasmids. The pOct-4(10x)TATA–Luc reporter plasmid contains 10 copies of the Oct-4-binding site upstream of a basal promoter–luciferase gene construct. The pFgf-4PromoterEnhancer–Luc reporter plasmid contains genomic DNA sequences from nucleotides −430 to −1 (for the promoter region) and +2555 to +3251 (for the enhancer region). The Oct-4-binding site in the enhancer region is indicated with the nucleotide sequence. (B) Transcriptional regulation by Nr5a2 with a pOct-4(10x)TATA–Luc reporter vector in differentiated cells. 293T cells were cotransfected with various amounts (0, 125, 250 and 500 ng) of expression vectors encoding Oct-4 or Nr5a2, the pOct-4(10x)TATA–Luc reporter vector, and the *Renilla* luciferase normalizing vector. For all assays, the total amount of transfected DNA was maintained by adjustment with empty vector. Firefly luciferase activity was normalized with *Renilla* luciferase activity to correct for transfection efficiencies. At least three independent transfections were performed, and the mean is plotted with the standard error (SE). Fold induction is relative to the empty expression vector. (C) Transcriptional regulation by Nr5a2 with a pOct-4(10x)TATA–Luc reporter vector in ES cells. ZHBTc4 ES cells expressing Nr5a2–EGFP or Oct-4–EGFP were cotransfected with the pOct-4(10x)TATA–Luc reporter vector and the *Renilla* luciferase normalizing vector. Firefly luciferase activity was normalized with *Renilla* luciferase activity to correct for transfection efficiencies. At least three independent transfections were performed, and the mean is plotted with the SE. Fold induction is relative to the empty expression vector. (D) Transcriptional regulation by the Nr5a2 with a pFgf-4PromoterEnhancer–Luc reporter vector in ES cells. ZHBTc4 ES cells expressing Nr5a2–EGFP or Oct-4–EGFP were cotransfected with the pFgf-4PromoterEnhancer–Luc reporter vector and the *Renilla* luciferase normalizing vector. Firefly luciferase activity was normalized with *Renilla* luciferase activity to correct for transfection efficiencies. At least three independent transfections were performed, and the mean is plotted with the SE. Fold induction is relative to the empty expression vector.


A
R^2 = 0.8001
Cdx2
Hand1
Nanog
Fgf-4
Sox2
Esrrb
Klf4
Nr5a2
Oct-4
B
R^2 = 0.8288
Cdx2
Hand1
Nr5a2
EGFP
C
Relative expression level
EGFP
Oct-4–EGFP
Nr5a2–EGFP #26
Nanog
Sox2
Rex-1
Fgf-4
D
Pri-miR-290 cluster
Relative expression level
EGFP
Oct-4–EGFP
Nr5a2–EGFP #26
Nr5a2–EGFP #41
Nr5a2–EGFP #73
E
Pri-miR-302 cluster
Relative expression level
EGFP
Oct-4–EGFP
Nr5a2–EGFP #26
Nr5a2–EGFP #41
Nr5a2–EGFP #73

we investigated whether Nr5a2 can replace Oct-4 function in ES cells. Nr5a2 is a nuclear receptor that is involved in lipid metabolism and modulates the expression of estrogen-related genes in some tissues [29]. This study showed that Nr5a2, like Oct-4, is a nuclear protein in ES cells, but it failed to rescue the stem cell phenotype or to maintain the self-renewal ability of *Oct-4*-null ES cells. In addition, a global gene expression analysis revealed differences between Nr5a2-expressing and Oct-4-expressing ES cells, suggesting that Nr5a2 function is not the same as Oct-4 function in ES cells.

The results reported here characterize the functions of Nr5a2 in ES cells. One of the remarkable properties of ES cells is their self-renewal capacity, which enables them to produce pluripotent daughter cells indefinitely [36]. Self-renewal is likely to be important for maintaining the stemness of ES cells, and Oct-4 plays a pivotal role in this process [33]. Downregulation of Oct-4 in ES cells inhibits proliferation by blocking cell cycle progression in G_0/G_1 [37]. Because Nr5a2 was reported to replace Oct-4 in the reprogramming of differentiated cells [27], the question arose as to whether Nr5a2 can also replace the Oct-4-mediated self-renewal function in ES cells. According to previous studies, the G_1 phase in ES cells is much shorter than in somatic cells [49–51], and Oct-4 controls the cell cycle progression of ES cells [37]. As shown in Figs 2 and 3, Oct-4 functions as a central mediator of ES cell self-renewal, as reported previously [33,37]. However, Nr5a2 did not support self-renewal, indicating that, unlike in the reprogramming process, Nr5a2 could not replace Oct-4 function in ES cells.

Oct-4-expressing ZHBTc4 ES cells possessed alkaline phosphatase activity (Fig. 4A), and were positive for Sox2 and SSEA-1 (Fig. 4B,C), whereas Nr5a2-expressing clones had lost alkaline phosphatase activity (Fig. 4A), and were negative for Sox2 and SSEA-1 (Fig. 4B,C). These results suggest that Nr5a2 failed to maintain the undifferentiated state. To determine the molecular signatures of Nr5a2-expressing ZHBTc4 ES cells, their global gene expression pattern was compared with that of Oct-4-expressing or EGFP-expressing ZHBTc4 ES cells. As shown in Fig. 6, Nr5a2-expressing ES cells are transcriptionally different from Oct-4-expressing ES cells, also suggesting that Nr5a2 function is not the same as Oct-4 function in ES cells.

