Conserved roles for Oct4 homologues in maintaining multipotency during early vertebrate development

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All vertebrate embryos have multipotent cells until gastrulation but, to date, derivation of embryonic stem (ES) cell lines has been achieved only for mouse and primates. ES cells are derived from mammalian inner cell mass (ICM) tissue that express the Class V POU domain (PouV) protein Oct4. Loss of Oct4 in mice results in a failure to maintain ICM and consequently an inability to derive ES cells. Here, we show that Oct4 homologues also function in early amphibian development where they act as suppressors of commitment during germ layer specification. Antisense morpholino mediated PouV knockdown in Xenopus embryos resulted in severe posterior truncations and anterior neural defects. Gastrulation stage embryos showed reduced expression of genes associated with uncommitted marginal zone cells, while the expression of markers associated with more mature cell states was expanded. Importantly, we have tested PouV proteins from a number of vertebrate species for the ability to substitute Oct4 in mouse ES cells. PouV domain proteins from both Xenopus and axolotl could support murine ES cell self-renewal but the only identified zebrafish protein in this family could not. Moreover, we found that PouV proteins regulated similar genes in ES cells and Xenopus embryos, and that PouV proteins capable of supporting ES cell self-renewal could also rescue the Xenopus PouV knockdown phenotype. We conclude that the unique ability of Oct4 to maintain ES cell pluripotency is derived from an ancestral function of this class of proteins to maintain multipotency.

KEY WORDS: Gastrulation, Self-renewal, Embryonic stem cell, Lineage commitment, Pou protein, Pou5f1, Xenopus

INTRODUCTION

Germ layer specification involves an elaboration of intrinsic cell-autonomous factors and non-autonomous signalling molecules. Embryonic cells prior to gastrulation have the capacity to respond to various signalling pathways and are therefore uncommitted (Gardner and Beddington, 1988; Snape et al., 1987). This pluripotent feature of early embryonic cells is mirrored in vitro by embryonic stem (ES) cells (Smith, 2001). ES cells are self-renewing pluripotent cell lines derived from the ICM of the mammalian blastocyst that, in mouse, have been shown to be capable of populating all the lineages of the foetus and adult (Beddington and Robertson, 1989). Unlike ES cells, early embryonic cells cannot be considered stem cells as they do not self-renew indefinitely. However, mammalian embryos still need to maintain a population of non-committed cells through a significant amount of cell division to generate the number of cells necessary to complete gastrulation. Although lower vertebrates undergo reductive rather than proliferative cell division during early cleavage (Frederick and Andrews, 1994; Kane et al., 1992), they still require active mechanisms to ensure against premature cellular differentiation. Indeed, studies in Xenopus have shown that the future ectoderm cells and the ventral marginal zone cells are multipotent until late gastrulation (Domingo and Keller, 2000; Okabayashi and Asashima, 2003).

A number of factors have been identified that are necessary for the maintenance of ES cell pluripotency, including the cytokines LIF and BMP4, and transcription factors such as Nanog and Oct4 (encoded by Pou5f1, referred to here as Oct4) (Chambers, 2004).

In addition to its role in ES cells, Oct4 is also required for the maintenance of the germ cell lineage (Kehler et al., 2004) and thus appears to have an in vivo role in maintaining multipotency. The ICM and then the epiblast express Oct4 until gastrulation, when Oct4 remains expressed only in the posterior epiblast and the primitive streak, but not in cells that have undergone mesendoderm induction (Yeom et al., 1996; Yoshimizu et al., 1999). This expression pattern is consistent with Oct4 having a role in maintaining cells as multipotent until it is the appropriate time for them to become committed to mesoderm, endoderm or ectoderm.

In the absence of Oct4, ES cells lose the capacity to self-renew and subsequently differentiate into extra-embryonic trophoderm (Niwa et al., 2000). Similarly, Oct4+/− embryos die at peri-implantation stages because of the conversion of ICM into trophoderm (Nichols et al., 1998). The involvement of Oct4 in embryonic versus extra-embryonic fate decisions and its role in the maintenance of primordial germ cells (PGCs) has led to the proposal that Oct4 arose in mammals to perform these specific functions (Pesce et al., 1998). Alternatively, Oct4 and the molecular programmes it regulates might represent an older evolutionary innovation designed to maintain a non-committed cell population in the early embryo until the appropriate time for induction of the germ layers.

