Intrinsic factors and the embryonic environment influence the formation of extragonadal teratomas during gestation

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Abstract

Background: Pluripotent cells are present in early embryos until the levels of the pluripotency regulator Oct4 drop at the beginning of somitogenesis. Elevating Oct4 levels in explanted post-pluripotent cells in vitro restores their pluripotency. Cultured pluripotent cells can participate in normal development when introduced into host embryos up to the end of gastrulation. In contrast, pluripotent cells efficiently seed malignant teratocarcinomas in adult animals. In humans, extragonadal teratomas and teratocarcinomas are most frequently found in the sacrococcygeal region of neonates, suggesting that these tumours originate from cells in the posterior of the embryo that either reactivate or fail to switch off their pluripotent status. However, experimental models for the persistence or reactivation of pluripotency during embryonic development are lacking.

Methods: We manually injected embryonic stem cells into conceptuses at E9.5 to test whether the presence of pluripotent cells at this stage correlates with teratocarcinoma formation. We then examined the effects of reactivating embryonic Oct4 expression ubiquitously or in combination with Nanog within the primitive streak (PS)/tail bud (TB) using a transgenic mouse line and embryo chimeras carrying a PS/TB-specific heterologous gene expression cassette respectively.

Results: Here, we show that pluripotent cells seed teratomas in post-gastrulation embryos. However, at these stages, induced ubiquitous expression of Oct4 does not lead to restoration of pluripotency (indicated by Nanog expression) and tumour formation in utero, but instead causes a severe phenotype in the extending anteroposterior axis. Use of a more restricted T(Bra) promoter transgenic system enabling inducible ectopic expression of Oct4 and Nanog specifically in the posteriorly-located primitive streak (PS) and tail bud (TB) led to similar axial malformations to those induced by Oct4 alone. These cells underwent induction of pluripotency marker expression in Epiblast Stem Cell (EpiSC) explants derived from somitogenesis-stage embryos, but no teratocarcinoma formation was observed in vivo.

Conclusions: Our findings show that although pluripotent cells with teratocarcinogenic potential can be produced in vitro by the overexpression of pluripotency regulators in explanted somitogenesis-stage somatic cells, the in vivo induction of these genes does not yield tumours. This suggests a restrictive regulatory role of the embryonic microenvironment in the induction of pluripotency.

Keywords: Extragonadal teratoma, Pluripotency, Oct4, Nanog, Brachyury, Inducible expression

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Background
In mammals, pluripotency, the ability to generate derivatives of all three embryonic germ layers, is an essential characteristic of cells in the preimplantation and very early postimplantation epiblastas as well as their in vitro counterparts, embryonic stem (ES) and Epiblast Stem (EpiSC) cells respectively. The maintenance of a pluripotent state is mediated by the activity of a core transcriptional network dominated by the principal pluripotency factors Oct4, Nanog and Sox2 (reviewed in [1]. Shortly after the start of gastrulation, although cell fate becomes regionalised [2], epiblast cells remain pluripotent until the beginning of somitogenesis [3]. We have previously shown that ectopic Oct4 expression is the minimal requirement for reinstating pluripotency in normally non-pluripotent somitogenesis-stage embryonic cells explanted in vitro in EpiSC conditions [3]. Furthermore, simultaneous ectopic expression of only four pluripotency factors is sufficient to reprogram embryonic fibroblasts or adult somatic cells to an ES cell-like pluripotent state [4, 5].

A key feature of all pluripotent cells is their capacity to seed teratocarcinomas after engraftment into permissive sites in the adult such as the kidney capsule. Teratocarcinomas comprise differentiated tissues derived from all three germ layers as well as a self-renewing pluripotent component, embryonal carcinoma (EC) cells [6]. In humans, teratocarcinomas and teratomas, their more benign versions lacking obvious EC cells, are classified as germ cell tumours as, by puberty, they mainly occur in the testis and ovary (for a review see [7]). Shortly after the start of gastrulation, although cell fate becomes regionalised [2], epiblast cells remain pluripotent until the beginning of somitogenesis [3]. Nanog and Sox2 (reviewed in [1]. Shortly after the start of gastrulation, although cell fate becomes regionalised [2], epiblast cells remain pluripotent until the beginning of somitogenesis [3]. We have previously shown that ectopic Oct4 expression is the minimal requirement for reinstating pluripotency in normally non-pluripotent somitogenesis-stage embryonic cells explanted in vitro in EpiSC conditions [3]. Furthermore, simultaneous ectopic expression of only four pluripotency factors is sufficient to reprogram embryonic fibroblasts or adult somatic cells to an ES cell-like pluripotent state [4, 5].

The predominant occurrence of extragonadal teratomas/teratocarcinomas in human neonates suggests that pluripotent cells are tumour-forming even during gestation. Here we sought to define whether the mouse embryo is permissive for teratocarcinoma formation on ectopic expression of pluripotency factors. We found that although injected ES cells efficiently induce tumour formation in midgestation embryos, ubiquitous or axial progenitor-specific ectopic expression of pluripotency factors in somitogenesis-stage embryos did not lead to the generation of tumours. Instead, severe developmental abnormalities occurred along the developing axis. Our findings suggest that while the somitogenesis-stage embryonic environment can suppress the neoplastic potential of cells ectopically expressing individual pluripotency factors, once pluripotency is established, pluripotent cells are capable of seeding extragonadal teratomas during gestation.