Oct-4 encodes a POU transcription factor that is expressed by all pluripotent cells during embryogenesis and is also abundantly expressed in ES, embryonic

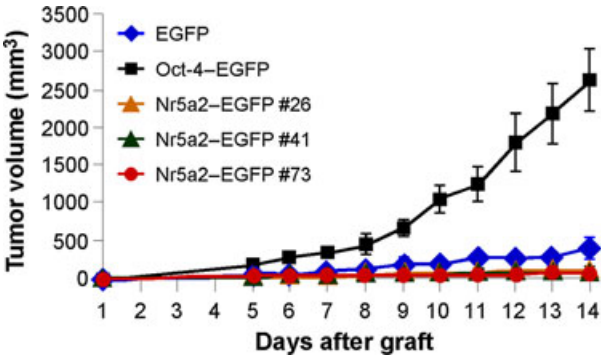


Fig. 7. Characterization of the effects of ZHBTc4 ES cells expressing Nr5a2 in nude mice. Approximately 1.0×10^7 ZHBTc4 ES cells expressing Nr5a2–EGFP (#26, #41, and #73), Oct-4–EGFP or EGFP alone were suspended in 100 μL of NaCl/P_i and injected into 8-week-old athymic nude mice. Tumor development was observed at 1-day intervals for a total of 2 weeks. The volume of palpable tumors derived from ZHBTc4 ES cells expressing Nr5a2, Oct-4 or EGFP was measured from 5 days after injection and plotted as mean increase ± standard error.

Fig. 6. Gene expression analysis in ZHTc4 ES cells expressing Nr5a2. (A) Scatter plots comparing the global gene expression patterns between Nr5a2–EGFP-expressing and Oct-4–EGFP-expressing ZHTc4 ES cell. Arrows indicate the expression levels of endogenous *Nanog*, *Fgf-4*, *Esrrb*, *Sox2*, *Klf4*, *Cdx2*, and *Hand1*. Fold changes of ≥ 2 are indicated above and below the lines parallel to the diagonal. Not all two-fold changes are statistically significant. (B) Scatter plots comparing the global gene expression patterns between Nr5a2–EGFP-expressing and EGFP-expressing ZHTc4 ES cells. Arrows indicate the expression levels of endogenous *Cdx2* and endogenous *Hand1*. Fold changes of ≥ 2 are indicated above and below the lines parallel to the diagonal. Not all two-fold changes are statistically significant. (C) Quantitative real-time PCR analyses of the expression of ES cell-specific markers. Real-time PCR analyses of *Nanog*, *Sox2*, *Rex-1* and *Fgf-4* mRNAs were performed in ZHBTc4 ES cells expressing EGFP, Oct-4–EGFP, or Nr5a2–EGFP. *Nanog*, *Sox2*, *Rex-1* and *Fgf-4* expression was normalized to that of the β-actin gene. Values represent the mean ± standard error (SE) of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. (D) Quantitative real-time PCR analyses of expression of the Pri-miR-290 cluster. Real-time PCR analysis of the Pri-miR-290 cluster was performed in ZHBTc4 ES cells expressing EGFP, Oct-4–EGFP, or Nr5a2–EGFP. Pri-miR-290 cluster expression was normalized to that of the β-actin gene. Values represent the mean ± SE of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown. (E) Quantitative real-time PCR analyses of expression of the Pri-miR-302 cluster. Real-time PCR analysis of the Pri-miR-302 cluster was performed in ZHBTc4 ES cells expressing EGFP, Oct-4–EGFP, or Nr5a2–EGFP. Pri-miR-302 cluster expression was normalized to that of the β-actin gene. Values represent the mean ± SE of triplicate assays. Similar results were obtained in three separate experiments, and representative data are shown.

germ and embryonic carcinoma cell lines. Oct-4 is a transcriptional regulator of genes involved in maintaining the undifferentiated pluripotent state in ES cells [16]. Oct-4 functions as a master switch during differentiation by regulating gene expression in pluripotent cells or in cells that have pluripotent potential [52]. Oct-4 is an essential transcriptional regulator of genes involved in maintaining the undifferentiated pluripotent state of ES cells. The results of the present study showed that Nr5a2 is unable to activate the transcription of Oct-4-responsive genes such as *Nanog*, *Sox2*, *Rex-1*, and *Fgf-4* (Fig. 6C). In addition, the ES cell-specific Pri-miR-290 and Pri-miR-302 miRNA clusters, which are activated in undifferentiated ES cells, were detected in Oct-4-expressing ZHBTc4 ES cells. However, expression of these ES cell-specific miRNAs was not detected in Nr5a2-expressing ZHBTc4 ES cells (Fig. 6D,E). From these results, it is clear that the orphan nuclear receptor Nr5a2 cannot substitute for Oct-4 function in ES cells, even though it can replace Oct-4 in the derivation of iPSCs from mouse somatic cells.

