Dynamic methylation and expression of Oct4 in early neural stem cells

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Abstract

Neural stem cells are a multipotent population of tissue-specific stem cells with a broad but limited differentiation potential. However, recent studies have shown that over-expression of the pluripotency gene, *Oct4*, alone is sufficient to initiate a process by which these can form 'induced pluripotent stem cells' (iPS cells) with the same broad potential as embryonic stem cells. This led us to examine the expression of *Oct4* in endogenous neural stem cells, as data regarding its expression in neural stem cells *in vivo* are contradictory and incomplete. In this study we have therefore analysed the expression of *Oct4* and other genes associated with pluripotency throughout development of the mouse CNS and in neural stem cells grown *in vitro*. We find that *Oct4* is still expressed in the CNS by E8.5, but that this expression declines rapidly until it is undetectable by E15.5. This decline is coincident with the gradual methylation of the *Oct4* promoter and proximal enhancer. Immunostaining suggests that the Oct4 protein is predominantly cytoplasmic in location. We also found that neural stem cells from all ages expressed the pluripotency associated genes, *Sox2*, *c-Myc*, *Klf4* and *Nanog*. These data provide an explanation for the varying behaviour of cells from the early neuroepithelium at different stages of development. The expression of these genes also provides an indication of why Oct4 alone is sufficient to induce iPS formation in neural stem cells at later stages.

Key words methylation; Nanog; neural stem cells; Oct4; pluripotency.

Introduction

Neural stem cells (NSCs) are a class of tissue-specific somatic stem cell. During normal development they produce all the cell types of the brain including numerous subtypes of neurons and glial cells. As stem cells, they also self-renew to maintain a population of progenitors capable of giving rise to a range of cell types throughout development and adulthood. However, it has become clear that the phenotype of these cells varies as the nervous system matures (Merkle & Alvarez-Buylla, 2006). Initially, the neuroepithelial cells of the neural tube and early brain can self-renew and are capable of forming all cell types of the CNS. By late foetal stages, only a smaller population of radial glial cells, derived from the earlier neuroepithelial cells, retain this capacity. Finally, in mature animals, astroglial cells, concentrated in the subventricular region of the lateral ventricles and the subgranular region of the hippocampus, are the main NSCs of the brain. Although these three stages of NSCs differ significantly in their morphology and gene expression, they all share a common lineage and the ability to self-renew and to give rise to multiple cell types. Despite these similarities, the types of cells produced when these NSCs give rise to later progenitors changes with time and there is evidence that this is due to cell autonomous changes in the NSCs themselves (Falk & Sommer, 2009; Kriegstein & Alvarez-Buylla, 2009). In addition, during early embryogenesis, the cells of the neuroepithelium undergo significant changes, most evident in their different growth requirements *in vitro*. When the neuroepithelium first forms, the cells cannot be isolated as neurospheres under standard culture conditions. At these stages cells can be grown as spheres, but like ES cells, they require Leukemia inhibitory factor (LIF). These 'primitive NSCs' not only exhibit features of NSCs, but also share other markers in common with embryonic stem cells (Hitoshi et al., 2004). Between E7.5 and E8.5,

the cells of the neuroepithelium switch to a definitive NSC state when they can no longer grow in LIF but, like later NSCs, they now require fibroblast growth factor (FGF). There is then another transition to epidermal growth factor (EGF) dependence. It has been suggested that these sequential changes are, in part at least, under epigenetic control (Allen, 2008).

Recent studies have shown that pluripotency can be induced in many different somatic cell types by the over-expression of very few 'pluripotency' genes, in particular *Sox2*, *Oct4* (*Pou5f1*), *c-Myc* and *Klf4*, to produce induced pluripotent stem cells (iPS) (Takahashi & Yamanaka, 2006; Wernig et al., 2007). NSCs are unusual in this respect, as they can be reprogrammed to iPS cells by the over-expression of *Oct4* alone (Kim et al., 2009a,b). It seems that active expression of these 'pluripotency' genes is central to the conversion of any cell to an iPS cell. To date, it appears that endogenous NSCs do, in fact, express *Sox2* and *c-Myc* (Eminli et al., 2008). Indeed, there is evidence that they also express *Klf4* (Kim et al., 2008, 2009b). However, there are few reports in which *Oct4* expression has been analysed in NSCs and these provide conflicting evidence (Tropepe et al., 2001; Okuda et al., 2004; Lengner et al., 2007; Akamatsu et al., 2009; Chin et al., 2009; Takehara et al., 2009). It remains unclear, therefore, to what extent *Oct4* is expressed in the developing CNS and in NSCs in particular.

In this paper, we have carried out a detailed analysis of the expression and methylation of *Oct4* in NSCs derived from developing mouse embryos. We have also analysed the expression of the other key genes associated with pluripotency (*Sox2*, *Klf4, c-Myc* and *Nanog*).

Experimental procedures

Cell culture

Primary neurosphere cultures were established from the forebrain regions of CD1 mice (Charles Rivers, UK) and were maintained in growth medium comprising; Neurobasal medium (Gibco, Invitrogen, http://www.invitrogen.com), DMEM F12 (Gibco, Invitrogen), Penicillin⁄Streptomycin (Sigma, http://www.sigmaaldrich.com), B27 (1×) and N2 (1×) supplements (Gibco, Invi-trogen), FGF (20 ng mL^{-1}) and EGF (20 ng mL^{-1}) (Invitrogen). All expression and methylation analyses were carried out on neuro-spheres at passages 3–5. The mouse embryonic stem cell line E14 IV was maintained in GMEM medium containing foetal calf serum (10%), non-essential amino acids (1×), LIF (1×), mercapto-ethanol (0.1 mM), glutamine (2 mM) and sodium pyruvate (1 mM). The human neural stem⁄progenitor cell line ReN VM (Millipore, http://www.millipore.com) was cultured as neurospheres in growth medium or adherently on laminin (15 ng mL^{-1})-coated flasks in complete ReNcell NSC Maintenance Medium (Millipore), containing FGF (20 ng mL^{-1}) and EGF (20 ng mL^{-1}).