Results
Induction of teratomas by pluripotent cells
Manual injection of ES cell suspensions into conceptuses (either the yolk sac or amniotic cavity) at E9.5 led to the appearance after birth of extragonadal teratocarcinomas in 2/3 injected pups (Fig. 1a, b) (7 embryos were injected in total of which 5 were born and 3 pups survived to weaning), showing that ectopic pluripotent cells can seed teratomas during embryogenesis. No tumours were observed when non-pluripotent primary somatic cells from E8.5-14.5 embryos were injected into age-matched embryos (BL6 or other) (approx. 200 embryos were assessed after birth, data not shown). Furthermore, a survey of the literature showed that, while reports of spontaneously occurring extragonadal teratomas are extremely rare, they can occur upon injection of ES cells to blastocysts (9/13 reported mice). In one case, they arose from a cell line known to harbour a large chromosome 8 deletion [16], a karyotypic abnormality frequently encountered in ES cell lines [17]. Interestingly, such tumours were generally found in young mice under 9 weeks old (11/13 mice; of which 8 were 1.5-4 week old pups/weanlings). Tumours were detected in multiple sites in the body (Fig. 1b), including two instances of tumours in the tail. In humans, extragonadal teratomas are often apparent at birth or in the early neonatal period (Fig. 1c), and while they are also found at multiple sites, some of which overlap with those in mice, the predominant location is at the base of the spine, in the sacrococcygeal region. Collectively, these data indicate that persistence of pluripotent cells after the natural shutdown of embryonic pluripotency is likely to result in the formation of extragonadal teratocarcinomas/teratomas shortly after birth and suggest that the posterior midline (the primitive streak and tail bud) represents a
Fig. 1

Mouse teratoma incidence

| Strain | History/treatment | Location | Sex | Age (weeks) | Mouse number | Authors | Tumour origin |
|--------|-------------------|----------|-----|-------------|--------------|---------|---------------|
| CAG-GFP/+Ju09(B6<->B6) | YSiamniotic cavity* ES cells in E9.5 | liver | f | 9 | 1 | This work | ES cell |
| CAG-GFP/+Ju09(B6<->B6) | YSiamniotic cavity* ES cells in E9.5 | liver | f | 9 | 1 | This work | ES cell |
| CAG-GFP/+Ju09(B6<->B6) | YSiamniotic cavity* ES cells in E9.5 | peritoneum | f | 9 | 2 | This work | ES cell |
| Brc2/+(129<->B6) | Chimera | cecum | m | 4 | 3 | Blackshear et al 1999 | nd |
| Hsp70+/+(129<->B6) | Chimera | tail | f | 4 | 3 | Blackshear et al 1999 | nd |
| E14Tg2a(129<->B6) | Chimera | thoracic | f | 3.5 | 5 | Blackshear et al 1999 | nd |
| E14Tg2a(129<->B6) | Chimera | perigenital | m | 3 | 6 | Hardy et al 1990 | ES cell |
| E14Tg2a(129<->B6) | Chimera | cecum | m/fern | 3 | 7 | Hardy et al 1990 | nd |
| E14Tg2a(129<->B6) | Chimera | perigenital | m/fern | 3 | 7 | Hardy et al 1990 | ES cell |
| CP1rep(129<->MF1) | Chimera | cervical | nd | 2 | 8 | Wilton 1991 | ES cell |
| CP1rep(129<->B6) | Chimera | thoracic | nd | 1.5 | 9 | Wilton 1991 | nd |
| (C3H/HeHx101/h)xPT | Spontaneous | liver | f | 7 | 10 | Philips and Hulse 1982 | |
| (C3H/HeHx101/h)xPT | Spontaneous | tail | f | 7 | 10 | Philips and Hulse 1982 | |
| wild | Spontaneous | face | m | 10 | 11 | Dunn and Andervont 1963 | |
| wild | Spontaneous | mesentery | m | 10 | 11 | Dunn and Andervont 1963 | |
| wild | Spontaneous | thigh | m | 4 | 12 | Dunn and Andervont 1963 | |
| BTBR TF/Nev | Spontaneous | liver | f | 50 | 13 | Artzt and Damjanov 1978 | |

*2/3 mice surviving weaning had tumours

**Bold type:** macroscopic tumours detected in mice ≤ 2 months old

**Red type:** tumours in tail

Human teratoma incidence

Fetuses+neonates (n=484)

- Sacrococcygeal: 214
- Cervical: 71
- Intracranial: 41
- Oro-naso-pharyngeal: 40
- Cardiac: 13
- Gastric: 14
- Orbital: 8
- Mediastinal: 13

Babies+children (n=716)

- Sacrococcygeal: 176
- Cervical: 48
- Intracranial: 26
- Oro-naso-pharyngeal: 29
- Cardiac: 25
- Gastric: 15
- Orbital: 14
- Mediastinal: 11
- Facial: 10

Fig. 1 (See legend on next page.)
permissive environment for the manifestation of a pluripotent phenotype.