Nr5a2 is a highly expressed gene in ES cells, and its expression is rapidly downregulated upon differentiation [53,54]. Like disruption of *Oct-4*, disruption of *Nr5a2* leads to embryonic lethality at the epiblast stage of development [53]. In a broader context, our findings suggest that Nr5a2 may play different roles in reprogramming and self-renewal processes. Several studies have shown that nuclear receptors play diverse roles in the regulation of ES cell pluripotency; for instance, forced expression of Nr5a1 modulates the proliferation of ES cells [55–57]. Thus, further studies should be performed to investigate whether or not Nr5a2 has a regulatory role in maintenance of ES cell pluripotency. Our data show that, although Nr5a2 could replace Oct-4 in the reprogramming of murine somatic cells to pluripotent stem cells [27], Nr5a2 could not substitute for Oct-4 function during ES cell self-renewal, and the tumor volume of Nr5a2-expressing ZHBTc4 ES cells was smaller than that of EGFP-expressing ZHBTc4 ES cells. These findings support previous research showing that Nr5a2 is a potent epiblast stem cell reprogramming factor but not an ES cell self-renewal factor [58]. However, the findings of the current study do not explain why sustained expression of Nr5a2 is not sufficient to maintain ES cell self-renewal. Recent evidence suggests that Nr5a2 directly binds to the proximal enhancer and the proximal promoter regions of *Oct-4*, and regulates *Oct-4* expression through these sites [53]. Hence, it is possible that Nr5a2 may simply promote reprogramming by activating endogenous *Oct-4* expression. Further research should be performed to investigate whether N45a2 can still promote reprogramming in the absence of endogenous *Oct-4*.

Oct-4 expression is upregulated in some cancer stem cells [59,60]. Recent studies have shown that knockdown of Oct-4 blocks the tumorigenic potential of cancer stem cells [59,60]. In addition, some cancer stem cells express multidrug resistance genes, such as that encoding ATP-binding cassette transporter G2/breast cancer resistance protein 1 [61,62]. ATP-binding cassette transporter G2 is an ATP-binding cassette transporter and cell surface marker that causes multidrug resistance [61,63]. Therefore, more studies are needed to investigate whether cancer stem cells also express *Nr5a2*, and whether the expression of this gene can maintain cancer stem cell-like properties in human cancers. In addition, it would be interesting to investigate whether knockdown of Nr5a2 also blocks the tumorigenic potential of cancer stem cells. The identification of target genes that regulate the biochemical properties of cancer stem cells could also provide targets for rational drug design for therapeutic intervention. However, further investigation is needed to determine whether Nr5a2 is a good therapeutic target for blocking tumor development.

This study clearly demonstrates that Nr5a2 cannot replace Oct-4 function in ES cells. Although Nr5a2 can substitute for Oct-4 in the reprogramming of somatic cells to pluripotent cells, the expression of Nr5a2 alone is not sufficient to maintain stem cell self-renewal (Fig. 2) and the undifferentiated state (Fig. 4). Thus, because of its pivotal role in ES cell self-renewal, Oct-4 still remains unique and irreplaceable by other factors in maintaining the undifferentiated pluripotent state of ES cells.

Experimental procedures

Materials and general methods

Restriction endonucleases, calf intestinal alkaline phosphatase and T4 DNA ligase were purchased from New England Biolabs. PfuTurbo polymerase was purchased from Stratagene. The preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation, bacterial transformations and SDS/PAGE of proteins were carried out according to standard methods [64]. Subclones generated from PCR products were sequenced by use of the chain termination method with dsDNA templates to verify the sequences.

Constructs

To construct pCAG–IP/Nr5a2–EGFP, encoding a fusion protein of Nr5a2 and EGFP, the Nr5a2 (1–882) fragment

was amplified from a J1 mouse ES cell cDNA library by PCR with the primers 5′-Nr5a2-1 (5′-GATCGAATTCATGTCTGCTAGTTTGGATACTG-3′; *Eco*RI site underlined) and 3′-Nr5a2-882 (5′-CGGTGGATCCGTCTGGGTACTC-3′; *Bam*HI site underlined), and cloned into the pCR2.1–TOPO vector (Invitrogen) to yield pCR2.1–TOPO/Nr5a2 (1–882). The Nr5a2 (803–1680) fragment was also amplified from a J1 mouse ES cell cDNA library by PCR with the primers 5′-Nr5a2-803 (5′-GATCGAATTCTGCCACCTCACAGCAGC-3′; *Eco*RI site underlined) and 3′-Nr5a2-882 (5′-CGGTGGATCCCGGGCTCTTTTGGCATG-3′; *Bam*HI site underlined), and cloned into the pCR2.1–TOPO vector to yield pCR2.1–TOPO/Nr5a2 (803–1680). To generate pNr5a2 (1–822)–EGFP and pNr5a2 (803–1680)–EGFP, pCR2.1–TOPO/Nr5a2 (1–882) and pCR2.1–TOPO/Nr5a2 (803–1680) were digested with *Eco*RI and *Bam*HI, and the Nr5a2 fragments were cloned into the corresponding sites of pEGFP N1(Clontech). To generate full-length pNr5a2–EGFP, pNr5a2 (803–1680)–EGFP was digested with *Bst*XI and *Bam*HI, and the fragment was cloned into the *Bst*XI and *Bam*HI sites of pNr5a2 (1–822)–EGFP. Finally, to construct pCAG–IP/Nr5a2–EGFP, pNr5a2–EGFP was digested with *Xho*I and *Not*I, and the Nr5a2–EGFP fragment was cloned into the corresponding sites of the pCAG–IP vector. Details regarding the construction of pCAG–IP/EGFP and pCAG–IP/Oct-4–EGFP have been previously reported [35].

