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# A genome-wide screen in EpiSCs identifies Nr5a nuclear receptors as potent inducers of ground state pluripotency

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## SUMMARY

In rodents, the naïve early epiblast undergoes profound morphogenetic, transcriptional and epigenetic changes after implantation. These differences are maintained between blastocyst-derived embryonic stem (ES) cells and egg cylinder-derived epiblast stem cells (EpiSCs). Notably, ES cells robustly colonise chimaeras, whereas EpiSCs show little or no contribution. ES cells self-renew independently of mitogenic growth factors, whereas EpiSCs require fibroblast growth factor. However, EpiSCs retain the core pluripotency factors Oct4 and Sox2 and the developmental barrier dividing them from unrestricted pluripotency can be surmounted by a single reprogramming factor. This provides an opportunity to identify molecules that can reset the naïve state. We undertook a forward genetic screen for effectors of EpiSC reprogramming, employing *piggyBac* transposition to activate endogenous gene expression at random and selecting for undifferentiated colonies in the absence of growth factor signalling. Three recovered clones harboured integrations that activate the closely related orphan nuclear receptor genes *Nr5a1* and *Nr5a2*. Activity of *Nr5a1* and *Nr5a2* was confirmed by direct transfection. Reprogrammed colonies were obtained without transgene integration and at 10-fold higher frequency than with other single factors. Converted cells exhibited the diagnostic self-renewal characteristics, gene expression profile and X chromosome activation signature of ground state pluripotency. They efficiently produced adult chimaeras and gave germline transmission. Nr5a receptors regulate *Oct4* transcription but this is insufficient for reprogramming. Intriguingly, unlike previously identified reprogramming molecules, Nr5a receptors play no evident role in ES cell self-renewal. This implies a different foundation for their capacity to reset pluripotency and suggests that further factors remain to be identified.

**KEY WORDS:** Embryonic stem cells, Pluripotency, Reprogramming, Mouse

## INTRODUCTION

Mouse embryonic stem (ES) cells and post-implantation epiblast stem cells (EpiSCs) are considered to represent two phases in the ontogeny of the pluripotent epiblast (Rossant, 2008). ES cells are obtained from the initial naïve population that emerges in the mature blastocyst (Brook and Gardner, 1997; Evans and Kaufman, 1981; Nichols et al., 2009). They contribute extensively to chimaeras, demonstrating retention of early epiblast identity and associated unbiased developmental potential. EpiSCs are derived by culturing post-implantation egg cylinder epiblast (Brons et al., 2007; Tesar et al., 2007). They can differentiate into some cell types in culture, including germ cells (Hayashi and Surani, 2009), and can produce teratomas. However, they colonise blastocyst chimaeras poorly, if at all (Guo et al., 2009; Tesar et al., 2007). Intriguingly, stem cells derived from human embryos show features more in common with rodent EpiSCs than with ES cells (Nichols and Smith, 2009; Rossant, 2008).

ES cells and EpiSCs express the central pluripotency factors Oct4 (Pou5f1 – Mouse Genome Informatics) and Sox2 at similar levels, but diverge in other important respects. EpiSCs do not express, or have lower levels than ES cells, of many pluripotency-associated transcription factors, and they show upregulation of early lineage specification markers (Guo et al., 2009; Hanna et al.,

2009; Tesar et al., 2007). They also exhibit a differentiated epigenetic feature in female cells: the decoration of one X chromosome with the silencing mark H3K27me3 (Guo et al., 2009; Silva et al., 2003). EpiSCs do not respond productively to the ES cell self-renewal cytokine leukaemia inhibitory factor (LIF) (Brons et al., 2007) and they cannot be propagated in fully defined ES cell culture conditions, known as 2i, in which the mitogen-activated protein kinase (Erk; Mapk1) cascade and glycogen synthase kinase 3 (Gsk3) are selectively inhibited (Guo et al., 2009; Ying et al., 2008). ES cells are maintained in a relatively homogenous ‘ground state’ by application of these two inhibitors (Silva and Smith, 2008; Wray et al., 2010; Ying et al., 2008). EpiSCs, by contrast, appear to be variable both between and within cell lines. This might reflect both their origin from heterogeneous egg cylinder populations and the use of undefined culture environments, which may either select for, or induce, particular phenotypes. EpiSC-like cells derived on feeders in serum or serum replacement have been reported to acquire ES cell-like features without genetic manipulation (Bao et al., 2009; Greber et al., 2010). This is proposed to represent epigenetic reversion or dedifferentiation. However, the potential heterogeneity and developmental status of these EpiSC cultures are uncertain. Furthermore, EpiSCs on feeders can differentiate into primordial germ cells that may then undergo conversion into embryonic germ (EG) cells (Hayashi and Surani, 2009). Consequently, it is unclear what process underlies such spontaneous conversion. Importantly, for the present study, when EpiSCs are derived and maintained in defined conditions using fibroblast growth factor (FGF) and activin without feeders, they do not spontaneously acquire ES cell properties (Brons et al., 2007) and on transfer to 2i they very rarely form undifferentiated colonies (Guo et al., 2009). Robust conversion can be triggered, however,

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by transfection with single genes: either *Klf4*, *Klf2* or *Nanog* (Guo et al., 2009; Hall et al., 2009; Hanna et al., 2009; Silva et al., 2009). Reprogramming is dependent upon withdrawal of the EpiSC self-renewal factors FGF and activin, and is promoted by 2i in combination with LIF (Yang et al., 2010).

Interestingly, despite their closer developmental proximity to naïve pluripotency and their endogenous expression of Oct4 and Sox2, the efficiency of generating induced pluripotent stem (iPS) cells from transfected EpiSCs in defined culture is not demonstrably higher than from fibroblasts. Therefore, in addition to illuminating features of pluripotency and developmental restriction, characterising the transition from EpiSC to iPS cell might also contribute to an understanding of somatic cell reprogramming. To identify factors that can surmount the molecular roadblock between EpiSCs and ground state pluripotency we undertook a genome-wide screen.