Oct4 is a Class V POU domain protein, containing both a POU specific domain (POUs) and a POU homeodomain (POUh) (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1990). Based on the degree of conservation within these domains and the linker region, the POU domain proteins have been divided into five classes. Class V POU domain proteins (referred to here as PouV proteins) are present in a number of other species and have been implicated in early embryonic development (Bachvarova et al., 2004; Frank and Harland, 1992; Hinkley et al., 1992; Takeda et al., 1994; Whitfield et al., 1993). To date, a single PouV protein has been identified in teleost fish (Burgess et al., 2002), zebrafish Pou2,
referred to here as DrPou2. Interestingly, DrPou2 has recently been shown to have a role in endoderm induction (Lunde et al., 2004; Reim et al., 2004).

Although no self-renewing ES cell lines have been generated from lower vertebrates, we considered whether the mechanisms governing self-renewal were conserved. Here, we report that a PouV gene from Xenopus laevis, Xlpou91, has the capacity to maintain murine ES cells in the absence of Oct4. As Xenopus has neither induced PGCs nor extra-embryonic development, we believe that ES cell pluripotency may be based in part on conserved, rather than mammalian-specific, aspects of vertebrate development. Moreover, two other Xenopus genes, Xlpou25 and Xlpou60, and axolotl-Oct4 (AmOct4, also referred to as Arox4) have some ability to rescue ES cell self-renewal, whereas the zebrafish protein Pou2 has none. Knockdown phenotypes of the three Xenopus XlpouV genes results in elevated expression of endodermal, organizer and neural markers, aspects of which can be rescued by axolotl and mouse Oct4. These data support our hypothesis that PouV proteins represent a class of transcription factor required for the maintenance of multipotent non-committed cell populations in both Xenopus and mouse.

MATERIALS AND METHODS

Xenopus embryo manipulations and biological assays

Xenopus laevis embryos were obtained by in vitro fertilization, staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994) and cultured according to Slack et al. (Slack et al., 1984). Embryos were injected at the two-cell stage in both blastomeres with RNA and/or morpholino oligonucleotides. Dextran fluorescein (Molecular Probes) was used as a lineage label (Brickman et al., 2000).

Animal caps were dissected from stage 8 blastula and cultured in the presence or absence of partially purified activin (a gift from J. Smith). Activin units (U) are defined by Cooke et al. (Cooke et al., 1987). In situ hybridization was carried out as described in Brickman et al. (Brickman et al., 2000). Morpholino oligonucleotides were purchased from GeneTools. Sequences were as follows: Xlpou25 MO, 5'-ACATGGTGTCAGACGGCTGTTCAACAGGC-3'; Xlpou60 MO, 5'-GTGACAATATGGGCTGGTCCATC-3'; Xlpou91 MO, 5'-GAAATCGTTGTTGTATACATGAC-3'. The control morpholino was the standard provided by GeneTools.

The whole-mount TUNEL staining was carried out as previously described (Hensey and Gautier, 1997).

In vitro translation

Wild-type XlpouV mRNA or non-complementary (NC) XlpouV mRNA were used as a template in a Red Nova Lysate Translation kit (Novagen) according to the manufacturer’s instructions in the presence of 35S-methionine. Translation productions were fractionated by SDS-PAGE and visualized by autoradiography.

Expression constructs and transcription

Wild-type Xlpou25, Xlpou60, mouse Oct4, DrPou2 and AmOct4, and non-complementary (NC) Xlpou25, Xlpou60 and Xlpou91 expression constructs were generated by PCR in pCAGIP. Plasmids for RNA injection were linearized with NotI and mRNA generated according to Smith (Smith, 1993). cDNAs were inserted into pCAGIP (Niwa et al., 2002; Niwa et al., 1991) for ES cell rescue.