Reverse transcription PCR analysis

Total RNA was extracted from cell pellets using TRI reagent (Sigma) and digested with DNAse1 (Invitrogen) to prevent geno-mic DNA contamination. cDNA synthesis was carried out using random primers (Promega, http://www.promega.com) with SuperScript III Reverse Transcriptase (Invitrogen). PCR was carried out using Platinum Taq polymerase (0.04 U μL^{-1}, Invitro-gen), forward primer (1 μM), reverse primer (1 μM), PCR buffer (1×), MgCl$_2$ (1.5 mM) and dNTP (0.8 mM). QPCR was carried out using Brilliant Sybr Green master mix (Stratagene, http://www.stratagene.com) on the MxPro 3005XP system. The QPCR reaction contained master mix (1×), Rox reference dye (300 nM), forward and reverse primers (150 nM each). Minus RT controls were routinely performed. Specific primers used are shown in Table 1. *Beta actin* was used as the loading control for all QPCR analysis.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization on mouse embryos was car-ried out using dioxygenin (DIG)-labelled riboprobes. Riboprobes were synthesized from plasmids that contained the target gene sequence using DIG-labelled nucleotides (Roche, http://www.roche.com) and RNA polymerase (Promega). The riboprobes were purified through G50 columns (GE healthcare, http://www4.gelifesciences.com). For hybridization, embryos were firstly treated with prewarmed 10 μg μL^{-1} proteinase K⁄PBST [phosphate-buffered saline (PBS) containing 0.1% Tween-20] at 37 °C with varying incubation times (15–30 min) depending on the size and the age of embryos and then fixed in 4% parafor-maldehyde in PBS for 20 min at room temperature. Embryos were prehybridized at 65 °C for at least 1 h and then the prehy-bridization solution was replaced with hybridization solution

Table 1 RT-PCR primer sequences.

Target gene⁄ref	Forward primer (5′-3′)	Reverse primer (5′-3′)
Oct4 primary PCR	TAGGTGAGCCGTCTTTCCAC	CTCGAACCACATCCTTCTCT
Oct4 secondary PCR	GTGAGCCGTCTTTCCACCAGG	TGATTGGCGATGTGAGTGAT
Oct4 QPCR	GTGAGCCGTCTTTCCACCAGG	GGGTGAGAAGGCGAAGTCTG
Nanog (Robertson et al., 2006)	ATGAAGTGCAAGCGGTGGCAGAAA	CCTGGTGGAGTCACAGAGTAGTTC
Sox2 (Takahashi & Yamanaka, 2006)	AAGTACACGCTTCCCGGAGGCTTG	AGTGGGAGGAAGAGGTAACCAC
c-Myc (Takahashi & Yamanaka, 2006)	GAGTGCATTGACCCCTCAGT	AATTCAGGGGATCTGGTCACG
Gapdh	GGGTGGAGCCAAACGGGTC	GGAGTTGCTGTTGAAGTCGCA
Beta Actin	GTATGCCTCGGTCGTACCA	CTTCTGCATCCTGTCAGCAA
Clathrin	GACAGTGCCATCATGAATCC	TTTGTGCTTCTGGAGGAAAGAA

containing 1 : 200 to 1 : 600 dilution of riboprobes and incubated overnight at 65 °C. The embryos were sequentially washed in each diluted mix of hybridization buffer (75, 50 and 25% in 2× SSC) for 10 min at 65 °C. Further washes were carried out with 2× SSC containing 0.1% Tween-20 at 65 °C and 0.2× SSC containing 0.1% Tween-20 for 15 min at 65 °C (×4). Embryos were then sequentially washed at room temperature in dilutions (25, 50 and 75%) of MABT (100 mM maleic acid pH 7.5, 150 mM NaCl, 0.1% Tween-20) with 0.2× SSC. Secondary antibodies were preabsorbed by diluting in 2% Boehringer Blocking Regent™ and left at 4 °C with occasional shaking. The embryos were blocked with 2% Boehringer Blocking Regent™ (Roche) in MAB (100 mM maleic acid pH 7.5, 150 mM NaCl) at room temperature for at least 60~90 min with gentle shaking. The blocking reagent was then replaced by 2% Boehringer Blocking Reagent™ containing diluted secondary anti-DIG-AP antibodies. All embryos were incubated at 4 °C overnight. The antibody solution was removed and embryos were washed eight times with MABT at room temperature for 15 min. Embryos were then stained with BM purple (Roche), until colour developed. The colour reactions were stopped by washing embryos in PBST with 20 mM EDTA (3×) at room temperature. Embryos were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and stored in 80% glycerol for imaging.

Immunocytochemistry

Immunohistochemical analysis was carried out using standard protocols and following manufacturer's recommendations. Cultured cells were grown on chamber slides, fixed in 4% paraformaldehyde-PBS for 15 min at room temperature and permeabilized in 0.2% Triton X-100-PBS (Sigma). Frozen sections of 12 μm were cut, placed onto slides and dried for 30 min at room temperature. Primary antibodies were mouse anti-OCT4 (SC-5279, Santa Cruz, http://www.scbt.com), rabbit anti-NANOG (ab21603, Abcam, http://www.abcam.com) and mouse anti-NESTIN (611658, BD Biosciences, http://www.bdbiosciences.com). Cells were incubated for 30–60 min in either 3% bovine serum albumin (BSA), 0.1% Triton X-100 or 10% sheep serum blocking solutions at room temperature and then incubated in 3% BSA with 1 : 100 primary antibody overnight at 4 °C. Cells were washed in PBS (3× 15 min each) and incubated in 3% BSA with 1 : 100 secondary antibody fluorescein-conjugated secondary antibody (Fl-1000 or Fl-2000, Vector, http://www.vectorlabs.com) for 1 h at room temperature. A final wash step in PBS (3× 15 min each) was carried out before mounting with 4,6-diamidino-2-phenylindole (DAPI) Vectashield (Vector).