## MATERIALS AND METHODS

### Ethics statement

Mouse studies were carried out in a designated facility under licences granted by the UK Home Office.

### Cell culture

EpiSCs derived from E5.5 mouse embryos (Guo et al., 2009) were cultured on fibronectin in N2B27 medium (Ying and Smith, 2003) with activin A (20 ng/ml) and FGF2 (12.5 ng/ml) prepared in-house. ES cells and iPS cells were cultured in 2i/LIF medium (Ying et al., 2008) comprising N2B27 with MEK inhibitor (1  $\mu$ M PD0325901), Gsk3 inhibitor (3  $\mu$ M Chir99021) and 200 units/ml LIF (Smith, 1991). LIF/BMP4 medium is N2B27 with LIF (100 units/ml) and 5 ng/ml BMP4 (R&D Systems). Immunostaining and blastocyst injection were performed as described (Guo et al., 2009).

### EpiSC reprogramming screen

Between 0.5 and  $0.7 \times 10^6$  EpiSCs carrying the *Oct4-GFPiresPuro<sup>R</sup>* transgene (Guo et al., 2009) were plated per well of a 6-well tissue culture plate in EpiSC culture media. The following day, cells were transfected using Lipofectamine 2000 (Invitrogen) and 1 day later the contents of each well were replated in a 10-cm plate. Hygromycin selection (200  $\mu$ g/ml) was applied for 3 days in EpiSC culture conditions to enrich for transfectants. Cultures were then transferred into 2i/LIF. Puromycin (1  $\mu$ g/ml) was applied to eliminate Oct4-negative differentiated cells before colony picking. *Rex1-GFPiresPuro<sup>R</sup>* knock in ES cells were generated by gene targeting.

### Vector construction, insertion site identification and PCR

The MSCV 5'LTR with a splice donor site from exon 1 of mouse *Foxf2* was amplified by PCR from T2/Onc (Dupuy et al., 2005) and inserted into the *NotI/PacI* sites of pGG131 (Guo et al., 2009) to produce the pGG134 gene-trap activation vector.

Insertion junctions were amplified using Splinkerette PCR (Guo et al., 2009; Mikkers et al., 2002). PCR products from screen 1 were cloned using the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen). Bacterial clones with PCR products were then re-amplified by colony PCR using HMsP2/Sp2 primers for both 5' terminal repeat (TR) and 3'TR junctions. Colony PCR products were treated with exonuclease I (New England Biolabs) to degrade single-stranded oligonucleotides, followed by ethanol precipitation. PCR products from screen 2 were separated by gel electrophoresis and individual bands were recovered. Sequencing was performed using HMsP3 and Sp3 primers for both 5'TR and 3'TR on an Applied Biosystems 3730xl DNA Analyser. Sequence results were annotated manually by BLAT search.

Open reading frames were amplified by PCR using the primers indicated below from cDNA prepared from Epi-iPS cells in which Nr5a1 or Nr5a2 were activated by the MSCV enhancer insertion. Sequence-verified products were inserted into *PB* expression vectors by Gateway cloning. For transient expression, open reading frames were sub-cloned into pPyCAG expression constructs (Chambers et al., 2003).

*Nr5a1* transgene was detected with primers CAG-For and Sf1-ex4-rev by genomic PCR. *Ube1* was amplified as a genomic DNA loading control using Ube1XA and Ube1XB primers.

Primers for PCR and RT-PCR (5' to 3'):

MSCV-For,  
ATCCGGATCCTTAATTAATAAGACCCACCTGTAGGTTT;  
LUNSD\_Rev,  
TTATGCGCCGCCAATGTATCTTAACGCGCGATGG;  
attB1-Nr5a1-ORF-For,  
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGACTATT  
CGTACGACGAGGAC;  
attB2-Nr5a1-ORF-Rev,  
GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAAGTCTGCTTGG  
CCTGCAGCATC;  
attB1-Nr5a2-ORFV2-For,  
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCTGCCC  
AAAGTGGAGACGGA;  
attB1-Nr5a2-ORFV1-For,  
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGTCTGCTA  
GTTTGGATACTGGAG;  
attB2-Nr5a2-ORF-Rev,  
GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAGGCTCTTTTGG  
CATGCAGCATC;  
Nr5a2-Ex2-For, GCACCAGGGTCAGAGACTCGCCA;  
Nr5a2-Ex3-For, ATGCTGCCCAAAGTGGAGACGGAA;  
Nr5a2-Ex4-For, CTCCTATGATGAAGATCTGGAAGAG;  
Nr5a2-Ex7-Rev, GGCTCGAATGAGGGCTTTCTTCTG;  
Nr5a2-V1-Ex1, ATGTCTGCTAGTTTGGATACTGGAG;  
Nr5a2-V2-Ex1, GTATCATCAGTTCAATACTAGATA;  
PB3'TR-Sp3, TTACGCACTATCTTTCTAGG;  
PB5'TR-Sp3, CGTCACAATATGATTATCTTTCTAGG;  
HMsP3, GTGGCTGAATGAGACTGGTGTGCGAC;  
CAG-For, GGCAACGTGCTGGTTGTTGTGCTGT;  
Sf1-ex4-rev, CCCTGTCTCCAGCTGAAGCCA;  
Ube1XA, TGGTCTGGACCCAACTGCTGCCACA; and  
Ube1XB, GGCAGCAGCCATCACATAATCCAGATG.

Other primers are described elsewhere (Guo et al., 2009; Hall et al., 2009).