Embryonic stem cell culture and transfection

ES cells were cultured according to Li et al. (Li et al., 1995). For the rescue experiments, 2 × 10⁷ ZHBTc4 ES cells were electroporated with 100 µg of linearized plasmid DNA followed by culture with or without 2 µg/ml tetracycline (Tc) (Sigma) for 2 days. Cells were then cultured in 2 µg/ml puromycin (Sigma) with or without Tc for 7 days. The resulting colonies were staining for alkaline phosphatase (AP) activity (Sigma-Aldrich) or expanded as clonal lines.

Luciferase reporter assays

ZHBTc4 ES cells (1 × 10⁷) were plated on a 24-well plate with 2 µg/ml Tc. Twenty-four hours later, 75 ng of reporter plasmids and 150 ng of the test plasmid was transfected according to Brickman et al. (Brickman et al., 2001). For Xenopus embryos, 50 ng of reporter plasmid with or without morpholino was injected into both blastomeres of a two-cell stage embryo.

RNA isolation and real-time RT-PCR

Total RNA was prepared from pools of 15 embryos, 25 animal caps or 10⁶ ES cells using Absolutely RNA RT-PCR (Stratagene). RNA (1 µg) was used as a template for cDNA synthesis. Real-time RT-PCR was performed using a LightCycler (Roche) and the LightCycler FastStart DNA MasterPLUS SYBR Green 1 (Roche). Standard curves were generated either from diluted cDNA derived from control embryos or from plasmid. Samples were normalized to ornithine decarboxylase (Odc) for Xenopus embryos and β-actin for ES cells. PCR primers and conditions can be provided on request.

RESULTS

Protein identity, chromosomal synteny and expression profiles indicate conservation between XlpouV proteins and Oct4

To compare the protein sequences of mammalian, amphibian and zebrafish PouV proteins, we performed a clustal-based alignment. Identity is immediately apparent in the POUs and POUh domains, and outside these regions there exists only weak homology (Fig. 1A). As both the POU domain and either the N- or C-terminal activation domains of Oct4 have been shown to be required for the maintenance of ES cell self-renewal (Niwa et al., 2002), we looked at these regions specifically. Both global and regionally focused alignments of these proteins failed to reveal any conserved motifs within these regions indicative of shared functional properties that might be present within a subset of this family (Fig. 1A; Table 1).

The identification of three Xenopus laevis PouV proteins, all of which are conserved in Xenopus tropicalis, has been confirmed by extensive database analysis. The three genes are arranged in tandem and are within syntenic groups conserved between Xenopus, mouse and zebrafish (Burgess et al., 2002) (Fig. 1B).

Analysis of mRNA expression by in situ hybridization for Xlpou60, Xlpou25 and Xlpou91 was performed (Fig. 1C). Xlpou60 mRNA is maternally expressed and restricted to the animal hemisphere in both unfertilized oocytes and early cleavage stage embryos. Expression remains in the animal and marginal zones of the embryo but is reduced by the late blastula stage and undetectable by the early gastrulation stages. Xlpou25 and Xlpou91 are first transcribed at the onset of zygotic transcription in the animal and...
marginal zone, similar to Xlpou60. Xlpou25 and Xlpou91 continue to be expressed throughout the process of gastrulation but only in cells that have not undergone involution. Xlpou25 and Xlpou91 expression is then rapidly downregulated as cells begin the process of involution and commitment to their germ layer fate (Fig. 1C). Thus, the composite expression pattern of the Xenopus PouV genes is reminiscent of Oct4.

Later during development, we observe two additional domains of Xlpou25 and Xlpou91 expression in the developing anterior neural tissue and in the posterior neural tube (see Fig. 5A,D). We believe these expression domains to be de novo activation independent of the earlier gastrula expression as transcripts are undetectable at the intervening developmental stages.

Xenopus PouV proteins can rescue self-renewal in Oct4 depleted ES cells

As Oct4 is an absolute requirement for ES cell self-renewal, we could assay the ability of the different PouV family members to substitute for Oct4 through the use of an inducible Oct4 knockout ES cell system (Niwa et al., 2000). Briefly, ZHBTc4 ES cells have both alleles of the endogenous Oct4 gene inactivated, and expression of Oct4 is maintained within these cells by a tetracycline (Tc) regulatable Oct4 transgene.