Oct4 methylation analysis

Methylation analysis of the Oct4 promoter and proximal region was carried out using bisulphite sequencing and pyrosequencing. Genomic DNA was bisulphate-converted using the EZ DNA methylation kit (Zymo, http://www.zymoresearch.com). PCR was carried out as stated in Hattori et al. (2004), with the following modification: 200 nM forward primer (ProF 5′-TGG GTT GAA ATA TTG GGT TTA TTT), 200 nM reverse primer (Pro R 5′-CTA AAA CCA AAT ATC CAA CCA TA), 0.8 mM dNTP, 1.5 mM MgCl₂, 1× PCR buffer and 0.05 U μL^{-1} Platinum Taq polymerase (Invitrogen). The PCR cycling condition was 94 °C for 10 min, followed by 45 cycles of 94 °C for 60 s, 55 °C for 60 s and 72 °C for 40 s,

with a final 5-min extension at 72 °C. An amplicon of 530 bp was purified and cloned into pCRII TA cloning vector (Invitrogen) and sequenced. The Oct4 bisulphite PCR was modified for pyrosequencing. The target amplicon of 376 bp was amplified using forward primer (ProF1 5′-AGA GGG TGT AGT GTT AAT AGT T) and a biotinylated reverse primer (Pro R 5′-CTA AAA CCA AAT ATC CAA CCA TA). The biotin-labelled strand of the amplicon was isolated and pyrosequencing was carried out using three sequencing primers, F1 (SEQF1-5′-GTG TTA ATA GTT TTT GTG G), F2 (SEQF2-5′-AAG GGT TGT TTT GTT TAG A) and F3 (SEQF3-5′-GAG GGA GAG GTG AAA T). Pyrosequencing was carried out at Wolfson Sequencing services, University College London, UK.

Results

Oct4 gene expression in Mouse NSCs

As a first step in determining whether Oct4 is expressed during brain development, we used RT-PCR to analyse mouse brain tissue of different ages. Several pseudogenes and splice variants exist for Oct4 (Pou5F1) (Takeda et al., 1992; Pain et al., 2005; Mizuno & Kosaka, 2008) and therefore it was necessary to ensure that PCR products were amplified from the splice variant that has been shown to code for the full-length version of the Oct4 protein responsible for 'stemness' properties (Cauffman et al., 2006; Liedtke et al., 2007; Atlasi et al., 2008; Mizuno & Kosaka, 2008). All primers were therefore designed to include exon 1 and to span introns, and all PCR products were sequenced to verify that they were not derived from a pseudogene (the relationship of the primers used to the exons of Oct4 is shown in Fig. 1A). Of the tissues examined, Oct4 was only detected in brain tissue obtained from embryonic day 8.5 (E8.5) embryos, even when a second round of PCR cycles using nested primers was applied (Fig. 1B). As NSCs will progressively represent a smaller proportion of the cells present as the brain grows, the absence of PCR products when analysing older brain samples might be explained by the gradual decline in the proportion of NSCs in the samples being analysed. To determine whether Oct4 expression was indeed in the NSC population, we isolated NSCs from these various ages of brain and screened these using the same RT-PCR approach. The earliest stage we could analyse was E9, the first stage when cells of the CNS will grow under standard neurosphere culture conditions. This semi-quantitative RT-PCR showed Oct4 was robustly expressed in NSCs derived from embryos at E9, and was expressed at a much lower level, requiring a second round of PCR cycles with nested primers for its detection in NSCs derived from E13.5 embryos (Fig. 1C). By E15.5, expression was barely detectable and by P3, no product was detected. These data show that Oct4 is expressed at a readily detectable level at E8.5 but that expression in NSCs rapidly declines between E9 and E15.5. To verify this decline in expression
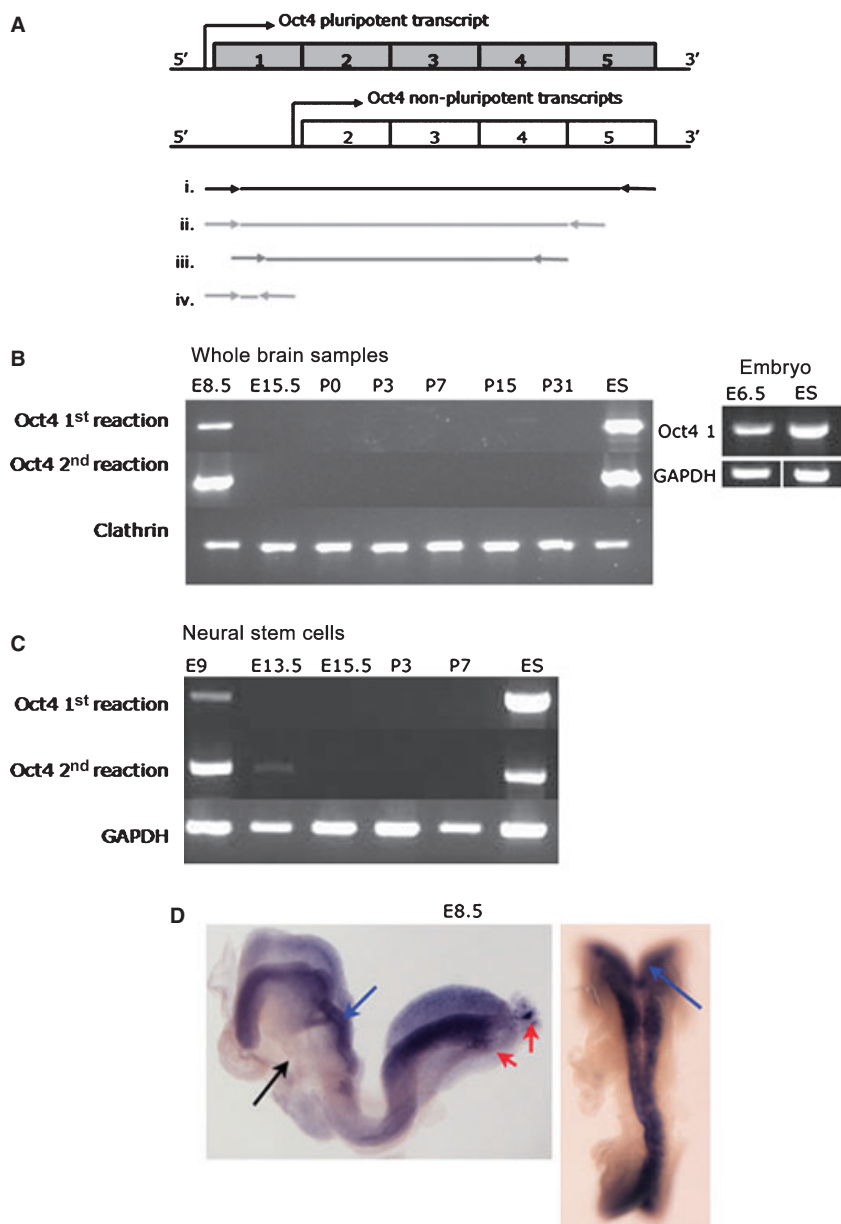
Fig. 1 *Oct4* expression analysis at embryonic and postnatal stages of brain and neural stem cell development. (A) Schematic representation of *Oct4* transcripts. Only the transcript including exon 1 has been shown to play a central role in pluripotency. Primers shown below were therefore designed incorporating exon1. (i) Amplification of full length *Oct4* transcript (used in E); (ii) Primary RT-PCR (used in B,C); (iii) Secondary nested RT-PCR (used in B,C); and (iv) QPCR primer locations. (B) RT-PCR from whole brain samples, except E6.5 which were whole embryos and ES cells as positive control. Products are shown from first PCR reaction and after a second nested PCR reaction. *Clathrin* was included as loading control for the amount of RNA in the reactions. (C) RT-PCR from neural stem cells, all populations used at passage 3–6, ES cells included as positive control. *GAPDH* included as loading control. (D) Whole mount *in situ* hybridization using a full length *Oct4* riboprobe on E8.5 embyros. *Oct4* expression identified in the neural plate (blue arrows) and PGC (red arrows), not in the non-neural tissue (black arrow). E, embryonic stage; P, postnatal stage.