Coincidence of pluripotency transcription factors in the posterior of somitogenesis-stage embryos

We hypothesised that if the acquisition or persistence of pluripotency in somatic cells in post-gastrulation stage embryos correlates with teratocarcinoma formation in extragonadal sites then it is likely to occur in locations exhibiting pluripotency factor expression. We thus examined the presence of the main pluripotency/reprogramming factors in somitogenesis stage embryos. We have shown that *Nanog* expression disappears around the end of gastrulation while *Oct4* becomes absent from somatic tissues by the ~10-somite (s) stage [3] although, like *Nanog*, its expression is retained in the PGCs [18, 19]. Expression of *Klf4*, one of the original factors necessary for reprogramming to induced pluripotency, was undetectable during early somitogenesis (data not shown). However, *in situ* hybridisation analysis revealed that *Klf4* transcripts were present at later somite stages (32-35 s) predominantly in the posterior end of the embryo up to the TB (Fig. 2a). Furthermore, we found by real-time qPCR that the chordo-neural hinge (CNH), a site within the TB shown to harbour progenitor cells with neuromesodermal (NM) potency [20], also exhibited *Klf4* expression (Fig. 2b). The same region was also found by qPCR to be positive for *Sox2* expression while being devoid of *Oct4* and *Nanog* transcripts (Fig. 2b). These data combined with the reported presence of the other main reprogramming factor c-Myc in the posterior end of the embryo [21] indicate that there is a coincidence of pluripotency/reprogramming factors in this location, although two major pluripotency transcription factors, *Oct4* and *Nanog*, are absent.

Ubiquitous induction of Oct4 expression *in vivo* causes severe developmental abnormalities but does not reactivate the pluripotency indicator Nanog

Given the presence of several critical reprogramming factors in somatic cells of somitogenesis stage embryos we next tested the effects of restoring the expression of the principal pluripotency determinant *Oct4* during these stages. We employed a mouse line (*TgOct4*) carrying an *Oct4* transgene that is ubiquitously expressed in response to Doxycycline (Dox) administration [22]. We have previously shown that ectopic *Oct4* induction in this line at somitogenesis stages leads to reactivation of
the pluripotency indicator Nanog within 24 h (hr) after explantation in EpiSC culture conditions and subsequent re-establishment of pluripotency evidenced by the derivation of stable EpiSC lines and formation of teratocarcinomas from grafts of tissue pieces to the adult kidney capsule [3]. We thus tested whether pluripotency is reactivated in Dox-treated embryos in utero. A 24 h treatment of pregnant females with Dox led, as expected, to the robust upregulation of Oct4 in transgenic embryos (Fig. 3a, c), and whole mount in situ hybridisation showed widespread Oct4 expression (Fig. 3a). However, we observed no reactivation of Nanog expression, as assessed either visually using a Nanog-GFP reporter transgene (data not shown), or by qPCR (Fig. 3d). A slight upregulation of Sox2 in the lateral area of the somites was detected (Fig. 3b), but otherwise there was no ectopic Sox2 expression (Fig. 3b, e). These results indicate that ubiquitous induction of ectopic Oct4 expression in somitogenesis-stage embryos does not result in widespread reactivation of the pluripotency transcriptional network.

Although Dox-treated transgenic embryos showed no evidence of pluripotency network reactivation, they exhibited consistent developmental abnormalities (Fig. 4). The most prominent was a failure of dorsal closure of the anterior neural tube (Figs. 3b, 4). Similar brain malformations have been reported by [23] after ubiquitous

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Fig. 3 Induction of ubiquitous ectopic Oct4 expression. a Top: Oct4 mRNA expression in wild type (wt) and induced (+Dox) or un-induced (−Dox) TgOct4 transgenic (TG) somitogenesis embryos analysed by whole mount in situ hybridisation. A high magnification image of Oct4 expression in the PGCs of the transgenic, un-induced E8.5 embryo is also shown on the right corresponding to the boxed area. Bottom: representative sections showing Oct4 expression in the wild type (left) and the induced transgenic (right) embryos. The position of the sections relative to the embryo is indicated by the lines on the top left image. b Top: Sox2 mRNA expression in wild type (wt) and induced (+Dox) or un-induced (−Dox) TgOct4 transgenic (TG) somitogenesis embryos analysed by whole mount in situ hybridisation. Middle: High magnification images depicting Sox2 expression in the neural tube. Arrow: Ectopic lateral Sox2 expression in induced embryo. Bottom: representative sections showing Sox2 expression in the wild type (left) and the induced transgenic (right) embryos. The position of the sections relative to the embryo is indicated by the lines on the top left image. Arrow: Ectopic Sox2 expression in induced embryo. c-e Log expression levels of Oct4, Nanog and Sox2 in pooled pre- and somitogenesis stage TgOct4 embryos in the presence (white bars) or absence of Doxycycline (black bars). Error bars represent s.e.m. (n = 3). Note error bars in (c) are smaller than the resolution of the figure. In all cases Oct4-induced embryos were treated with Doxycycline for 24 h prior to the indicated recovery time point. E14, E14tg2a ES cells; MEF, mouse embryonic fibroblasts; PS, prestreak; ES, early streak; OB, no allantoic bud; LB, late allantoic bud; LHF, late headfold; s, somite.
Oct4 expression. However, we also observed a phenotype in the posterior parts of the axis not reported by these authors. Therefore we initiated a series of experiments to explore this phenotype. Treatment of heterozygous TgOct4 embryos (after mating with wild type mice) from E7.5-E10.5 led to severe retardation with very little somitogenesis evident (Fig. 4). Shorter treatments from E8.5-E9.5 or E9.5-E10.5 produced phenotypes in the posterior part