Using this system, we could measure the extent of Oct4 rescue conveyed by the different PouV proteins in three ways: (1) the generation of ‘ES cell like’, alkaline phosphatase (AP) positive colonies (the rescue index); (2) long-term self-renewal (generation of clonal cell lines); and (3) expression of ES cell specific markers. To ensure that differences in ES cell phenotypes were not due to expression level or a global defect in transcriptional regulatory activity, we first tested the ability of all PouV proteins to activate transcription of octamer binding reporter genes. Co-transfection of the PouV proteins with an Fgf4 enhancer reporter in ZHBTc4 ES cells all resulted in similar levels of induced transcription (Fig. 2A) with the exception of mouse Oct4 (V267P), a variant of Oct4 known to be unable to bind DNA when expressed in ES cells (Niwa et al., 2002). We also tested the activity of these proteins by co-transfection with a second reporter gene that contained six reiterated copies of the octamer binding motif (Niwa et al., 2002). Interestingly all three Xenopus proteins were potent activators in this context, whereas
mouse Oct4, AmOct4 and DrPou2 were less active to varying degrees (Fig. 2A). The ability of all of these proteins to activate transcription from the Fgf4 enhancer reporter indicates that any differences in function they display in ES cells was not due to variability in protein stability.

ES cells normally give rise to AP-positive colonies with a characteristic undifferentiated morphology. In the absence of Oct4, ES cells differentiate into trophoblast. Typical examples of the morphology of cells resulting from transfection of the PouV genes into ZHBTc4 cells in the presence or absence of Oct4 expression are shown in Fig. 2B. All PouV proteins tested, except mouse Oct4 (V267P) (data not shown) and DrPou2, showed some degree of rescue of the Oct4-null phenotype. Interestingly, only cells transfected with Xlpou91 produce colonies with a morphology and growth rate indistinguishable from ES cells. This result was confirmed quantitatively by culturing half of each transfection in the absence of Oct4 and dividing the number of undifferentiated (AP-positive) colonies growing in the absence of Oct4 by the number of undifferentiated colonies growing in the presence of Oct4. These values, rescue indices, are shown in Fig. 2C.

A rigorous test of whether these genes can support long-term self-renewal is to derive and expand ES cell lines in which the introduced genes are stably expressed in the absence of Oct4. The experimental strategy for expanding and analysing the resultant clonal cell lines is shown in Fig. 3A. For each transfection that produced colonies in the absence of Oct4, colonies were picked and expanded to generate clonal lines. All colonies from the mouse Oct4 transfection survived for 10 passages (p10), as did those transfected with Xlpou91. However only 17% and 80% of colonies from the Xlpou25 and Xlpou60 transfection, respectively, survived 10 passages. In cell culture conditions that normally sustain ES cell self-renewal (+LIF) and in the absence of mouse Oct4 expression (Fig. 3B, left panel), both Xlpou91 and mouse Oct4 lines appear undifferentiated, while Xlpou60 and AmOct4 lines contained a significant number of differentiated cells. Xlpou25 lines were almost all differentiated, with clumps of cells that appear to grow as colonies. DrPou2 cell lines, similar to the parental ZHBTc4 cell line, could not be maintained in the absence of mouse Oct4.

ES cell expansion is dependent on the cytokine LIF and, in its absence, ES cell lines rapidly differentiate (Smith et al., 1988; Williams et al., 1988). We observed that all of our cell lines also differentiated upon LIF withdrawal (Fig. 3B, centre panel) and thus the ability of these rescued cell lines to differentiate is not blocked by the presence of the amphibian PouV proteins. ES cells differentiate in response to increased Oct4 protein levels (Niwa et al., 2000), an effect also observed in our PouV cell lines when the Oct4 transgene was reactivated (Fig. 3B, right panel).