### Real-time RT-PCR

Total RNA was prepared using the RNeasy Mini Kit (Qiagen) with DNaseI treatment. First-strand cDNA was synthesised using Superscript III reverse transcriptase (Invitrogen) with poly(dT) priming. Real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems) in a 7900HT Fast Real-Time PCR system (Applied Biosystems). Gene expression was determined relative to *Gapdh* using the  $\Delta$ Ct method. Standard deviation was calculated from PCR triplicates. TaqMan assays: *Nr5a1*, Mm00446826\_m1; *Nr5a2*, Mm00446088\_m1; *Oct4*, Mm00658129\_gH; *Klf4*, Mm00516104\_m1; *Klf2*, Mm01244979\_g1; *Klf5*, Mm00456521\_m1; *Nanog*, Mm02384862\_g1; *Rex1*, Mm03053975\_g; *Fgf5*, Mm00438615\_m1; *Lefty*, Mm00438615\_m1; brachyury (*T*), Mm01318252\_m1; *Nr0b1*, Mm00431729\_m1; *stella*, Mm00836373\_g1; *Gapdh*, 4352339E.

### Immunoblotting

Cells were harvested in SDS buffer (2% SDS, 10% glycerol, 60 mM Tris-HCl, pH 6.8) with Complete Protease Inhibitor Cocktail tablets (Roche). Samples were sonicated and protein concentration was determined by BCA assay (Pierce, Thermo Scientific). Total protein (11.5  $\mu$ g) was fractionated on a 4-12% Bis-Tris Novex gel, electroblotted onto nitrocellulose membranes, and the membranes probed sequentially either anti-Nr5a1 (R&D, PP-N1665-00) or anti-Nr5a2 (R&D, PP-H2325-00) and then anti- $\alpha$ -tubulin (Abcam). Blots were incubated with horseradish peroxidase-coupled anti-mouse IgG and developed with ECL Plus (Amersham). Membranes were stripped between probing by incubation at 70°C for 20 minutes in 50 mM Tris, pH 6.8, 2% SDS.

## RESULTS

### A piggyBac insertional activation screen identifies EpiSC reprogramming factors

We prepared a *piggyBac* (*PB*) gene-trap vector containing the murine stem cell virus (MSCV) enhancer/promoter (Dupuy et al., 2005) (Fig. 1A). PBBase-mediated transposition (Guo et al., 2009; Wang et al., 2008) was used to integrate the vector throughout the genome of Oct4GFP reporter (O4G) EpiSCs (Guo et al., 2009). Hygromycin selection for *PB* vector integration was maintained for 7 days. We speculated that transient co-expression of Klf4 might facilitate reprogramming. Therefore, we co-transfected a pool of EpiSCs with *PB* gene-trap construct plus circular Klf4 plasmid. After 3 days in FGF plus activin, transfectants were replated in 2i/LIF, which is both non-permissive for continued EpiSC propagation (Guo et al., 2009) and inductive/selective for iPS cells (Silva et al., 2008; Yang et al., 2010). Puromycin was added after 6 days in 2i/LIF to select for Oct4-positive cells. We picked two colonies on day 13 in 2i/LIF and 7 colonies on day 18. These colonies all expanded with ES cell-like morphology and stable GFP expression (Fig. 1B and Table S1 in the supplementary material).

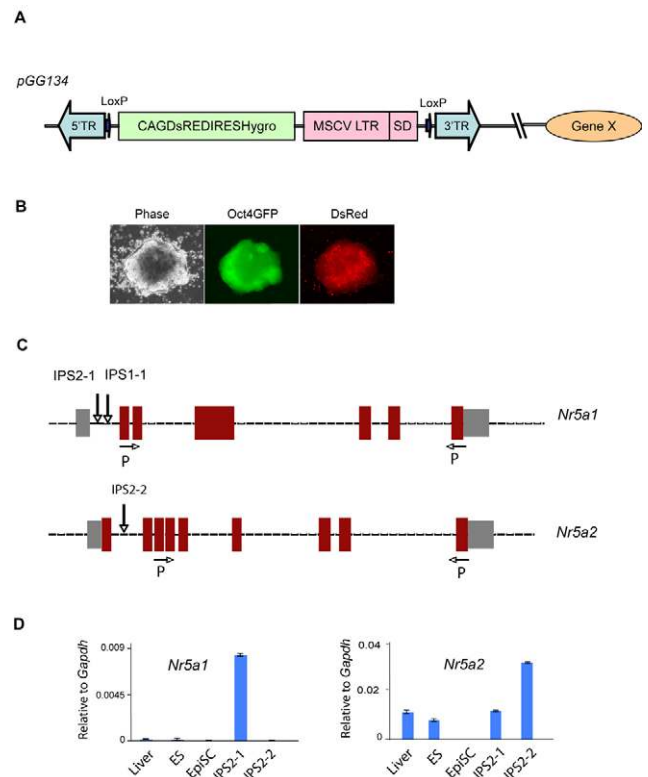
Integration sites were analysed using Splinkerette PCR (see Fig. S1A and Table S2 in the supplementary material). Among these, a potentially productive insertion was identified before the first coding exon of the *Nr5a1* gene on chromosome 2 (Fig. 1C). Unexpectedly, however, we detected integration of the Klf4 plasmid in several clones recovered from the screen (see Fig. S1B in the supplementary material). The Klf4 plasmid does not contain *PB* terminal repeats and is therefore not a substrate for transposition. We surmised that genomic double-strand breaks created by PBBase might facilitate plasmid integration. Indeed, we found that co-transfection of circular Klf4 plasmid with PBBase and a control *PB* vector was sufficient for low-frequency integration of the *Klf4* transgene and subsequent generation of EpiSC-derived iPS (Epi-iPS) cell clones in 2i/LIF.

We therefore performed a second screen without inclusion of Klf4 plasmid. We transfected  $2.5 \times 10^6$  EpiSCs. Conservative assumptions of 40% transfection efficiency and 30% plating efficiency with an average of four integrations per cell gave an estimate of  $1 \times 10^6$  insertions, or 15 times gene density. Nine colonies were picked and expanded. Splinkerette PCR indicated that these represent three independent clones, each with two to five integrations (see Fig. S1C in the supplementary material). One clone contained a vector insertion upstream of *Nr5a1* at a location distinct from that isolated in the first screen (Fig. 1C). A second clone harboured the gene-trap vector at the *Nr5a2* locus on chromosome 1. *Nr5a1* and *Nr5a2* are closely related genes that encode nuclear receptors also known as steroidogenic factor 1 (SF1) and liver receptor homologue 1 (Lrh1), respectively (Fayard et al., 2004; Galarneau et al., 1996; Hoivik et al., 2009; Lala et al., 1992; Morohashi et al., 1992).