| Treatment   | Genotype       | Severe Phenotype* | Mild Phenotype** | No Phenotype |
|-------------|----------------|-------------------|------------------|--------------|
| +Dox (E7-10)| TgOct4/+       | 5/5               | 0/0              | 0/0          |
| +Dox (E8-9) | TgOct4/+       | 3/32              | 32/32            | 26/32        |
| +Dox (E9-10)| TgOct4/+       | 1/10              | 2/10             | 7/10         |
| -Dox (E8-9) | TgOct4/TgOct4  | 0/0               | 0/0              | 3/3          |
| +Dox (E8-14)| +/- (MF1 background) | 0/0               | 0/0              | 8/8          |

* Open/deformed neural tube, axis truncation, oedema, retardation
** Smaller somites, abnormal neuroectoderm expansion, bleeding

Fig. 4 Phenotypic consequences of ubiquitous ectopic Oct4 expression. a Timings/duration of Dox administration. b-f Wildtype untreated (b, e) or 24 h Dox-treated TgOct4 embryos (c, d, f) recovered at E9.5. (e, f) high magnification views of wildtype (e) or Dox-treated TgOct4 embryos showing affected somites. g-j Wildtype untreated (g, i) or 24 h Dox-treated TgOct4 embryos (h, j) recovered at E10.5. (i, j) high magnification views of wildtype (i) or Dox-treated TgOct4 embryos showing affected neural tube. k, l 72-h Dox-treated TgOct4 E10.5 embryo. (k) high magnification view showing kinked neural tube. WT, wild type; s, somite; n, neural ectoderm. Arrowheads indicate the position of the somite pair formed at the time of Oct4 induction. Black arrowheads: 24 h induction (e8-9 or e9-10); white arrowheads: induction from e7-10. Scale bar: 500 μm (b-d); 250 μm (e, f, i-l); 800 μm (g, h). The observed phenotypes are summarised in the table.
of the axis with little perturbation to the axial regions formed prior to treatment (Fig. 4). In axial regions formed after Dox administration, a kinked neural tube, suggestive of excess neural tissue, and small somites were evident (Figs. 3b, 4). Therefore, ectopic Oct4 expression during somitogenesis disrupts axis elongation, and in particular may interfere with the balance of neural and mesodermal tissues emanating from the TB region.

Development of a novel T(Bra)-based tool for inducible gene expression in the PS and tail bud
The severity of the embryonic phenotype produced by ubiquitous Oct4 expression precluded assessment of tumour formation over a longer period. Therefore we developed a system that would allow targeted and inducible transgene expression specifically at the midline of the extending axis, i.e. the site where extragonadal teratomas are most often observed in humans (Fig. 1b). We established a feeder-free E14tg2a-derived ES cell line which contains a randomly integrated cassette (Tps/tb-rtTA) consisting of the PS/tb-specific 1.2 kb T(Bra) promoter fragment [24] driving the reverse tetracycline transactivator rtTA2S-M2 gene [25]. To conditionally express heterologous genes, we targeted a Tetracycline Responsive Element (TRE) module coupled to a gene of interest at the Hprt locus (Fig. 5a). This strategy has been shown to be very efficient for achieving introduction of single copy transgenes into a locus offering a defined chromatin environment for stable ectopic gene expression [26].

We tested the spatiotemporal specificity of our system by examining the Dox-dependent expression of a TRE-dsRed2 fluorescent reporter introduced into the Hprt locus in the Tps/tb-rtTA ES cell line. We first investigated the dynamics of dsRed2 induction in vitro by culturing the resulting Tps/tb-rtTA/HPRT-TRE-dsRed2 reporter ES cell line (hereafter referred to as Tps/tb-RED) in the absence of LIF and in presence of serum. We found that these conditions promote the induction of T(Bra) positive, PS-like cells within 3–4 days (Additional file 1: Figure S1) in line with previously published data [27–29]. Culture of Tps/tb-RED ES cells in −LIF/+serum media and in the presence of Dox promoted the induction of dsRed2+ cells with dsRed2 expression peaking at day 4 of differentiation (17.5 ± 2.7 %; Fig. 5b). A 10-fold reduction in the levels of Dox correlated with a decreased induction of dsRed2 protein (17.5 ± 2.7 %; Fig. 5b). We observed no leakiness in dsRed2 expression in the absence of Dox (Fig. 5b).