Fig. 3C shows the average expression levels calculated from the individual cell lines derived for each different PouV mRNA transfection. The very low standard deviations of these values illustrate that each clonal cell line, within the set, expressed similar levels of their particular PouV mRNA, indicating that ES cell self-renewal has a specific crucial threshold for each individual PouV protein. Interestingly, the expression level of Xlpou91 transcripts was similar to that of mouse Oct4, whereas the number of transcripts produced in the Xlpou25 and Xlpou60 rescued cell lines proved to be on average 15 fold and 116 fold higher, respectively (Fig. 3C).

Quantitative molecular marker analysis was performed on RNA from the rescued cell lines by real-time RT-PCR (Fig. 3D). We first confirmed that Oct4 expression had been suppressed in these lines. The values presented in Fig. 3D represent the mean level of gene expression calculated from at least two independent cell lines.
following rescue using the different PouV proteins. Thus, although the real-time PCR technique we employed detected very low residual levels of Oct4 expression on average, we have cell lines supported by each PouV protein that do not express detectable levels of Oct4 (data not shown). Moreover, we have confirmed that this residual level of Oct4 mRNA does not produce detectable levels of Oct4 protein, as determined by immunohistochemistry (data not shown).

In agreement with the degree of rescue produced by the individual Xenopus proteins, Xlpou91 cell lines displayed the highest expression levels of the ES cell markers Sox2, Rex1, Fgf4 and Nanog. The higher degree of differentiation displayed in Xlpou25 and Xlpou60 supported cell lines (Fig. 3B) is consistent with the reduced levels of ES cell gene expression observed in these cells.

As there is no trophoblast lineage in Xenopus, we considered whether Oct4 might have a broader function than ES cell self-renewal. RNAi-mediated knockdown of Oct4 in both mouse and human ES cells suggested that Oct4 suppressed the endodermal marker Gata4 (Hay et al., 2004), while expression of both brachyury and Gata4 appeared to be lost following Oct4 shut down in ZHBTc4 ES cells (Niwa et al., 2000). Owing to this discrepancy, we thought it important to examine the response of both mesodermal and endodermal markers to variations in Oct4 levels.

We found that Gata4 expression is maintained in the absence of Oct4, while brachyury expression was rapidly downregulated in the ZHBTc4 ES cell line following depletion of Oct4 (Fig. 4E). We also observe that re-expression of the Oct4 transgene in the mouse
Oct4, Xlpou25 and Xlpou91 ES cell lines results in reduced expression of Gata4 and Mixl1 and an increase in brachyury expression (Fig. 4F). It has previously been shown that both an increase or decrease in Oct4 levels leads to ES cell differentiation (Niwa et al., 2000). Marker analysis in our cell lines helps to explain this phenomenon by demonstrating that enhanced Oct4 drives ES cells towards a brachyury-positive state, whereas reduced levels of Oct4 leads to both endodermal and trophoblast differentiation.

**PouV protein depleted Xenopus have a truncated body axis and anterior defects**

The ability of the Xenopus PouV proteins to rescue Oct4-deficient ES cells suggests a conserved function for the PouV proteins predating mammalian evolution. To gain insight into the embryonic function of PouV proteins in Xenopus, we depleted the levels of XlpouV proteins through the use of antisense morpholino oligonucleotides (MO). An in vitro translation assay demonstrated that the MOs block translation of the wild-type mRNAs but not the non-complementary (NC) Xlpou mRNAs containing non-coding changes to the sequence around the ATG that do not match the morpholino sequence (Fig. 4A). An in vivo luciferase assay confirmed that MO treatment significantly reduced the levels of functional PouV protein in Xenopus embryos (Fig. 4B). The residual luciferase activity is presumably caused by the presence of other octomer binding proteins such as XOct1 and XBrn3 during Xenopus embryogenesis (King and Moore, 1994).

Embryos injected with control MO, Xlpou25 MO or Xlpou60 MO alone developed with no observable phenotype. Protein knockdown with Xlpou91 MO alone, Xlpou25 and Xlpou91 MO, or with a combination of all three MOs (XlpouV MO) resulted in a failure in axis elongation, of varying severity (Fig. 4C, Table 2). Although the most prominent phenotype was posterior truncation, we also observed an anterior defect consisting of reduced head and eye size. The depletion of all three XlpouV proteins resulted in the most severe phenotype (Fig. 4C, Table 2).