and to gain insight into the level of *Oct4* expression relative to ES cells, we next applied qRT-PCR. After relative quantification, the *Oct4* expression in E9 NSCs was found to be 10^5-fold lower than that in ESCs. Thus, it seems that *Oct4*, a key marker of pluripotent cells, is expressed in neural stem cells during development but this expression decreases rapidly as the embryo matures.

To gain more detailed insight into the location of populations of cells expressing *Oct4*, whole-mount *in situ* hybridization was carried out on early mouse embryos. Expression was only detected at E8.5 when it was seen throughout the entire CNS (Fig. 1D). There was no striking variation in expression either rostrocaudally or mediolaterally, implying that, at this stage, all cells of the neuroepithelium expressed *Oct4* to a similar extent.

Immunohistochemical analysis of Oct4 protein in mouse NSCs

To verify the expression of Oct4 in NSCs and because its subcellular distribution is critical to its function, we carried out immunofluorescence analysis on sections from embryos and neurospheres derived from different ages and regions of mouse brains. The Oct4 antibody used has been shown to bind to the region of Oct4 encoded by exon 1. This therefore provides an assay of the full-length protein that is involved in pluripotency and it is widely used in studies of Oct4 (Cauffman et al., 2006; Atlasi et al., 2008; Liedtke et al., 2008). Analysis of cryo-sections of embryos showed that, in addition to strong expression in the primordial germ cells (PGCs) of the genital ridge,

expression of Oct4 could be weakly detected in the cytoplasm of neural plate cells in E8.5 and E9.5 embryos, consistent with RNA levels determined by RT-PCR and *in situ* hybridization (Fig. 2). We next investigated the expression and subcellular distribution of Oct4 in cultured neurospheres isolated from mouse brains of various ages. Oct4 protein was barely detectable in NSCs using immunofluorescence at E9.5, and was not observed in NSCs isolated from later developmental stages (data not shown). E8.5 neurospheres were not tested as these cells do not readily grow as neurospheres.

### *Oct4* gene methylation in NSCs

Oct4 expression is known to be repressed by methylation of CpG dinucleotides within its promoter, the proximal enhancer and distal enhancer regions (Feldman et al., 2006; Li et al., 2007). It has also been shown previously that *Oct4* expression can be induced by treatment of adult NSCs with the DNA methyltransferase inhibitor, 5-azacytidine and histone deacetylase inhibitor, TSA (Ruau et al., 2008). We hypothesized that the expression of *Oct4* at very early stages of CNS development might be due to a reduced level of methylation and that loss of expression as the embryo matures would reflect gradual methylation. We therefore carried out a combination of bisulphite sequence analysis and pyrosequencing of CpGs within the promoter and proximal enhancer (shown schematically in Fig. 3A). ES cells were analysed as a control in which methylation is known to be generally absent.

Bisulphite sequencing of individual clones identified a gradual methylation of CpGs, from the unmethylated pattern in ES cells to the highly methylated pattern in P15 NSCs (Fig. 3B). This was confirmed by pyrosequencing, which allows a larger number of amplicons to be analysed, providing a reliable quantitative value for each sample (Fig. 3C). Both pyrosequencing and sequencing of individual bisulphate-treated clones revealed a similar gradual methylation from when *Oct4* expression was detectable until it was absent by postnatal stages. It is interesting that both bisulphite and pyrosequencing analysis show that there is only a small increase in methylation after E8.5, but the level of *Oct4* expression falls dramatically in NSCs from later stages, even though most CpG sites at the embryonic stages are still unmethylated. However, the changes in methylation are not uniform across all CpGs. The CpG sites at −165, −189 and −227 in particular are highly methylated in NSCs after embryonic stage E8.5.