We examined expression of *Nr5a1* and *Nr5a2* in EpiSCs, ES cells and reprogrammed clones. Neither *Nr5a1* nor *Nr5a2* transcripts were detected in EpiSCs (Fig. 1D). *Nr5a1* mRNA was undetectable in ES cells, but was present at low levels in the reprogrammed IPS2-1 clone with insertion at the *Nr5a1* locus. *Nr5a2* was expressed in ES cells and in both clones. Increased expression was apparent in the IPS2-2 clone with the *Nr5a2* insertion (Fig. 1D). These data indicate that the insertions activate endogenous Nr5a genes.

### Direct transfection with *Nr5a1* or *Nr5a2* converts EpiSCs to naïve pluripotency

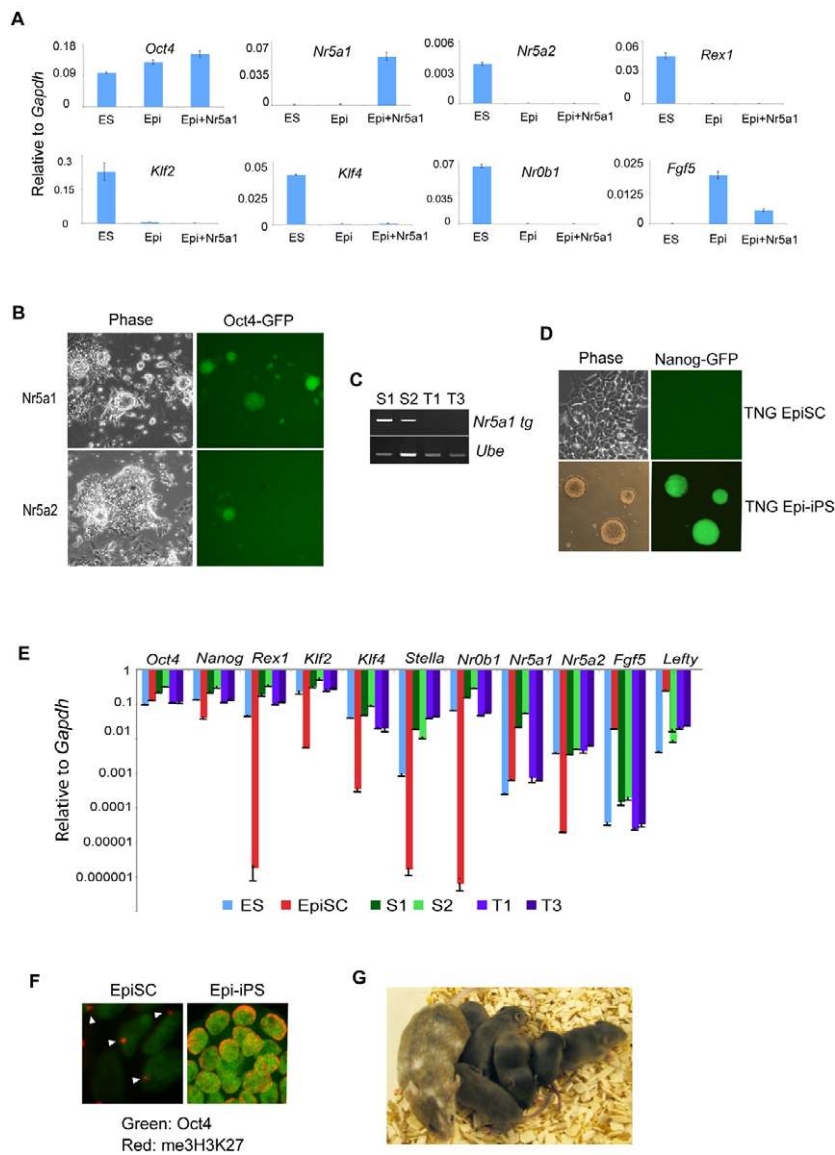
To test directly whether Nr5a receptors can reprogramme EpiSCs we prepared expression constructs in a *PB* vector. O4G EpiSCs were transfected and selected in hygromycin. In FGF plus activin,



**Fig. 1. Isolation of *Nr5a1* and *Nr5a2* by piggyBac mutagenesis screen.**

(A) Diagram of endogenous gene activation by randomly integrated *piggyBac* vector containing the MSCV enhancer. This construct can activate nearby genes in either orientation, although the splice donor can only be utilised 3' of the insertion site. (B) Primary Oct4-GFP-positive colony obtained in 2i/LIF after *PB*-MSCV vector transposition. (C) Insertions at mouse *Nr5a1* and *Nr5a2* loci. IPS1-1 was recovered from screen 1; IPS2-1 and IPS2-2 were recovered from screen 2. Red boxes, coding exons; white boxes, non-coding exons; P, primer location for open reading frame amplification. (D) qRT-PCR analysis of *Nr5a1* and *Nr5a2* expression in EpiSCs, ES cells and two iPS cell clones recovered from the screen.