To determine the identity of dsRed2-expressing cells on day 4 after LIF removal in vitro, the expression of PS markers was examined in flow sorted dsRed2+ and dsRed2− populations. Sorted dsRed2+ cells were enriched for all PS markers tested compared to their dsRed2− counterparts (Fig. 5c). Conversely, expression of neural markers such as Sox1 and Pax6 was underrepresented in dsRed2+ cells (Fig. 5c). All dsRed2− cells were also T(Bra)-immunopositive, although T(bra) expression was more widespread than that of dsRed2 (Fig. 5d). This suggests that at least some T(Bra)+/dsRed2− cells may have a ventral node/notochord identity, since the promoter is inactive in this population. However, we cannot exclude the possibility that these cells may also reflect a lower sensitivity of detection of dsRed fluorescence or differences in turnover between the dsRed2 and T(Bra) proteins. Collectively, these results indicate that dsRed2 induction takes place in vitro specifically within cells exhibiting a PS/mesodermal identity and are consistent with our previous findings that the Tps/tb promoter becomes activated within the PS quite early, at the beginning of gastrulation [30].

We investigated the induction of the dsRed2 reporter in Dox-treated somitogenesis-stage (E8.5-11.5) chimeric embryos generated using Tps/tb-RED ES cells modified to carry a ubiquitously-expressed GFP transgene (cell line C2). We found that dsRed2 was expressed in the PS, tail bud and recently emerged mesoderm at all stages tested (Fig. 5e-h). At E8.5, expression was present up to and including the epiblast close to the node (in the node-streak border), but absent in the ventral node and notochord (Fig. 5e). While early on E8.5 (<5 somites), expression was almost exclusively mesodermal, later E8.5 embryos (>8 somites) showed expression in emerging neurectoderm as well as mesoderm (Fig. 5f). At E10.5-11.5, dsRed2 fluorescence was visible in both the ectodermal and mesodermal components of the TB as well as the emerging neurectoderm and mesoderm (Fig. 5g-h). The highest intensity dsRed2 expression was seen in the presomitic mesoderm, consistent with the relatively long maturation time of this fluorescent protein [31]. Thus the expression of dsRed2 accurately reports activation of T(Bra) expression in the PS and TB and is in agreement with previously published findings [28, 32, 33]. Examination of the anterior limits of expression at E8.5 and E10.5-11.5 suggests that dsRed2 protein persists for around 24 h after its initial transcriptional activation in the PS and TB. Together these data indicate that our transgenic system comprises a reliable platform for inducible heterologous gene expression in the PS and TB.

Expression of Oct4 and Nanog in the primitive streak/tail bud after the start of somitogenesis does not result in neoplasia
We next examined whether expression of pluripotency factors in the primitive streak/tail bud after gastrulation
Fig. 5 (See legend on next page.)
is sufficient to reactivate pluripotency in vivo potentially leading to the formation of teratocarcinomas in the mid-line. We introduced a TRE-Oct4-2A-Nanog-2A-Venus cassette into the Hprt locus in Tps/tb-rtTA ES cells (Fig. 6a) thus allowing the simultaneous expression of three proteins: Oct4, Nanog and Venus, within cells in which the Tps/tb promoter is active.

Differentiation of Tps/tb-rtTA-Oct-Nanog-Venus ES cells in the absence of LIF resulted in robust induction of Venus expression correlating with the emergence of PS-like cells in vitro (Additional file 2: Figure S2). These ES cells were injected into blastocysts and chimeras were recovered during gestation or after development to term. Dox treatment starting at E8.5 drove efficient Venus expression and transgene induction in the PS/TB (Fig. 6b, c). Since we previously showed that Nanog reactivation serves as an early indicator of successful pluripotent EpiSC derivation [3], we tested whether induction of these transgenes in explants reactivated endogenous Nanog on culture in Activin/Fgf EpiSC growth conditions. Explantation of E9.5 chimeric tail buds to EpiSC culture, their embryonic expression neither reactivated endogenous Nanog nor led to or the development of teratomas.

**Discussion**

**Embryonic environment and tumorigenicity of pluripotent cells**

Human teratomas/teratocarcinomas typically occur in the gonads of postpubertal individuals and hence are classed as germ cell tumours and considered of germ cell origin [8]. However in prenatal and neonatal infants, teratomas most frequently occur in extra-gonadal locations [35, 36]. Since around 50% of sacrococcygeal teratomas are detectable by ultrasound scan at 18–20 weeks [36], these tumours may arise early during gestation. The predominant view regarding their aetiology is that PGCs fail to migrate properly towards the genital ridges and through evasion of apoptosis give rise to germ cell tumours [7, 8, 37]. However, the occurrence of extra-gonadal teratomas in locations distant from the canonical route of germ cell migration (i.e. the brain and mediastinum) raises the possibility that at least some may be of a somatic cell origin. Since pluripotent cells of somatic origin are capable of normal development in gastrulation-stage embryos [15], yet form teratocarcinomas in an adult environment [38, 39], there must be a stage at which pluripotent cells become neoplastic. Here we provide proof-of-principle that introduction of established pluripotent cells into midgestation (E9.5) embryos after normal pluripotency shutdown leads to the development of teratomas. Thus, the midgestation environment is prohibitive for the integration of established pluripotent cells, and permissive for their growth as teratocarcinomas.