**Xenopus PouV proteins function in anterior and posterior neural development**

Xlpou25 and Xlpou91 are expressed in the developing anterior neural tissue (Fig. 5A) and later in the posterior neural tube (Fig. 5C). The abnormal anterior appearance of the XlpouV MO-treated embryos suggested a possible role in anterior neural patterning. Fig. 5B demonstrates the dramatically reduced expression of Fgf8 at the midbrain hindbrain boundary (MHB) of XlpouV-depleted embryos. The other anterior domains of Fgf8 expression (the prospective hatching gland and an epidermal crescent outside the neural plate area) were not so severely affected (data not shown), while the posterior periblastoporal domain of Fgf8 expression was also reduced. En2, a marker specific to the MHB region was initiated appropriately at the early neurula stage but then became dramatically reduced at later stages (Fig. 5B). This defect also extended to the anterior hindbrain as expression of KroX20 in rhombomeres 3 and 5 was initiated but subsequently not maintained in rhombomere 3 (Fig. 5B).

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**Table 2. Percentage phenotypes resulting from XlpouV protein depletion**

<table>
<thead>
<tr>
<th>Morpholino (ng/embryo)</th>
<th>Severe AP truncation (%)</th>
<th>Moderate AP truncation (%)</th>
<th>Anterior defect (%)</th>
<th>Normal (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xlpou25</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>89</td>
<td>109</td>
</tr>
<tr>
<td>Xlpou60</td>
<td>–</td>
<td>–</td>
<td>0</td>
<td>89</td>
<td>109</td>
</tr>
<tr>
<td>Xlpou91</td>
<td>–</td>
<td>40</td>
<td>5</td>
<td>41</td>
<td>147</td>
</tr>
<tr>
<td>40</td>
<td>–</td>
<td>40</td>
<td>58</td>
<td>35</td>
<td>36</td>
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<tr>
<td>40</td>
<td>–</td>
<td>10</td>
<td>10</td>
<td>25</td>
<td>14</td>
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<td>40</td>
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<td>50</td>
<td>149</td>
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<td>40</td>
<td>40</td>
<td>20</td>
<td>68</td>
<td>26</td>
<td>61</td>
</tr>
<tr>
<td>Control (120 ng)</td>
<td>40</td>
<td>40</td>
<td>68</td>
<td>26</td>
<td>61</td>
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</table>

Abbreviations: AP, anterior-posterior axis; n, number of embryos.
Interestingly, we observed a dramatic loss of for a number of gastrulation stage markers in XlPouV-depleted during cell specification. Fig. 6A shows RNA in situ hybridization suggests that Xlpou depletion might have resulted in defects early late gastrulation (Fig. 5E). To investigate whether the loss of posterior tissue was due to inhibition of differentiation (Cao et al., 2004; Constance Lane et al., 2004; Ying et al., 2003). We also observed a loss of Fgf8 and brachyury (Xbra) expression, genes normally expressed in the marginal zone. Expression of marker genes associated with both endoderm (Mix2, Sox17a and endoderm) and the organizer region [goosecoid (Gsc), chordin and cerberus] were all expanded (Fig. 6A,B). These results were confirmed by real-time RT-PCR analysis of XIpouV-depleted embryos (Fig. 6B). Depletion of individual XIPouV proteins revealed some differences in function within the Xenopus gene family. For example, depletion of Xlpou25 protein alone did not result in the loss of Xbra and Bmp4 expression or an increase in Sox17a expression (data not shown). This difference in the function of Xlpou25, compared with Xlpou60 and Xlpou91, may explain its reduced capacity to rescue ES cell self-renewal.