The expression of other 'pluripotency genes' in mouse NSCs

In recent years a number of genes have been shown to be central to the ability of cells to behave as pluripotent stem cells. Other than *Oct4*, these include *Sox2*, *c-Myc*, *Klf4* and *Nanog*. The expression of *Sox2* is well established as a marker for NSCs, and *c-Myc* and *Klf4* expression has been reported in NSCs (Kim et al., 2008, 2009b). However, *Nanog* expression has been reported to be negative in NSCs (Chambers et al., 2003; Kim et al., 2008, 2009b). We have shown that *Oct4* was expressed in NSCs in early embryos and therefore we reanalysed the expression of these four genes in NSCs during mouse development. As anticipated, *Sox2* and *c-Myc* and *Klf4* all showed robust expression at all stages analysed (Fig. 4).
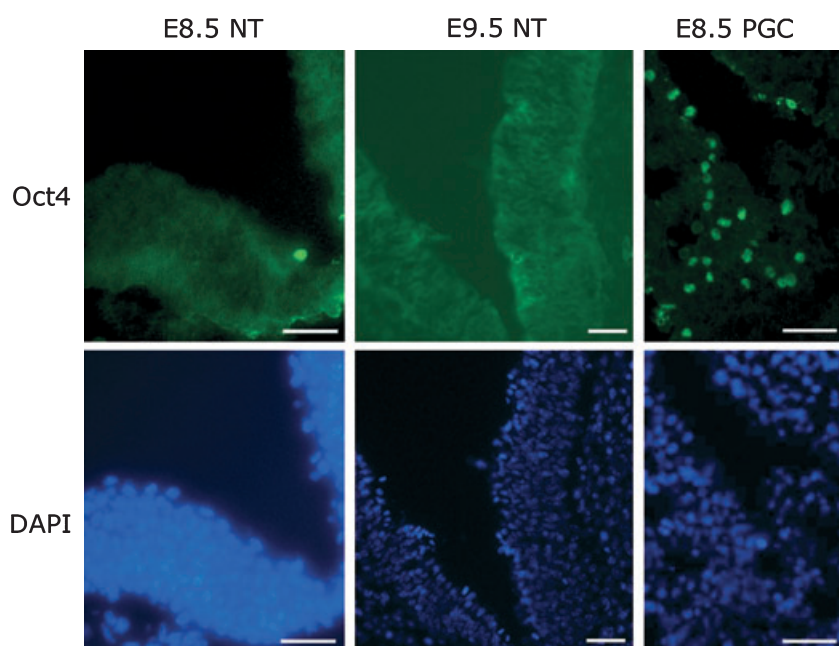
Fig. 2 Oct4 immunoreactivity in mouse embyronic cranial neuroepithelium. Oct4 was identified in the E8.5 and E9.5 neural plate. Although this was predominantly cytoplasmic, some staining also seen in the nuclei. Staining in PGCs was predominantly nuclear.
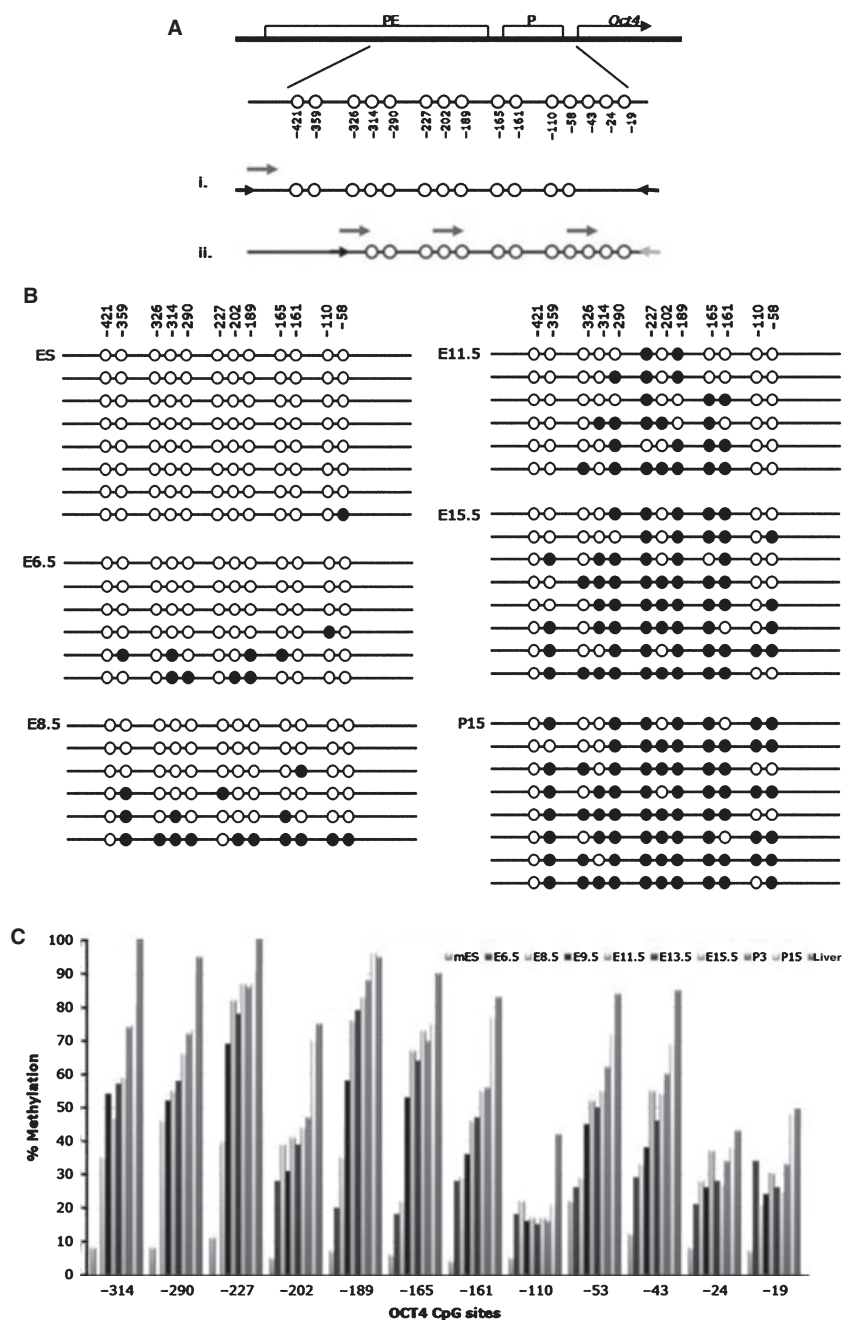
Fig. 3 *Oct4* DNA methylation analysis of neural stem cells at embryonic and postnatal stages. (A) Schematic representation of the CpG sites within the *Oct4* promoter (P) and proximal enhancer (PE). CpGs covered by (i) bisulphite sequencing and (ii) pyrosequencing (locations of primers shown by arrows, PCR primers in black and short arrows above the product represents the sequencing primers). (B) Bisulphite sequencing results on ES cells, E6.5 head, E8.5 head, E11.5 NSCs, E15.5 NSCs and P15 NSCs. Open circles represent unmethylated CpGs, closed circle represent methylated CpGs, with each line representing one sequenced clone. (C) Pyrosequencing results showing percentage methylation at each CpG site from E6.5 head, E8.5 head to embryonic and postnatal NSCs. ES cells and liver used as controls for unmethylated and highly methylated, respectively.