In conclusion, we find no evidence that ectopic expression of Oct4 and Nanog within the PS and TB during mid-late gestation results in tumour formation in vivo. Although both ubiquitous Oct4 expression, and tail bud-specific Oct4/Nanog expression reactivated pluripotency in vitro in EpiSC culture, their embryonic expression neither reactivated endogenous Nanog nor led to the development of teratomas.
Fig. 6 (See legend on next page.)
Embryonic pluripotency is extinguished in vivo around the start of somitogenesis, coincident with a rapid down-regulation first of Nanog and then of Oct4 expression [3]. Oct4 is critical for the maintenance of a pluripotent state in the postimplantation epiblast and ectopic Oct4 reactivation in post-pluripotent primary embryonic tissue after grafting beneath the adult kidney capsule leads to teratocarcinoma formation [3]. This led us to hypothesise that the failure of embryonic somatic cells to shut down Oct4 might result in extragranal teratomas. However, we did not observe tumour formation following ectopic induction of Oct4 alone or in combination with Nanog even at the posterior of the embryo, a site where the other main reprogramming factors, Sox2, Klf4 and c-Myc were found to be present (Fig. 2 and [21]). So far only cells that express pluripotency gene regulatory network factors, including Oct4, Sox2 and Nanog, have been shown to produce teratocarcinomas. In adult mice, expression of Oct4 alone leads to atypical epithelial neoplasias [22], whereas expression of all four reprogramming factors produces teratocarcinomas [12, 13]. Thus it is likely that a cell-of-origin of spontaneously arising teratomas would express all these genes or their functional equivalents. Both ubiquitous Oct4 [3] and ps/tb-specific Oct4/Nanog (Fig. 6e) are sufficient to promote the re-assembly of the pluripotency network in EpiSC culture, as evidenced by the reactivation of endogenous Nanog expression [3]. However, neither of these perturbations resulted in the in vivo reactivation of the pluripotency network or teratoma formation in vivo. This reinforces the conclusion that a permissive environment, as well as the expression of intrinsic factors, is important for the formation of teratomas.

The extracellular environment is known to be important in determining the behaviour of pluripotent cells. Mouse ES cells readily incorporate into preimplantation embryos at the stage from which they were isolated [14] and contribution to the resulting chimeras suggests that they respond correctly to differentiation cues provided by the embryo. Similarly, EpiSCs efficiently colonise cultured gastrulation-stage embryos [15]. However, ES cells cannot integrate in post-implantation embryos from which pluripotency is lost [15] or pre-implantation embryos [39]. Moreover, either pluripotent cell type, when introduced into adults, forms teratocarcinomas at very high frequencies. Thus, the ability of the organism to suppress neoplasia in a pluripotent cell type depends critically on the extracellular environment.

The failure of pluripotent cells to integrate correctly into a normal environment may be a necessary early step in the formation of a niche that is permissive for pluripotent cell maintenance. Such a niche could either be supplied by the surrounding embryonic cells, or by the pluripotent cells themselves, for example via the ability of differentiated derivatives of pluripotent cells to secrete self-renewal factors [40]. The latter possibility provides a potential explanation for the ability of established lines to form tumours in midgestation embryos, while expressing Oct4 in situ at the same stage does not. Together with the observation that fresh tissue grafted to the adult kidney capsule leads to teratocarcinoma formation, this suggests that the E8.5-9.5 embryonic environment lacks factors that promote a pluripotency-permissive niche.

What signalling pathways could constitute a pluripotency-permissive niche? In vitro, Oct4-induced Nanog reactivation in somitogenesis-stage EpiSC explants and subsequent acquisition of pluripotency is dependent on Nodal/activin signalling [3]. At the beginning of gastrulation, Nodal signalling is widespread in the posterior region of the embryo, but becomes highly restricted to the node coincident with the extinction of embryonic pluripotency at early-somite stages, and is absent after 7–8 somites [41]. Similarly, activin subunit transcripts are absent from the embryo proper during the critical period of pluripotency shutdown, till around E9.5, after which restricted expression domains appear in various regions [42, 43]. Moreover, Nodal signalling has been shown to be critical
for regulating germ cell pluripotency [44] and perturbations in its levels have been linked to the formation of germ cell tumours, including extragonadal ones [44–47].

Ectopic Oct4 expression perturbs normal development
The phenotypes produced by Oct4 expression are reproducible and dependent on the stage at which expression was induced. Ubiquitous Oct4 overexpression before closure of the cranial neural folds leads to an open brain phenotype, while expression thereafter affects mainly the parts of the anteroposterior axis that form after induction of the Oct4 transgene, where somites are smaller than normal and the neural tube is kinked. Induction of Oct4/Nanog in the PS/TB leads to rather similar phenotypes (failure of cranial neural tube closure and kinked posterior regions of the axis), suggesting both that the phenotypes observed are mainly due to Oct4 expression, rather than Nanog, and that these phenotypes result from ectopic pluripotency factor expression in the PS/TB. The combination of small somites and a kinked neural tube suggests that Oct4 expression during somitogenesis may hinder the production or expansion of mesoderm at the expense of neuroectoderm. Interestingly, depletion of Oct4 around E7.5 severely affects both neural tube closure and somitogenesis/posterior development and results in misregulation of a number of key signalling pathways such as TGFβ and Notch [48]. Together, these findings suggest that Oct4 plays a critical role in morphogenetic processes possibly by influencing proliferation [48] and/or adhesion [49].