stable transfectants showed no overt phenotype shift and the marker profile was unchanged (Fig. 2A). Upon transfer to 2i/LIF most cells died, but within 10 days over 100 GFP-positive undifferentiated colonies emerged per well of  $1 \times 10^4$  cells (Fig. 2B). We noted that expression of dsRed from the *PB* vector was downregulated in these colonies, indicative of transgene silencing. We therefore investigated transient expression by transfecting O4G EpiSCs with *PB*-*Nr5a1* only. Transfected cells were transferred to 2i/LIF after 48 hours. Vector-only transfections yielded no colonies, whereas  $\sim 100$  colonies per  $1 \times 10^4$  plated cells were obtained from *Nr5a1* transfection. Expanded colonies lacked detectable transgene integration or expression (Fig. 2C). Similar results were obtained by transient transfection of Nanog-GFP (TNG) EpiSCs. The Nanog reporter is barely detectable in EpiSCs, in line with low levels of *Nanog* transcript, but is bright in ES cells and iPS cells. Five or six days after transfection with *Nr5a1*, clusters of GFP-positive cells became visible within emerging colonies (Fig. 2D). *Nr5a2* is expressed as a different isoform in ES cells compared with somatic cells owing to the use of an internal promoter (Gao et al., 2006). The gene-trap insertion does not induce the longer, somatic cell transcript (see Fig. S2A,B



**Fig. 2. Nr5a transfection reprogrammes EpiSCs to naive pluripotency.** (A) Gene marker profile of *Nr5a1*-transfected EpiSCs maintained in FGF and activin. (B) Emerging Oct4-GFP-positive colonies 5 days after transfer of Nr5a stable transfectants to 2i/LIF. (C) Detection of *Nr5a1* transgene (*Nr5a1 tg*) by genomic PCR in Epi-iPS cell clones generated by stable (S) transfection but not by transient (T) transfection. Amplification of endogenous *Ube1* (*Ube*) provides a loading control. (D) Nanog-GFP is barely detectable in TNG EpiSCs but is intense in Nr5a1-reprogrammed derivatives 10 days after transient transfection and culture in 2i/LIF. (E) Gene marker profiles of reprogrammed cells generated by stable (S) or transient (T) transfection with Nr5a receptors. (F) Nuclear foci of H3K27me3 immunostaining (arrowheads) indicate an epigenetically modified X chromosome in EpiSCs, which is not exhibited by derivative Epi-iPS cells generated by transient expression of Nr5a1. (G) Germline chimaera and pups from mating with C57BL/6 male. Agouti pups are produced from Epi-iPS cell-generated oocytes. Epi-iPS cells were generated by transient expression of Nr5a1.

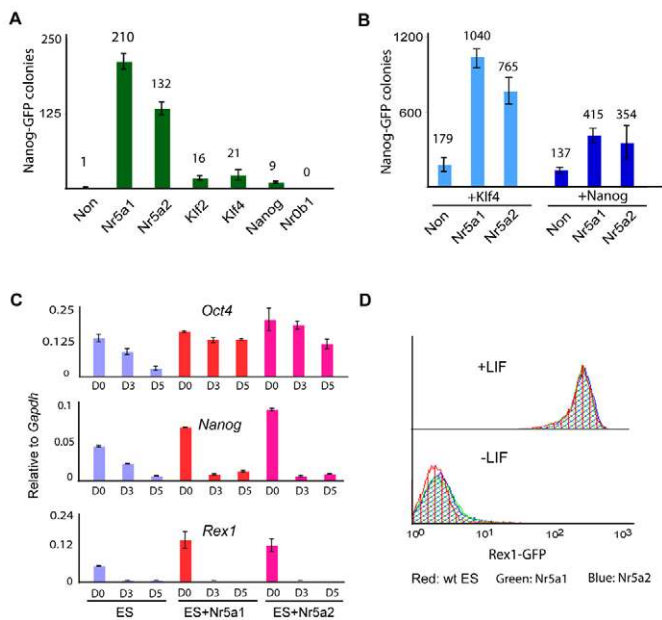
in the supplementary material). Nonetheless, we tested both isoforms and found that either can reprogramme EpiSCs (see Fig. S2C in the supplementary material).

We expanded cells obtained by transient transfection and interrogated them for expression of ES cell and EpiSC markers. In all cases, we found that the lineage-specific markers *Lefty* (*Lefty1*) and *Fgf5* were downregulated, and ES cell-specific markers, such as *Rex1* (*Zfp42*), *Nr0b1*, *stella* (*Dppa3*), *Klf2* and *Klf4*, were upregulated (Fig. 2E). Immunostaining for the H3K27me3 silencing mark on the X chromosome showed that the nuclear focus in the XX EpiSCs was erased in derivative Nr5a1-reprogrammed cells (Fig. 2F).

Propagation in 2i/LIF with appropriate marker expression is a good surrogate for ground state pluripotency, but the definitive criterion is contribution to healthy chimaeras. We injected two clones of O4G cells reprogrammed by transient expression of Nr5a1 into C57BL/6 blastocysts. Twenty of 25 live pups showed overt coat colour chimaerism, with 14 from one clone and six from the other. Three female chimaeras mated with C57BL/6 males produced agouti offspring, demonstrating transmission of the EpiSC genome (Fig. 2G).

### Nr5a receptors are potent EpiSC reprogramming factors but are not ES cell self-renewal factors

By utilising the MSCV enhancer/promoter, which drives moderate transcription and is subject to silencing, the screen was tailored to isolate factors that do not require high or sustained expression. This could explain why we did not isolate Nanog, Klf2 or Klf4, which have previously been shown to effect EpiSC reprogramming by stable transfection (Guo et al., 2009; Hall et al., 2009; Hanna et al., 2009). To examine directly whether Nr5a receptors are more potent, we prepared a series of transient expression constructs driven by the powerful CAG unit (Chambers et al., 2003; Niwa et al., 1991). In addition to known reprogramming factors, we included the orphan nuclear receptor Nr0b1 (Dax1), which is a well-defined target of Nr5a1 in steroidogenic cells (Hoivik et al., 2009), is regulated by Oct4/Sox2 in ES cells (Sun et al., 2008), and directly interacts with Oct4 (van den Berg et al., 2010). We transiently transfected wells of  $2 \times 10^4$  TNG EpiSCs and then cultured cells in 2i/LIF for 9 days (Fig. 3A). An average of over 200 Nanog-GFP-positive Epi-iPS cell colonies were obtained from Nr5a transfection, a frequency of at