Analysis of *Nanog* is complicated by the presence of a number of pseudogenes and retrogenes. Therefore, to test whether *Nanog* was expressed in NSCs, a method of RT-PCR (Robertson et al., 2006) was utilized to discriminate between the pseudogenes (no amplification), retrogenes (band size shift and RsaI digestion) and the genuine transcript, with all PCR products sequenced. *Nanog* was shown to be expressed consistently in whole brain tissue at all developmental stages analysed and the retrogene *NanogPd* was also detected in samples of E15.5, P7 and P15 (Fig. 5A). *Nanog* was also detected in NSCs derived from embryonic,

postnatal and mature mice (Fig. 5B). However, no expression of *NanogPd* was observed in any of the NSC samples.

We next used qRT-PCR to determine the level of *Nanog* transcripts present. Although we were able to detect *Nanog* RNA in all the tissues we analysed, the level was 10^4 lower relative to ES cells (Fig. 5C).

Because the above data suggested that *Nanog* transcripts were present in NSCs at a similar, but very low, level throughout development and adulthood, we also analysed a range of other tissues to determine whether this low level was a general feature of many tissues. *Nanog* was indeed

ES E11.5 E13.5 E15.5 P3 P31 NTC

Sox2
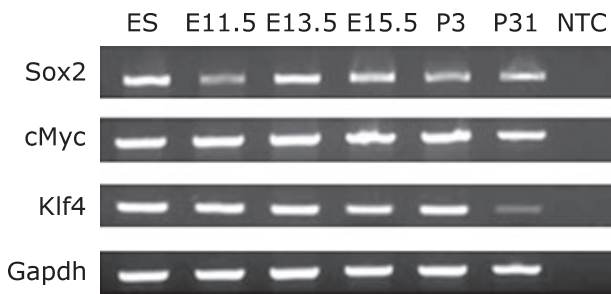
cMyc

Klf4

Gapdh

Fig. 4 *Sox2*, *c-Myc* and *Klf4* expression in embryonic and postnatal brain. RT-PCR results for brain samples, with positive control ES and NTC (no template control). GAPDH used as loading control for the amount of RNA in the reactions.

detected in lung and kidney tissue, but not in stomach (Fig. 5D). Kidney also expressed *NanogPd*. More specific adult brain regions were also analysed and both *Nanog* and *NanogPd* were expressed in all regions apart from the lateral ventricles, where only *Nanog* was expressed (Fig. 5D). RsaI digests of the *Nanog* PCR product confirmed that another retrogene, *NanogPc*, was not detected in any of the samples.

Immunohistochemical analysis of Nanog in NSCs

To determine whether the very low levels of *Nanog* transcripts we detected might be of significance, we analysed NSCs for the presence of the Nanog protein. The Nanog antibody used in this study has been shown by other groups to act as a marker for pluripotent cells (Storm et al., 2007; Shi et al., 2008; Singh et al., 2008; Silva et al., 2009). Our analysis demonstrated the presence of Nanog protein in the nuclei of NSCs (Fig. 5E). To determine whether the same might be true of human NSCs, we analysed the human REN-VM NSC line, which has been shown to retain all the characteristics of an NSC; self-renewal and the ability to differentiate into glia and neurones (Donato et al., 2007). This cell line was derived from foetal human ventral mesencephalon brain tissue. Our analysis using immunocytochemistry demonstrated clear expression of Nanog in both the cytoplasm and nuclei of these cells (Fig. 5F).

Discussion

Pluripotency is a feature of only very restricted populations of cells in the early vertebrate embryo. These cells are marked out by the expression of a number of 'pluripotency genes' that, when over-expressed in somatic cells, can initiate a process through which some of those cells can become pluripotent, generating so-called iPS cells. Although none of the genes alone provides a mark unique to endogenous pluripotent cells, the combined expression of the full set of genes is regarded as a unique feature of such cells. Our data demonstrate that the cells of the CNS

express several key genes typical of pluripotent stem cells, but *Oct4* expression is rapidly lost as the CNS matures, coincident with methylation of its promoter and proximal enhancer regions.

The expression of pluripotency genes in neural stem cells

Expression of *Sox2* and *c-Myc* in the developing CNS and NSCs is already well-described (Hirvonen et al., 1990; Graham et al., 2003; Eminli et al., 2008; Kerosuo et al., 2008; Kim et al., 2008) and *Klf4* expression has recently been demonstrated in both mouse and human NSCs (Kim et al., 2008, 2009a,b).

Because of the problem of alternative transcripts and pseudogenes, there is little definitive evidence regarding the expression of Oct4 in NSCs. We have used RT-PCR to show that full-length *Oct4* expression is maintained in the neural ectoderm beyond E8.5, when definitive NSCs are present, albeit at lower levels than in ES cells. Although our data suggested that Oct4 was predominantly localized to the cytoplasm, it was not excluded from the nucleus, so a functional role as a transcription factor remains possible. It may not be surprising that Oct4 protein is hard to detect despite significant levels of its transcript, as Oct4 protein has been shown to be processed by ubiquitination and rapidly turned over (Saxe et al., 2009; Wagner & Cooney, 2009).

Our observation that *Nanog* is expressed in the neuroepithelium and NSCs throughout development is contrary to the interpretation of most previous studies, but it does not in fact contradict their data. Previous reports have generally regarded levels of *Nanog* that are over 1000 times lower than that seen in ES cells, as negative. However, despite the fact *Nanog* RNA was present at a level 10^5-fold lower than in ES cells, this level was readily detectable in cells from several tissues. In addition, the presence of *Nanog* RNA in NSCs was reflected in the presence of nuclear Nanog protein (Fig. 4). Indeed, a recent study by Kim et al. (2009a) showed that endogenous *Nanog* expression in a foetal human NSC population was only approximately 14-fold lower than an ES cell line.