A transgenic tool for inducible expression in the PS and the tail bud
Various transgenic strategies for T-dependent replacement have been reported, including targeted replacements of the T gene [27], cells and mice containing integrated copies of cosmid [32, 50] or BAC constructs [29] spanning the T genomic region, and up to 2.4 kb upstream of the transcription start site [28, 32, 51]. The expression of our reporter in the primitive streak but not node and notochord is consistent with these studies that show up to 8.3 kb of upstream sequences contain only these regulatory elements. The expression of the dsRed2 and Oct/Nanog transgenes specifically in the primitive streak and tail bud and their nascent mesoderm derivatives demonstrates the utility of T-rtTA driver for PS and tail bud-specific expression of heterologous genes in vitro and in vivo. Targeting heterologous genes at the Hprt locus offers a convenient selectable strategy allowing integration of a single transgene copy at a single site in a permissive chromosomal domain thus eliminating unpredictable chromosomal position effects on expression due to random integration.

Conclusions
We have shown that established pluripotent cells are neoplastic if present after the time when pluripotency is normally shut down, i.e. at organogenesis stages. We also show that reactivation of Oct4, or Oct4 and Nanog, after pluripotency shutdown is not sufficient to reactivate pluripotency in vivo, and requires additional signals from the cellular microenvironment. It will be interesting in future to determine whether sites at which either Activin/Nodal/Fgf or LIF/BMP signalling is naturally present coincide with sites at which teratomas spontaneously arise in mice and humans.

Methods
Generation of reporter ES cell lines
To construct the Tps/tb-RED ES cell line, we first engineered a transgene in which a 1.2 kb Brachyury (T) proximal upstream regulatory element and promoter fragment [24] was linked to the sequence of the reverse tetracycline transactivator rtTA2S-M2 [25]. This transgene, Tps/tb-rtTA, was randomly integrated by electroporation into E14tg2a (129 background) ES cells. The resulting clones were screened for optimal expression levels of rtTA after LIF removal. The tet-responsive element (TRE) upstream of the dsRed2 fluorescent reporter gene or the Oct4-F2A-Nanog-T2A-Venus cassette were then introduced, by homologous recombination at the Hprt locus, into the clone expressing the highest levels of T-rtTA mRNA. Correctly targeted clones reconstruct a functional Hprt locus and were identified by selection in medium with hypoxanthine, aminopterin and thymidine (HAT) to generate Tps/tb;HprtTRE-dsRed2 or Tps/tb;HprtTRE-Oct/Nanog/Venus. The HAT resistant clones with the most robust induction of dsRed2 or Venus were then employed for detailed analysis. Subclones of the Tps/tb-RED ES cell line were also generated by introducing a randomly-integrated CAG-GFP plasmid and identifying clones with ubiquitous expression. A representative clone of each resulting ES cell line (Tps/tb;HprtTRE-dsRed2 , Tps/tb;HprtTRE-Oct/Nanog/Venus or Tps/tb; HprtTRE-dsRed2_CAG-GFP, (named C2)) was used to generate chimeric embryos.

Cell culture and differentiation
Feeder-free ES cells were routinely cultured in gelatinised flasks in GMEM-based medium supplemented with LIF. For induction of PS-like cells in vitro, ES cells were seeded at a density of 7.5×10³ cells/cm², cultured in ES medium without LIF for up to four days in the presence of Doxycycline (0.01-1 μg/ml; Sigma).

Mouse husbandry and in vivo transgene induction
Chimeric embryos were generated either by morula aggregation or blastocyst injection followed by transfer into pseudopregnant foster (CBA/BL6 F1) mothers using
standard procedures. For transgene induction oral doxycycline was administrated at a final concentration of 1–2 mg/ml in drinking water containing 5% sucrose. Mice were maintained on a 12 h light/12 h dark cycle. For timed matings, noon on the day of finding a vaginal plug was designated E0.5. To assess the effects of ectopic Oct4 activation, transgenic TgOct4 mice were either treated directly with Doxycycline or first crossed with wild type (129 or MF1 background) or Nanog:GFP [129-Nanog\(^{tm1(GFP-IRES-Puro)}\)] mice [52] before Doxycycline administration to pregnant mothers. ES cell suspensions were manually injected into the amniotic cavity of E9.5 embryos \textit{in utero} using a hand-pulled Pasteur pipette after laparotomy and exposure of the uterine horns under a general anaesthetic. The position of the amniotic cavity was visualised by trans-illumination with a fibre-optic light.

**Microdissection of tail bud regions**

Tail bud regions were microdissected with hand-pulled solid glass needles as described previously [20] and 7–10 regions pooled for RNA isolation.

**In situ hybridization**

Whole-mount \textit{in situ} hybridisation was performed as described [53]; proteinase K treatment was empirically adjusted between 8–16 min according to embryo size and stage. The riboprobes used were: Oct4 [54], Sox2 [55] and Klf4 which was designed by cloning a 900 bp fragment (base pair 972–1871, NM_010637.3) into a pCR®II-TOPO® vector (Life technologies). Embryos were sectioned in paraffin wax (7 μm).