The construct pOct-4(10x)TATA–Luc has been described previously [21]. The pFgf-4PromoterEnhancer–Luc construct was generated with the following steps. The mouse Fgf-4 enhancer, which contains an Oct-4-binding site (5′-ATGCTAAT-3′) [43], was cloned into the *Bam*HI–*Sal*I sites of the promoterless pGL3 Basic vector (Promega, Madison, WI, USA) by PCR amplification of the sequence from a BAC library (clone number RP23-294B14; Children's Hospital Oakland–BACPAC Resources, Oakland, CA, USA) to generate pFgf-4Enhancer–Luc. The amplification primers used for this purpose were 5′-fgf4enhancer (5′-GATCGGATCCCTCTTCCACATGTAGTATC-3′; *Bam*HI site underlined) and 3′-fgf4enhancer (5′-GATCGTCGACTGGGCTATGAGACCGTC-3′; *Sal*I site underlined). To generate pFgf-4PromoterEnhancer–Luc, the Fgf-4 proximal promoter region (from −430 to −1) was amplified from a BAC library (clone number RP23-294B14) by PCR with the primers 5′-fgf4promoter (5′-GATCCTCGAGGCCTCTGGGGCCAGACCAAG-3′; *Xho*I site underlined) and 3′-fgf4promoter (5′-GATCAAGCTTGCCCAGCCCTCCGGAGCAG-3′; *Hin*dIII site underlined), digested with *Xho*I and *Hin*dIII, and cloned into the *Xho*I–*Hin*dIII sites of pFgf-4Enhancer–Luc. To generate the pCMV–Tag2A/Oct-4 construct, pcDNA3/Oct-4 [21] was digested with *Kpn*I, blunted with Klenow fragment, and redigested with *Xho*I. The excised fragment was ligated into the *Eco*RV and *Xho*I sites of pCMV–Tag2A (Stratagene, La Jolla, CA, USA). To generate the pCMV–Tag2B/Nr5a2 construct, pEF–BOS/Nr5a2 was digested with *Not*I, blunted with Klenow fragment, and redigested with *Bam*HI. The excised fragment was ligated into the *Bam*HI and blunted *Eco*RI sites of pCMV–Tag2B (Stratagene).

Establishment of Nr5a2-expressing ZHBTc4 ES cell line

The establishment of Nr5a2–EGFP-expressing ZHBTc4 ES cells was performed as previously described [65]. In brief, to generate ES cell lines stably expressing Nr5a2–EGFP, pCAG–IP/Nr5a2–EGFP was linearized with *Sca*I, and 6 μg of linearized DNA was transfected into ZHBTc4 ES cells (1×10^7 cells) by electroporation with the MicroPorator (Digital Bio, Seoul, Korea). At 48 h postelectroporation, puromycin (Sigma Aldrich, St Louis, MO, USA) was added to a final concentration of 1 $\mu g \cdot mL^{-1}$ to select clones carrying stably integrated plasmid DNA. After selection of transfected ZHBTc4 cells, individual clonal cell lines were isolated by picking individual puromycin-resistant colonies. Details regarding the establishment of Oct-4–EGFP-expressing and EGFP-expressing ZHBTc4 ES cells have been previously reported [35].

Western blot analysis

Western blot analysis was performed with antibodies against EGFP (A11122; Invitrogen, Carlsbad, CA, USA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (V-18p; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and reactive bands were detected by chemiluminescence with the Western Lightening system (PerkinElmer Life Sciences, Waltham, MA, USA).

Subcellular localization

To localize Nr5a2 in ES cells, 2.5×10^5 ZHBTc4 ES cells stably expressing EGFP, Oct-4–EGFP or Nr5a2–EGFP were plated on 60-mm dishes, and cultured for 24 h in the presence of Dox (1 $\mu g \cdot mL^{-1}$). The cells were then washed in NaCl/P_i, and green fluorescence was detected with a fluorescence microscope (IX71; Olympus, Tokyo, Japan) equipped with a DP71 digital camera (Olympus).

ES cell growth curve

ZHBTc4 ES cells were grown as previously described [32,35]. Equal numbers of ZHBTc4 cells (5×10^3) were plated in duplicate in 0.2% gelatin-coated 35-mm dishes, and cultured in the presence of Dox (1 $\mu g \cdot mL^{-1}$). Cells were harvested at 1-day intervals over a total culture time of 6 days. The total number of cells in each individual plate was determined by hemocytometer counts, and the means were calculated.

Cell cycle analysis

For cell cycle analysis, cells were grown for 10 min at 37 °C in the presence of 10 μM BrdU prior to fixation with ethanol and staining with PI and APC (allophycocyanin)-conjugated antibody against BrdU (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Flow cytometry data were acquired with a FACSCalibur Flow Cytometer (BD Biosciences), and the data were analyzed with CELLQUEST (BD Biosciences).