**Fig. 3. Nr5a receptors are potent reprogramming factors in EpiSCs but are not ES cell self-renewal factors.** (A) Nanog-GFP colonies were manually counted after 9 days in 2i/LIF following transient transgene expression in TNG EpiSCs. The colony numbers shown are averaged from three wells, each plated with  $2 \times 10^4$  cells. (B) Nanog-GFP colonies in 2i/LIF reprogrammed from TNG EpiSCs by stable expression of *Klf4* or *Nanog* with and without transient *Nr5a* transfection. K4-S, stable *Klf4*; Ng-S, stable *Nanog*; K4+N1 or N2, stable *Klf4* plus transient *Nr5a1* or *Nr5a2*; Ng+N1 or N2, stable *Nanog* plus transient *Nr5a1* or *Nr5a2*. Data are means of triplicate assays. (C) *Oct4*, *Nanog* and *Rex1* mRNA expression in parental and *Nr5a* stable transfectant ES cells following withdrawal of LIF and differentiation in the presence of serum for 5 days. (D) Flow cytometry analysis of *Rex1*-GFP expression in ES cells cultured in serum plus LIF with puromycin or differentiated for 6 days after withdrawal of LIF and puromycin.

least 1% (assuming an EpiSC transient transfection efficiency of 50%, which is rather higher than routinely obtained). Slightly fewer colonies were obtained from TNG cells, possibly because the starting EpiSC population is more differentiated in the absence of selection. *Nr5a1* transfections consistently produced more colonies than *Nr5a2* transfections. An order of magnitude fewer colonies were obtained with *Nanog*, *Klf2* or *Klf4*, and no colonies were obtained from *Nr0b1* transfections.

We then investigated the combination of transient *Nr5a* with stable *Nanog* or *Klf4* transgene expression. EpiSCs stably transfected with either *Nanog* or *Klf4* convert at a frequency of 0.5–1% upon transfer to 2i/LIF. This was substantially increased upon transient transfection with *Nr5a* factors (Fig. 3B), by 4- to 6-fold in the case of *Klf4* transfectants and ~2-fold for *Nanog*. Furthermore, significantly more colonies were obtained than from parallel *Nr5a* transfections of parental EpiSCs (Fig. 3A). In the case of transient *Nr5a1* transfection into *Klf4*-expressing EpiSCs, the yield of over 1000 colonies corresponds to a frequency of ~10% of transiently transfected cells. These results indicate that *Nr5a* receptors act combinatorially with *Klf4* or *Nanog* to promote EpiSC reprogramming.

The previously identified EpiSC reprogramming factors *Klf4*, *Klf2* and *Nanog* are known to inhibit ES cell differentiation and sustain self-renewal without addition of LIF (Chambers et al.,

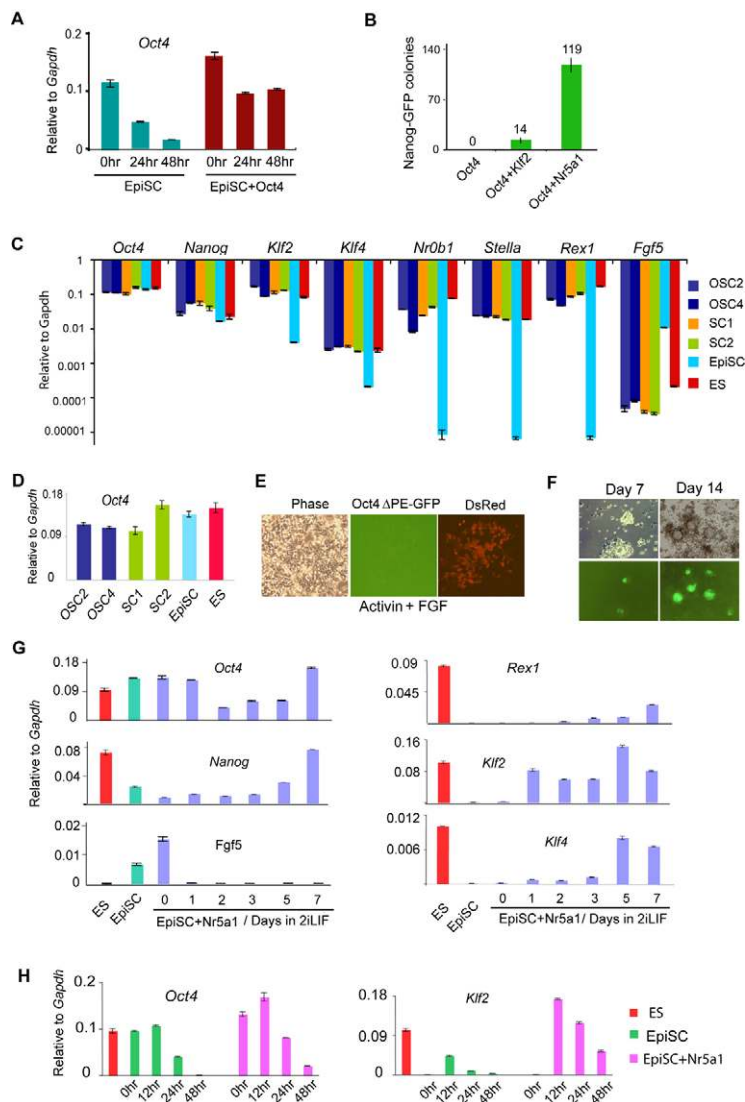
2003; Hall et al., 2009; Niwa et al., 2009). We tested whether forced expression of *Nr5a* receptors (see Fig. S3 in the supplementary material) could similarly confer reduced dependency on LIF. Upon LIF withdrawal, *Nr5a*-transfected ES cells maintained *Oct4* expression. However, they lost the expression of the ES cell markers *Nanog* and *Rex1* (Fig. 3C) and acquired a spread, differentiated morphology (data not shown). We also observed that *Rex1*-GFP knock-in reporter ES cells transfected with *Nr5a* lost GFP expression throughout the entire population after LIF withdrawal (Fig. 3D). Therefore, we find no evidence that *Nr5a* receptors sustain ES cell self-renewal even though they retard downregulation of *Oct4* (Gu et al., 2005).