**Flow cytometry**

Analysis of dsRed2 or Venus expression was performed using a FACS Calibur (BD Biosciences) cytometer. Cell sorting was performed using a FACSARia (BD Biosciences).

**EpiSC explantation**

EpiSC explantation was performed as previously described [3]. Briefly, after removal of extraembryonic membranes, the tail buds of chimeric embryos were dissected, dissociated in trypsin/pancreatin and explanted in EpiSC medium [39] supplemented with 20 ng/ml activin A (PeproTech) and 10 ng/ml bFGF (R&D) in the presence (1–3 μg/ml) or absence of Doxycycline on irradiated mouse embryonic fibroblasts (MEFs). The duration of culture under these conditions was 48 h.

**Gene expression analysis**

RNA isolation was performed using the RNEasy Mini or Micro kit (Qiagen) following the manufacturer’s instructions. On-column DNase I (Qiagen) digestion was carried out in all cases to eliminate genomic DNA contamination. The concentration and the purity of each sample were determined by UV spectroscopy using a NanoDrop ND-1000 (Thermo Scientific). Total RNA (25–250 ng) was reverse-transcribed into cDNA using random primers (Promega) and the SuperScript III reverse transcriptase kit (Invitrogen). Real-time RT-PCR was performed using a LightCycler 480 (Roche) and LightCycler 480 SYBR Green 1 Master Mix (Roche). All primers employed were designed to flank introns or span exon-intron boundaries and their sequences can be found in Additional file 3: Table S1. The optimal performance of each primer set was confirmed using 10-fold serial dilutions of plasmid preparations containing the corresponding target sequences. Serial dilutions of the targets were also employed to generate standard curves for determining copy numbers and values were normalized against the input determined for the housekeeping gene TATA-binding protein (TBP). All qPCR experiments were carried out using three technical replicates and at least two biological replicates.

**Immunostaining**

Immunostaining of cells and embryos was performed as described previously [3]. The following primary antibodies were used: anti-Brachyury (N-19, Santa Cruz, 0.4 μg/ml), anti-Foxa2 (M-20, Santa Cruz, 2 μg/ml), anti-Gsc (N-12; Santa Cruz, 2 μg/ml), anti-Nanog, 2.5 μg/ml (14-5761-80, eBioscience).

**Imaging**

Images were captured using a Zeiss Stemi SV11 or Nikon AZ100 dissecting microscope (for freshly dissected whole and \textit{in situ} hybridised embryos), an Olympus IX51 (for cultured cells), or an Olympus BX61 (for sections). Image processing was performed using Adobe Photoshop software. For higher resolution visualisation of dsRed2 or Venus/Nanog expression a Leica TCS SP2 inverted confocal microscope was employed (Leica Microsystems). Image acquisition and processing were carried out using the Leica ConfoCal (Leica Microsystems) and Volocity (Improvision) software packages, respectively. Nuclear segmentation and fluorescence signal quantification were performed as described previously [3].

**Skeletal preparations and histology**

Skeleton preparations and histological stains of tumour sections were prepared as described in [56].

**Ethics approval**

All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) act 1986 under Home Office Licence 60/4435. Experiments were approved...
by the University of Edinburgh Animal Welfare Ethical Review Board.

Additional files

Additional file 1: Figure S1. ES cells differentiated in the absence of LIF give rise to PS-like cells in vitro. (A) Time-course qPCR expression analysis of ES- and PS-organiser-specific markers in wild type E14 ES cells cultured in the presence of serum and absence of LIF for the indicated amount of time. (B) Immunocytochemistry of PS-organiser marker expression in wild type E14 ES cells cultured in the presence of serum and absence of LIF for 4 days. (JPEG 943 kb)

Additional file 2: Figure S2. The induction of PS-like cells in vitro correlates with Venus upregulation in Tps/tb-Oct-Nanog-Venus ES cells. (A) Flow cytometry analysis of Venus expression in Tps/tb-Oct-Nanog-Venus ES cells after 4 days of differentiation in the presence of serum/Dox and absence of LIF. (B) Fluorescence microscopy of Venus expression Dox-treated and untreated Tps/tb-Oct-Nanog-Venus ES cells after 4 days of differentiation in the presence of serum/Dox and absence of LIF. (JPEG 607 kb)

Additional file 3: Table S1. Primer sequences. (DOCX 14 kb)

Abbreviations

ES: Embryonic stem; EpSGC: Epiblast stem cell; EC: Embryonal carcinoma; PGCs: Primordial germ cells; TB: Tail bud; CNH: Chordoneural hinge; NM: Neurornesodermal; Dox: Doxycycline; TRE: Tetracycline responsive element; PS: Primitive streak.

Competing interests

The authors declare there are no competing interests involved in this study.

Authors’ contributions

CE, AT and VW designed the research; RD, DF, AM and AJHS contributed essential reagents; FAW performed the ifII4 in situ hybridization; SGK carried out the teratoma analysis; CE, WW and AT carried out other experiments and assembled the data; AT, CE and WW analysed the data and wrote the manuscript. All authors have read and approved the manuscript.

Authors’ information

Not applicable.

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