Overall it seems that early NSCs express all four 'pluripotency' genes but that *Oct4* and *Nanog* are present at very low levels compared to ES cells and Oct4 is generally located in the cytoplasm.

Oct4 methylation

It is well-established that methylation is a common mechanism for the silencing of *Oct4* expression. In the mouse embryo as a whole, *Oct4* is unmethylated at the blastula stage and undergoes *de novo* methylation by E6.5, remaining modified at similar levels as development proceeds (Gidekel & Bergman, 2002). Our report is the first study to
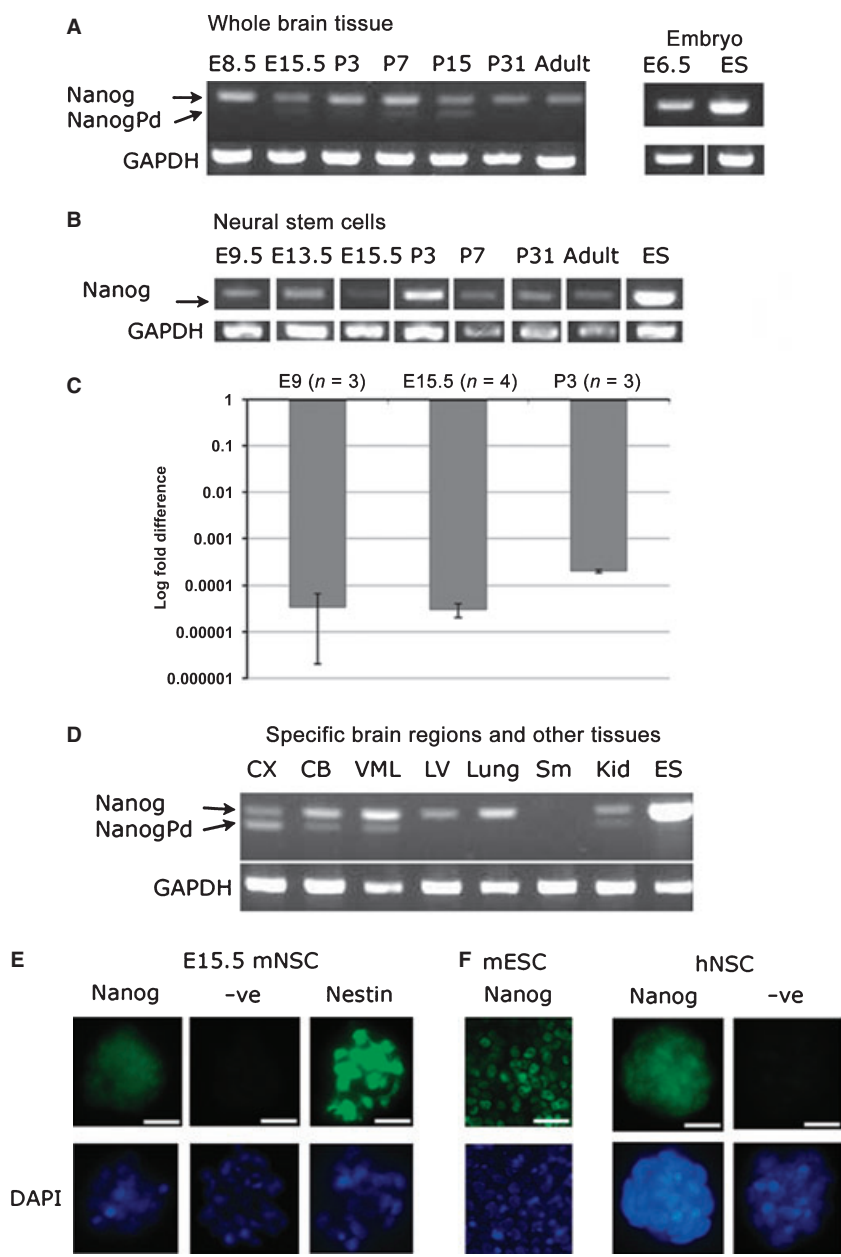
Fig. 5 Analysis of *Nanog* expression and protein localization in neural stem cells. (A) RT-PCR results for whole brain samples showing amplification of *Nanog* (upper band) and *NanogPd* pseudogene (lower band). (B) RT-PCR amplification of *Nanog* and *NanogPd* showed only *Nanog* transcript was expressed in mouse neural stem cells. (C) Relative fold change of *NANOG* expression in neural stem cells compared to ES cells. Each bar represents the average fold change of three independent cultures of cells, with error bars showing SD. (D) RT-PCR results for specific brain regions and other tissues. In all RT-PCR reactions, ES cells were used as the positive control and *GAPDH* as a loading control. (E) Immunostaining of mouse E15.5 NSC with Nanog, negative control of secondary antibody only and Nestin as a positive control. (F) Nanog immunostaining of human NSCs (hNSCs) with mouse ES (mESC) cells as positive control for nanog expression.

show that the *Oct4* gene is progressively methylated during the *in vivo* maturation of NSCs in the neuroepithelium of the CNS, coincident with downregulation of its expression. Our data suggest that methylation plays a significant role in the decreasing expression *Oct4* as the CNS matures.

It is notable, however, that not all CpGs analysed were methylated to the same extent. A core area of CpGs from −161 to −290 shows the most dramatic change from low methylation at E8.5 to high methylation after E8.5 (in particular CpGs −165, −189 and −227) and it is therefore possible that it is the methylation of these CpGs that is primarily responsible for the silencing of gene expression.

The functional significance of *Oct4* expression in neural stem cells

As NSCs are clearly not pluripotent, what is the significance of the expression of the genes that we have described? Why is *Oct4* still expressed in neural tissue at early embryonic stages and not immediately switched off when cells become neural precursors? One possibility is that a low level of Oct4 is necessary to regulate developmental events. Gerrard et al. (2005) analysed *OCT4* expression as human ES cells were differentiated into neurones *in vitro* (Gerrard et al., 2005). They found that the level of *OCT4* transcripts

declined slowly compared to the decline of other hESC markers, and inhibition of *OCT4* expression resulted in differentiation to extra-embryonic endoderm-like cells.