### Sustained expression of *Oct4* is not sufficient to reprogramme EpiSCs

*Oct4* is downregulated by 24 hours in parental EpiSCs transferred to 2i/LIF, but we noted that expression is maintained in *Nr5a* transfectants. *Nr5a1* and *Nr5a2* have been reported to bind to *Oct4* upstream elements and promote transcription (Barnea and Bergman, 2000; Gu et al., 2005). We tested whether maintenance of *Oct4* expression is sufficient to mediate reprogramming in 2i/LIF. Transient transfection with a *PB-Oct4* expression vector did not yield any iPS cells. We therefore generated stable transfectants driving modest overexpression of *Oct4* (Fig. 4A). Upon transfer to 2i/LIF these *Oct4*-EpiSCs maintained *Oct4* transgene expression while downregulating endogenous *Oct4* (Fig. 4A). They showed reduced cell death compared with parental EpiSCs, but differentiated. *Oct4*-GFP expression was lost completely and no iPS cell colonies were obtained. A caveat to this experiment is that increased *Oct4* can cause ground state cells to differentiate (Niwa et al., 2000), raising the possibility that iPS cells might be lost if *Oct4* overexpression continued after reprogramming. To test whether the *Oct4* transfectants could produce Epi-iPS cells, we transiently transfected them with *Nr5a1*. Epi-iPS cell colonies were readily recovered and expanded in 2i/LIF (Fig. 4B). They showed transcriptional resetting to the ground state (Fig. 4C). Interestingly, these cells no longer overexpressed *Oct4* (Fig. 4D), indicating either that the transgene is silenced upon reprogramming or that autoregulatory feedback is in operation. These observations indicate that although sustaining the expression of *Oct4* may be part of the contribution of *Nr5a* receptors to reprogramming, it is not sufficient.

We also investigated whether *Nr5a* receptors directly induce *Oct4* enhancer switching. In EpiSCs and ES cells, *Oct4* expression is directed by distinct proximal (PE) and distal (DE) enhancers, respectively (Tesar et al., 2007; Yeom et al., 1996). Potential *Nr5a* binding sites have been identified in both enhancers as well as in the *Oct4* proximal promoter (Barnea and Bergman, 2000; Gu et al., 2005). The *Oct4* $\Delta$ PE-GFP transgene is active in ES and iPS cells but not in EpiSCs (Bao et al., 2009; Yoshimizu et al., 1999). Stable transfection with *Nr5a1* did not activate  $\Delta$ PE-GFP in EpiSCs maintained in FGF plus activin (Fig. 4E). Upon transfer to 2i/LIF, GFP became apparent in patches of cells only after 5–6 days (Fig. 4F). Reporter expression then spread throughout the expanding colonies, similar to observations described above for *Nanog*-GFP. These observations confirm that the distal enhancer is activated upon reprogramming, but also show that it is not an immediate target of *Nr5a* receptors.

Like activation of the distal *Oct4* enhancer, most ES cell-specific markers were not acutely upregulated after transfer into 2i/LIF and their expression rose gradually over 7 days in reprogramming cells (Fig. 4G). An exception is *Klf2*, which was upregulated within 12



#### Fig. 4. Reprogramming action of Nr5a receptors is not attributable to direct regulation of Oct4, Nanog or Klf2.

(A) TaqMan analysis of total *Oct4* mRNA levels in empty vector and *Oct4* stable transfectant EpiSCs in FGF plus activin and following transfer to 2i/LIF. (B) Numbers of Nanog-GFP-positive colonies formed from TNG EpiSCs after transient transfection of *Oct4* stable transfectants with *Klf2* or *Nr5a1*. (C) Marker profiles of Epi-iPS cells generated by *Nr5a1* transient transfection of EpiSCs with or without the integrated *Oct4* transgene. OSC2 and OSC4 are clones with the *Oct4* transgene; SC1 and SC2 are empty vector stable transfectants. (D) Total *Oct4* mRNA expression in Epi-iPS cells generated by transient transfection with *Nr5a1* of EpiSCs with or without an integrated *Oct4* transgene. (E) *Oct4*ΔPE EpiSCs stably transfectants with *Nr5a1* lack detectable GFP when maintained in activin plus FGF. DsRed fluorescence indicates expression of the transgene construct. (F) *Oct4*ΔPE-GFP-positive cells emerging after transfer of Nr5a transfectants into 2i/LIF. (G) qRT-PCR analysis of gene expression dynamics during EpiSC reprogramming induced by stable transfection with *Nr5a1* and transfer to 2i/LIF. Red, ES cells in 2i/LIF; green, O4G EpiSCs in activin plus FGF; blue, Nr5a-transfected O4G EpiSCs in activin plus FGF (day 0) and after transfer to 2i/LIF. (H) qRT-PCR analysis of *Oct4* and *Klf2* expression in EpiSCs with and without *Nr5a1* transfection in activin plus FGF (0 hours) and in 2i/LIF (12–48 hours). *Nr5a1* transfection is shown in pink and empty vector transfection in green. Expression in ES cells is shown as a control (red).

hours (Fig. 4H). *Klf2* is an integral component of the core gene regulatory network of ground state pluripotency (Hall et al., 2009; Jiang et al., 2008; Smith, 2010) and can replace *Klf4* in reprogramming (Nakagawa et al., 2008). It has recently been suggested that the expression of endogenous *Klf2* might mediate conversion of feeder-maintained EpiSCs (Greber et al., 2010). However, acute upregulation of *Klf2* is not sufficient in our conditions as it was observed in non-transfected cells that subsequently died or differentiated. Nr5a transfection did increase this initial expression of *Klf2* in 2i/LIF (Fig. 4H). It has been suggested that *Klf2* could be a direct target of Nr5a receptors (Heng et al., 2010), and transfection with *Klf2* can enable EpiSC reprogramming in 2i/LIF (Hall et al., 2009). We therefore tested the possibility that a combination of *Klf2* and *Oct4* regulation could account for the reprogramming efficacy of Nr5a receptors by transfecting the *Oct4*-EpiSCs with *Klf2*. As observed in parental EpiSCs, we found that *Klf2* transient transfection yielded only a small number of colonies – an order of magnitude fewer than with *Nr5a1* (Fig. 4B).