Alkaline phosphatase staining and immunocytochemistry

Alkaline phosphatase was stained with an alkaline phosphatase staining kit (Sigma Aldrich), as described previously [35]. For immunocytochemistry, ZHBTc4 ES cells expressing EGFP, Oct-4–EGFP or Nr5a2–EGFP were washed in NaCl/P_i, fixed for 30 min at 37 °C with 3.7% paraformaldehyde solution (3.7% formaldehyde and 0.18% Triton X-100), and stained with antibody against Sox-2 (Y-17; Santa Cruz Biotechnology) or antibody against SSEA-1 (480; Santa Cruz Biotechnology). After washing with NaCl/P_i, the plates or slides were incubated with tetramethylrhodamine-conjugated secondary antibody (Sigma Aldrich). Fluorescence was detected with a fluorescence microscope (Olympus, IX71) equipped with a DP71 digital camera (Olympus).

Reporter gene assays

Dox (1 μg·mL^{-1})-treated ZHBTc4 ES cells stably expressing EGFP, Oct-4–EGFP or Nr5a2–EGFP, or 293T cells, were transiently transfected with the pOct-4(10x)TATA–Luc or pFgf-4PromoterEnhancer–Luc reporter plasmids with the MicroPorator (Digital Bio Technology) for ES cells, or the VivaMagic Reagent (Vivagen, Seoul, Korea) for 293T cells, according to the manufacturer's instructions. Luciferase assays were performed with the Dual-Luciferase Reporter Assay System according to the supplier's protocol (Promega). *Renilla* luciferase activity was used to normalize the transfection efficiency.

Microarray analysis

Total RNA was isolated from ZHBTc4 ES cells expressing Nr5a2–EGFP, Oct-4–EGFP or EGFP with TRIzol reagent (Invitrogen). RNA quality control and DNase digestion were performed with an Agilent 2100 Bioanalyzer (Agilent Technologies). Cy3-labeled (reference) and Cy5-labeled (sample) complementary RNAs were prepared and used to hybridize an Agilent Whole Mouse 44k Genome Oligo Array (Agilent Technologies, Santa Clara, CA, USA) with the Gene Expression Hybridization Kit (Agilent Technologies), according to the manufacturer's instructions. The microarray data of Nr5a2–EGFP-expressing, Oct-4–EGFP-expressing and EGFP-expressing ZHBTc4 ES cells (GEO datasets) have been deposited with the accession number GSE52034.

Quantitative real-time PCR

Quantitative real-time PCR was performed with an Applied Biosystems 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) and SYBR Green Master Mix (Takara, Shiga, Japan), as described previously [35]. As a control, the level of β-actin mRNA was determined by real-time PCR of each RNA sample, and was used to correct for experimental variation. The following primer sequences were used: Nanog forward, 5′-CACCCACC CATGCTAGTCTT-3′; Nanog reverse, 5′-ACCCTCAAAC TCCTGGTCCT-3′; Sox2 forward, 5′-GCACATGAACGG CTGGAGCAACG-3′; Sox2 reverse, 5′-TGCTGCGAGTA GGACATGCTGTAGG-3′; Rex-1 forward, 5′-TGTCCTC AGGCTGGGTAGTC-3′; Rex-1 reverse 5′-TGATTTTCTG CCGTATGCAA-3′; Fgf-4 forward, 5′-CGAGGGACAG TCTTCTGGAG-3′; Fgf-4 reverse, 5′-ACCTTCATGGTAG GCGACAC-3′; Pri-miR-290 cluster forward; 5′-ACCTGG CTCCTAGCACAAACA-3′; Pri-miR-290 cluster reverse, 5′-GGGCTATTGTAAAGCCAAAAGGTA-3′; Pri-miR-302 cluster forward, 5′-TTCACCCTCCGAGGACAGAA-3′; Pri-miR-302 cluster reverse, 5′-ACAGACATAAGCTT TACCTCCTTTACCT-3′; β-actin forward, 5′-GCTCGTCG TCGACAACGGCTC-3′; and β-actin reverse, 5′-CAAACA TGATCTGGGTCATCTTCTC-3′. Quantification of the relative expression levels of *Nanog*, *Sox2*, *Rex-1*, *Fgf-4*, Pri-miR-290 cluster, and Pri-miR-302 cluster was achieved by normalizing to the endogenous β-actin level with the ΔC_T method of quantification.

Teratoma formation assays in nude mice

Teratoma formation assays were performed in nude mice as previously described [66]. In brief, 6-week-old athymic nude mice (CD1 nu/nu; Charles River, Wilmington, MA, USA) were pretreated with Dox (10 μg·mL^{-1}) in their drinking water for 2 weeks before injection. Subsequently, 1.0×10^7 cells from each cell clone in 100 μL of NaCl/P_i was injected subcutaneously into 8-week-old nude mice anesthetized with Avertin (2,2,2-tribromoethanol; Sigma Aldrich). The Dox treatment was continued as above. To determine the tumor volume with external calipers, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined. Teratoma volumes based on caliper measurements were calculated with the modified ellipsoidal formula [tumor volume = 1/2(length × width^2)], as previously reported [67–69]. Mice that developed tumors were killed after 14 days. All procedures were carried out in accordance with the animal experimentation guidelines of Sogang University, Seoul, Korea.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This research was supported by a grant (A084697) from the Korea Healthcare Technology R&D Project funded by the Ministry of Health & Welfare, Republic of Korea, and by the Sogang Research Frontier Program (SRF 2012-14003) from Sogang University. We thank H. Niwa for providing ZHBTc4 ES cells and the pCAG–IP vector.