Although Oct4 is expressed in NSCs at early stages of development, this expression is low and the protein is predominantly in the cytoplasm. This brings into question the functional significance of this expression. Indeed, inactivation of *Oct4*, driven by *Nestin*-cre, had no discernible effect on brain development, suggesting that it is certainly not required for the basic features of NSCs once they are formed (Lengner et al., 2007). However, as *Nestin* is only robustly expressed from E8 in NSCs (Lothian & Lendahl, 1997; Kawaguchi et al., 2001), and it would then take time for the *Nestin*-cre used by Lengner et al. (2007) to be expressed and *Oct4* mRNA and protein levels to decline, it seems likely that the loss of Oct4 would have occurred only a little before the time we have seen its loss endogenously. Its role in the very early stages of NSC formation remains to be tested.

The higher levels of *Oct4* expression at the earlier stages of NSC development may explain observed differences in behaviour of the cells of the neural plate from E7.5 to E9.5. During this period, there is a change in the conditions under which cells of the neuroepithelium can be cultured *in vitro*. Prior to E8.5, cells from the neuroepithelium require LIF rather than EGF or FGF to be grown in culture, and these cells have been named 'primitive NSCs' (Hitoshi et al., 2004). These primitive NSCs not only exhibit features of neural stem cells, such as *Sox1* and *Nestin* expression, but also have features in common with stem cells capable of forming other cell lineages. A role of Oct4 in this progression is suggested from the data of Akamatsu et al. (2009), who studied the development of NSCs derived from ES cells in culture. They found that *Oct4* expression was increased in mice lacking the orphan nuclear receptor 'germ cell nuclear factor' gene (GCNF) and that primitive NSCs were inhibited from undergoing the transition to definitive NSCs. This block could be rescued by small interfering (si)RNA inhibition of Oct4 (Akamatsu et al., 2009). These data suggest that Oct4 may indeed be important in controlling the transition from primitive NSC to definitive NSC. A similar Oct4-positive population of cells was isolated by Takehara et al. from P0 mice, but this was achieved by growing cells in medium designed for ES cells, not for NSCs. Hence, it seems that a primitive population of cells may exist as late as birth, although it is not clear from their study if this primitive state was induced in cells by their unusual culture conditions.

Our data show that, even later in development, NSCs express three of the four 'Yamanaka' factors (Oct4 now being absent). This is likely to explain why NSCs are more readily reprogrammed to iPS cells than other somatic cell types (Eminli et al., 2008; Kim et al., 2009a,b). Addition of Oct4 alone is sufficient to initiate reprogramming such that some of those cells convert to cells exhibiting full pluripo-tency, with increased expression of *Nanog* and other pluripotency genes as a consequence (Kim et al., 2009a,b).

Oct4 and the origins of teratomas

A key assay of pluripotency is the ability of cells to form a teratoma (a tumour made up of components from all three embryonic germ layers) when transplanted into mice. Teratomas are in fact one of the most common forms of tumour seen in humans prenatally and in neonatal infants. Within the brain, teratomas make up about 50% of tumours diagnosed before or at birth, whereas they represent only a tiny proportion of brain tumours diagnosed after the first few months of life.

Although these tumours are classified as germ cell tumours, believed to arise from misplaced germ cell progenitors, we have hypothesized that they arise form endogenous progenitor cells in the developing brain. The data presented here provide a plausible explanation for their appearance so early in life and support this alternative hypothesis for their origins. As discussed above, simply over-expressing Oct4 in NSCs can be sufficient to convert them to a pluripotent state capable of forming a teratoma. We have shown that Oct4 expression is less robustly repressed in the NSCs of the early brain than at later stages and that this is coincident with methylation of its promoter. It therefore seems feasible that Oct4 expression would be more readily reactivated by demethylation at such early stages than at later stages. As intracranial teratomas normally present at birth and appear to develop at the expense of much of the brain's own normal development, they are likely to be initiated early during development consistent with the time when Oct4 methylation in NSCS is lowest.

Reactivation of Oct4 by demethylation would also be consistent with a role for global hypomethylation as an early event in cancer. Indeed, the earlier in life that a tumour forms, the less likely it is that its development is driven by a series of mutational events as seen in most adult cancers. Activation of key genes due to global epigenetic events is a strong alternative model for the mechanism by which these unusual tumours are initiated. Consistent with such a hypothesis, in contrast to virtually every other type of cancer examined, teratomas rarely exhibit any detectable cytogenetic abnormality.

However, high levels of Oct4 expression have not been described in these tumours. This is not surprising for two reasons. First, because such tumours go on to exhibit widespread differentiation into a range of different tissue types, any stem cell-like cells will represent a very small proportion of the tumour mass. Secondly, as in iPS cells, where over-expression of Oct4 is an initiating event for pluripotency after which its expression is down-regulated, any increase in Oct4 expression could be transient.

We would therefore propose a model in which rare disruption of global methylation in an early NSC could result

in up-regulation of Oct4 expression (along with other aberrant gene expression) and so trigger conversion to pluripotency, resulting in teratoma formation. This would be restricted to the earliest stages of brain development, when repression of Oct4 by methylation is not yet complete, explaining the restriction of teratomas to the first few months of human life.

Summary

We have shown that Oct4 is initially expressed as both RNA and protein in NSCs during early embryogenesis, but the level of expression drops rapidly from E8.5 to become undetectable by E15.5. This loss of *Oct4* expression is coincident with gradual methylation of its promoter and proximal enhancer region. Alongside the expression of other key genes associated with pluripotency this may explain the plasticity and other behavioural differences between NSCs at the different stages of CNS development and may explain why intracranial teratomas arise almost exclusively prior to birth.

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Author contribution

Shih-Han Lee: conception and design, collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. Jennie N. Jeyapalan: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. Vanessa Appleby: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. Dzul Azri Mohamed Noor: collection and/or assembly of data. Virginie Sottile: conception and design, final approval of manuscript. Paul J. Scotting: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

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