Collectively, these findings indicate that induction of previously identified reprogramming factors is not sufficient to explain the potent ability of Nr5a receptors to reset the naïve pluripotent state.

#### DISCUSSION

Reprogramming factors have primarily been discovered by testing candidate genes that are implicated in the pluripotent phenotype of ES cells (Feng et al., 2009; Silva et al., 2009; Takahashi and Yamanaka, 2006). In this study, a genomic screening approach has identified the potent activity of Nr5a nuclear receptors. Transient expression of these receptors in combination with 2i/LIF culture can reliably reset EpiSCs to ground state pluripotency. Furthermore, when combined with *Klf4*, reprogramming frequency is ~1 in 10 transfected cells. Although Nr5a receptors are known to regulate *Oct4* expression, our findings indicate that neither increasing nor maintaining *Oct4* levels is sufficient to reprogramme EpiSCs into naïve pluripotent stem cells.

The Nr5a class of nuclear receptors are thought to be able to function independently of ligand (Fayard et al., 2004). They are well-described regulators of steroid metabolism and endocrine homeostasis (Fayard et al., 2004; Hoivik et al., 2009). Nr5a1 also plays an essential role in sexual differentiation, whereas Nr5a2 has functions in primitive and definitive endoderm. Neither gene is essential in ES cells, nor for formation of the epiblast in vivo (Hoivik et al., 2009). Nr5a1 is not expressed in ES cells and there is no indication of loss of pluripotency or self-renewal capacity in

*Nr5a2*-deficient ES cells (Gu et al., 2005). In the embryo, deletion of *Nr5a2* causes disorganisation of the egg cylinder with loss of Oct4 and failed gastrulation (Gu et al., 2005; Pare et al., 2004). However, this phenotype is non-cell-autonomous and appears to be secondary to defective visceral endoderm development because *Nr5a2*-null cells are capable of progressing through gastrulation in tetraploid aggregation chimaeras (Labelle-Dumais et al., 2006). Consistent with a non-essential role in Oct4 regulation, EpiSCs lack both *Nr5a1* and *Nr5a2* yet show abundant Oct4.

Recent reports have indicated that EpiSCs may revert to ES cell status without genetic manipulation (Bao et al., 2009; Greber et al., 2010). In those studies, however, EpiSC cultures were undefined, being maintained on feeders with serum or serum replacement and without activin. EpiSCs may represent a heterogeneous mixture of cells under these conditions (Chou et al., 2008). Interestingly, these cultures show detectable levels of *Klf4* and *Klf2* and have similar levels of *Nanog* to ES cells (Greber et al., 2010; Tesar et al., 2007). By contrast, under defined, feeder-free, serum-free conditions using FGF and activin (Brons et al., 2007), *Klf2* and *Klf4* are not expressed and *Nanog* expression is 5- to 10-fold lower than in ES cells, whereas Oct4 and *Sox2* are fully maintained (Guo et al., 2009; Hall et al., 2009). From EpiSCs in these defined conditions the incidence of spontaneous generation of cells that can expand in rigorous 2i/LIF ground state culture is less than 1 in  $1 \times 10^{-6}$ . The relevance of EpiSC conversion to direct reprogramming is corroborated by a report, while our study was being finalised, that *Nr5a* factors can enhance the production of iPS cells from mouse fibroblasts (Heng et al., 2010). These authors assayed a panel of nuclear receptors in combination with the canonical reprogramming factors (Takahashi and Yamanaka, 2006). They also reported that *Nr5a* receptors can substitute for Oct4. However, in the case of EpiSCs, endogenous Oct4 is already present, yet *Nr5a* factors have a profound effect. Therefore, the major activity of *Nr5a* receptors in EpiSCs must be additional to inducing or replacing Oct4. It will be of great interest in future studies to determine which among the many proposed targets of *Nr5a* receptors (Heng et al., 2010) are functionally relevant to their reprogramming action and whether these are identical in the somatic cell and EpiSC contexts. The high frequency of EpiSC reprogramming triggered by *Nr5a* receptors, which is complete within 7 days, should also facilitate biochemical dissection.

Unlike other EpiSC reprogramming factors, *Nr5a* receptor deletion does not compromise ES cells (Gu et al., 2005), nor does their overexpression promote self-renewal or suppress differentiation (Fig. 3C). In fact, the silencing of integrated *Nr5a1* constructs and the greater efficiency of transient transfection suggest that continued high expression might even be deleterious in naïve pluripotent cells. The ability of *Nr5a* factors to induce pluripotency is therefore rather unexpected and likely to have a different origin from previously described factors. From inspection of UniGene EST profiles we observed that both *Nr5a1* and *Nr5a2* are expressed in mouse oocytes, zygotes and early cleavage stage embryos. It is therefore conceivable that these nuclear receptors might act as maternal reprogramming factors in the zygote and that this underlies their capacity to reset the pluripotent ground state. It will be interesting to examine the effect of oocyte-specific deletion of *Nr5a1* and *Nr5a2*.

In conclusion, these results validate EpiSC conversion in defined culture as an experimental system for interrogating molecular reprogramming and establish an unanticipated function of *Nr5a* receptors in the induction of pluripotency.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Author contributions

G.G. designed, executed, analysed and interpreted experiments. A.S. oversaw the study and wrote the paper.

#### Supplementary material

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