References

1 Yamanaka S & Blau HM (2010) Nuclear reprogramming to a pluripotent state by three approaches. *Nature* **465**, 704–712.
2 Takahashi K & Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–676.
3 Ho R, Chronis C & Plath K (2011) Mechanistic insights into reprogramming to induced pluripotency. *J Cell Physiol* **226**, 868–878.
4 Feng B, Jiang J, Kraus P, Ng JH, Heng JC, Chan YS, Yaw LP, Zhang W, Loh YH, Han J *et al.* (2009) Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. *Nat Cell Biol* **11**, 197–203.
5 Jauch R, Aksoy I, Hutchins AP, Ng CK, Tian XF, Chen J, Palasingam P, Robson P, Stanton LW & Kolatkar PR (2011) Conversion of Sox17 into a pluripotency reprogramming factor by reengineering its association with Oct4 on DNA. *Stem Cells* **29**, 940–951.
6 Aksoy I, Jauch R, Eras V, Bin AC, Chen J, Divakar U, Ng CK, Kolatkar PR & Stanton LW (2013) Sox transcription factors require selective interactions with Oct4 and specific transactivation functions to mediate reprogramming. *Stem Cells* **31**, 2632–2646.
7 Shu J, Wu C, Wu Y, Li Z, Shao S, Zhao W, Tang X, Yang H, Shen L, Zuo X *et al.* (2013) Induction of pluripotency in mouse somatic cells with lineage specifiers. *Cell* **153**, 963–975.
8 Okamoto K, Okazawa H, Okuda A, Sakai M, Muramatsu M & Hamada H (1990) A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. *Cell* **60**, 461–472.
9 Scholer HR, Dressler GR, Balling R, Rohdewohld H & Gruss P (1990) Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. *EMBO J* **9**, 2185–2195.
10 Rosner MH, Vigano MA, Ozato K, Timmons PM, Poirier F, Rigby PW & Staudt LM (1990) A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* **345**, 686–692.
11 Goto T, Adjaye J, Rodeck CH & Monk M (1999) Identification of genes expressed in human primordial germ cells at the time of entry of the female germ line into meiosis. *Mol Hum Reprod* **5**, 851–860.
12 Hansis C, Grifo JA & Krey LC (2000) Oct-4 expression in inner cell mass and trophectoderm of human blastocysts. *Mol Hum Reprod* **6**, 999–1004.
13 Burdon T, Smith A & Savatier P (2002) Signalling, cell cycle and pluripotency in embryonic stem cells. *Trends Cell Biol* **12**, 432–438.
14 Scholer HR, Balling R, Hatzopoulos AK, Suzuki N & Gruss P (1989) Octamer binding proteins confer transcriptional activity in early mouse embryogenesis. *EMBO J* **8**, 2551–2557.
15 Scholer HR (1991) Octamania: the POU factors in murine development. *Trends Genet* **7**, 323–329.
16 Brehm A, Ohbo K & Scholer H (1997) The carboxy-terminal transactivation domain of Oct-4 acquires cell specificity through the POU domain. *Mol Cell Biol* **17**, 154–162.
17 Scholer HR, Ciesiolka T & Gruss P (1991) A nexus between Oct-4 and E1A: implications for gene regulation in embryonic stem cells. *Cell* **66**, 291–304.
18 Chew JL, Loh YH, Zhang W, Chen X, Tam WL, Yeap LS, Li P, Ang YS, Lim B, Robson P *et al.* (2005) Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. *Mol Cell Biol* **25**, 6031–6046.
19 Butteroni C, De Felici M, Scholer HR & Pesce M (2000) Phage display screening reveals an association between germline-specific transcription factor Oct-4 and multiple cellular proteins. *J Mol Biol* **304**, 529–540.
20 Brehm A, Ohbo K, Zwerschke W, Botquin V, Jansen-Durr P & Scholer HR (1999) Synergism with germ line transcription factor Oct-4: viral oncoproteins share the ability to mimic a stem cell-specific activity. *Mol Cell Biol* **19**, 2635–2643.
21 Lee J, Rhee BK, Bae GY, Han YM & Kim J (2005) Stimulation of Oct-4 activity by Ewing's sarcoma protein. *Stem Cells* **23**, 738–751.
22 Lee J, Kim HK, Han YM & Kim J (2008) Pyruvate kinase isozyme type M2 (PKM2) interacts and cooperates with Oct-4 in regulating transcription. *Int J Biochem Cell Biol* **40**, 1043–1054.
23 Scholer HR, Ruppert S, Suzuki N, Chowdhury K & Gruss P (1990) New type of POU domain in germ line-specific protein Oct-4. *Nature* **344**, 435–439.
24 Yeom YI, Ha HS, Balling R, Scholer HR & Artzt K (1991) Structure, expression and chromosomal location of the Oct-4 gene. *Mech Dev* **35**, 171–179.

25 Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H & Smith A (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**, 379–391.
26 Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N & Yamanaka S (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol* **26**, 101–106.
27 Heng JC, Feng B, Han J, Jiang J, Kraus P, Ng JH, Orlov YL, Huss M, Yang L, Lufkin T *et al.* (2010) The nuclear receptor Nr5a2 can replace Oct4 in the reprogramming of murine somatic cells to pluripotent cells. *Cell Stem Cell* **6**, 167–174.
28 Fayard E, Auwerx J & Schoonjans K (2004) LRH-1: an orphan nuclear receptor involved in development, metabolism and steroidogenesis. *Trends Cell Biol* **14**, 250–260.
29 Lee YK & Moore DD (2008) Liver receptor homolog-1, an emerging metabolic modulator. *Front Biosci* **13**, 5950–5958.
30 Pare JF, Malenfant D, Courtemanche C, Jacob-Wagner M, Roy S, Allard D & Belanger L (2004) The fetoprotein transcription factor (FTF) gene is essential to embryogenesis and cholesterol homeostasis and is regulated by a DR4 element. *J Biol Chem* **279**, 21206–21216.
31 Heng JC, Orlov YL & Ng HH (2010) Transcription factors for the modulation of pluripotency and reprogramming. *Cold Spring Harb Symp Quant Biol* **75**, 237–244.
32 Niwa H, Masui S, Chambers I, Smith AG & Miyazaki J (2002) Phenotypic complementation establishes requirements for specific POU domain and generic transactivation function of Oct-3/4 in embryonic stem cells. *Mol Cell Biol* **22**, 1526–1536.
33 Niwa H, Miyazaki J & Smith AG (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* **24**, 372–376.
34 Atanasov AG, Leiser D, Roesselet C, Noti M, Corazza N, Schoonjans K & Brunner T (2008) Cell cycle-dependent regulation of extra-adrenal glucocorticoid synthesis in murine intestinal epithelial cells. *FASEB J* **22**, 4117–4125.
35 Lee J, Kim HK, Rho JY, Han YM & Kim J (2006) The human OCT-4 isoforms differ in their ability to confer self-renewal. *J Biol Chem* **281**, 33554–33565.
36 Odorico JS, Kaufman DS & Thomson JA (2001) Multilineage differentiation from human embryonic stem cell lines. *Stem Cells* **19**, 193–204.
37 Lee J, Go Y, Kang I, Han YM & Kim J (2010) Oct-4 controls cell-cycle progression of embryonic stem cells. *Biochem J* **426**, 171–181.
38 Evans MJ & Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156.
39 Tomioka M, Nishimoto M, Miyagi S, Katayanagi T, Fukui N, Niwa H, Muramatsu M & Okuda A (2002) Identification of Sox-2 regulatory region which is under the control of Oct-3/4-Sox-2 complex. *Nucleic Acids Res* **30**, 3202–3213.
40 Koestenbauer S, Zech NH, Juch H, Vanderzwalmen P, Schoonjans L & Dohr G (2006) Embryonic stem cells: similarities and differences between human and murine embryonic stem cells. *Am J Reprod Immunol* **55**, 169–180.
41 Mueller M, Cima I, Noti M, Fuhrer A, Jakob S, Dubuquoy L, Schoonjans K & Brunner T (2006) The nuclear receptor LRH-1 critically regulates extra-adrenal glucocorticoid synthesis in the intestine. *J Exp Med* **203**, 2057–2062.
42 Mueller M, Atanasov A, Cima I, Corazza N, Schoonjans K & Brunner T (2007) Differential regulation of glucocorticoid synthesis in murine intestinal epithelial versus adrenocortical cell lines. *Endocrinology* **148**, 1445–1453.
43 Yuan H, Corbi N, Basilico C & Dailey L (1995) Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. *Genes Dev* **9**, 2635–2645.
44 Rodda DJ, Chew JL, Lim LH, Loh YH, Wang B, Ng HH & Robson P (2005) Transcriptional regulation of nanog by OCT4 and SOX2. *J Biol Chem* **280**, 24731–24737.
45 Kuroda T, Tada M, Kubota H, Kimura H, Hatano SY, Suemori H, Nakatsuji N & Tada T (2005) Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. *Mol Cell Biol* **25**, 2475–2485.
46 Ben-Shushan E, Thompson JR, Gudas LJ & Bergman Y (1998) Rex-1, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site. *Mol Cell Biol* **18**, 1866–1878.
47 Ambrosetti DC, Basilico C & Dailey L (1997) Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein–protein interactions facilitated by a specific spatial arrangement of factor binding sites. *Mol Cell Biol* **17**, 6321–6329.
48 Marson A, Levine SS, Cole MF, Frampton GM, Brambrink T, Johnstone S, Guenther MG, Johnston WK, Wernig M, Newman J *et al.* (2008) Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell* **134**, 521–533.
49 Neganova I & Lako M (2008) G1 to S phase cell cycle transition in somatic and embryonic stem cells. *J Anat* **213**, 30–44.
50 Becker KA, Ghule PN, Therrien JA, Lian JB, Stein JL, van Wijnen AJ & Stein GS (2006) Self-renewal of

human embryonic stem cells is supported by a shortened G1 cell cycle phase. *J Cell Physiol* **209**, 883–893.
51 Fluckiger AC, Marcy G, Marchand M, Negre D, Cosset FL, Mitalipov S, Wolf D, Savatier P & Dehay C (2006) Cell cycle features of primate embryonic stem cells. *Stem Cells* **24**, 547–556.
52 Ovitt CE & Scholer HR (1998) The molecular biology of Oct-4 in the early mouse embryo. *Mol Hum Reprod* **4**, 1021–1031.
53 Gu P, Goodwin B, Chung AC, Xu X, Wheeler DA, Price RR, Galardi C, Peng L, Latour AM, Koller BH *et al.* (2005) Orphan nuclear receptor LRH-1 is required to maintain Oct4 expression at the epiblast stage of embryonic development. *Mol Cell Biol* **25**, 3492–3505.
54 Wagner RT, Xu X, Yi F, Merrill BJ & Cooney AJ (2010) Canonical Wnt/beta-catenin regulation of liver receptor homolog-1 mediates pluripotency gene expression. *Stem Cells* **28**, 1794–1804.
55 Wagner RT & Cooney AJ (2013) Minireview: the diverse roles of nuclear receptors in the regulation of embryonic stem cell pluripotency. *Mol Endocrinol* **27**, 864–878.
56 Yazawa T, Kawabe S, Inaoka Y, Okada R, Mizutani T, Imamichi Y, Ju Y, Yamazaki Y, Usami Y, Kuribayashi M *et al.* (2011) Differentiation of mesenchymal stem cells and embryonic stem cells into steroidogenic cells using steroidogenic factor-1 and liver receptor homolog-1. *Mol Cell Endocrinol* **336**, 127–132.
57 Jadhav U & Jameson JL (2011) Steroidogenic factor-1 (SF-1)-driven differentiation of murine embryonic stem (ES) cells into a gonadal lineage. *Endocrinology* **152**, 2870–2882.
58 Guo G & Smith A (2010) A genome-wide screen in EpiSCs identifies Nr5a nuclear receptors as potent inducers of ground state pluripotency. *Development* **137**, 3185–3192.
59 Chen YC, Hsu HS, Chen YW, Tsai TH, How CK, Wang CY, Hung SC, Chang YL, Tsai ML, Lee YY *et al.* (2008) Oct-4 expression maintained cancer stem-like properties in lung cancer-derived CD133-positive cells. *PLoS ONE* **3**, e2637.
60 Atlasi Y, Mowla SJ, Ziaee SA & Bahrami AR (2007) OCT-4, an embryonic stem cell marker, is highly expressed in bladder cancer. *Int J Cancer* **120**, 1598–1602.
61 Dean M, Fojo T & Bates S (2005) Tumour stem cells and drug resistance. *Nat Rev Cancer* **5**, 275–284.
62 Hu L, McArthur C & Jaffe RB (2010) Ovarian cancer stem-like side-population cells are tumourigenic and chemoresistant. *Br J Cancer* **102**, 1276–1283.
63 Diestra JE, Scheffer GL, Catala I, Maliepaard M, Schellens JH, Scheper RJ, Germa-Lluch JR & Izquierdo MA (2002) Frequent expression of the multi-drug resistance-associated protein BCRP/MXR/ABCP/ABCG2 in human tumours detected by the BXP-21 monoclonal antibody in paraffin-embedded material. *J Pathol* **198**, 213–219.
64 Sambrook J & Russell DW (2001) Molecular Cloning: a Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York.
65 Lee J, Kim JY, Kang IY, Kim HK, Han YM & Kim J (2007) The EWS–Oct-4 fusion gene encodes a transforming gene. *Biochem J* **406**, 519–526.
66 Kim S, Lim B & Kim J (2010) EWS–Oct-4B, an alternative EWS–Oct-4 fusion gene, is a potent oncogene linked to human epithelial tumours. *Br J Cancer* **102**, 436–446.
67 Jensen MM, Jorgensen JT, Binderup T & Kjaer A (2008) Tumor volume in subcutaneous mouse xenografts measured by microCT is more accurate and reproducible than determined by 18F-FDG-microPET or external caliper. *BMC Med Imaging* **8**, 16.
68 Euhus DM, Hudd C, LaRegina MC & Johnson FE (1986) Tumor measurement in the nude mouse. *J Surg Oncol* **31**, 229–234.
69 Tomayko MM & Reynolds CP (1989) Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol* **24**, 148–154.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Doc. S1. Supplemental materials and methods.

Fig. S1. Schematic drawing of the Tet-off system in ZHBTc4 ES cells.

Fig. S2. Flag-tagged Nr5a2 is defective in its ability to confer self-renewal ability.

Fig. S3. Reporter assays of the effect of Nr5a2–EGFP on transcription from the *CYP11A1* gene promoter in 293T cells containing the pCYP11A1–Luc reporter plasmid.

Table S1. Genes showing more than two-fold higher expression in ZHBTc4 ES cells expressing Nr5a2–EGFP than in ZHBTc4 ES cells expressing Oct-4–EGFP.

Table S2. Genes showing more than two-fold lower expression in ZHBTc4 ES cells expressing Nr5a2–EGFP than in ZHBTc4 ES cells expressing Oct-4–EGFP.

Table S3. Genes showing more than two-fold higher expression in ZHBTc4 ES cells expressing Nr5a2–EGFP than in ZHBTc4 ES cells expressing EGFP.

Table S4. Genes showing more than two-fold lower expression in ZHBTc4 ES cells expressing Nr5a2–EGFP than in ZHBTc4 ES cells expressing EGFP.