Fig. 6C shows the post-gastrulation consequences of XIpouV depletion. All three Xlpou proteins are expressed in the animal hemisphere and following their depletion we found that the neural plate marker Sox2 and a pro-neural gene, Ngnr1, were both upregulated. Marginal zone cells from Xlpou-depleted embryos express high levels of organizer and anterior endoderm markers, consistent with the precocious conversion of uncommitted marginal zone into a committed cell fate. In agreement with the early conversion of an abnormally large region of this tissue to early endoderm, we found an expansion of later Hex expression, which normally marks the earliest endoderm to involute during gastrulation. In normal stage 35 tadpoles, Hex expression is

The expression of both Xlpou25 and Xlpou91 in the posterior neural tube prompted us to investigate the effects of XlpouV depletion on the expression of Xcad3, a member of the caudal (Cdx) gene family. Xcad3 is also expressed in the posterior neural tube at this stage and was of particular interest as it is a homologue of mouse Cdx2, a gene whose expression was shown to be increased following mouse Oct4 depletion in ES cells (Niwa et al., 2000). Depletion of Pou protein in Xenopus embryos also resulted in an expansion of Xcad3 expression (Fig. 5D). We also observed an increase in Xcad3 expression during gastrulation, although at a lower level (data not shown).

**XIPouV protein depletion disrupts the expression of genes associated with early lineage commitment**

To investigate whether the loss of posterior tissue was due to increased cell death of late gastrulating marginal zone cells, whole-mount TUNEL was performed on XlpouV-depleted embryos and control MO-treated embryos. We observed no difference between XlpouV-depleted embryos and control MO-treated embryos during late gastrulation (Fig. 5E).

The loss of posterior tissues in the absence of cell death suggests that Xlpou depletion might have resulted in defects early during cell specification. Fig. 6A shows RNA in situ hybridization for a number of gastrulation stage markers in XIpouV-depleted embryos. Interestingly, we observed a dramatic loss of Bmp4 and Xom (Xvent2) expression, both genes associated with the
restricted to the descendants of this tissue in the liver, whereas in Xlpou-depleted embryos there is both an expanded liver domain and ectopic Hex expression (Fig. 6C). These embryos also exhibit a dramatic reduction in posterior myosin-light chain 1-3 (MLC1-3) expression. This result is consistent with a premature conversion of uncommitted marginal zone cells to organizer or anterior endoderm early in gastrulation, leaving an insufficient number of cells to form the posterior somites. Thus, the anterior somites appear normal but towards the posterior of the embryo the expression of MLC1-3 is decreased to a point where no segmented somites are visible (Fig. 6C).

Overexpression of XPouV proteins produced the opposite effects on gene expression to that of Xlpou depletion. At the gastrulation stage, overexpression of all three *Xenopus* PouV proteins enhanced the expression of *Xbra* and *Bmp4* and reduced the expression of *Mixer* and goosecoid (Fig. 6D). These results paralleled those obtained from the depletion or overexpression of PouV proteins in ES cells (Fig. 3E,F) and indicate that mammalian and *Xenopus* PouV proteins have similar downstream targets and may function through similar molecular pathways.

**Rescue of knockdown phenotype**

To confirm that the changes in gene expression were specific to depletion of PouV proteins, we co-injected XlpouV MO with XlpouV RNAs non-complementary (NC) to the MO-targeted sequence. Luciferase assays demonstrated that the NC proteins activated transcription as efficiently as the wild-type proteins (data not shown). As expected, some specificity in the ability of the

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**Fig. 6. Depletion of XPouV proteins affects germ layer induction.** (A) Two-cell stage embryos were injected in both blastomeres with 60 ng of control MO or 20 ng of each of the XlpouV MOs. In situ analyses were performed on stage 10.25 embryos. Embryos are positioned with the vegetal hemisphere upwards and the dorsal lip towards the top, apart from Bmp4 and Xom, which are positioned animal hemisphere upwards. (B) Real-time RT-PCR analysis of XlpouV protein-depleted embryos. Embryos were injected with control MO or XlpouV MO as in Fig. 6A and RNA extracted at stage 10.25. The relative change in gene expression was calculated by dividing the XlpouV MO values by the control MO values. Experiments were carried out in triplicate. (C) Embryos were injected with control MO or XlpouV MO as in Fig. 6A. Sox2 and Ngnrt expression was analysed at stage 13 and Hex and MLC1-3 expression at stage 35 embryos. (D) Two-cell stage embryos were injected in both blastomeres with 250 pg of each RNA and in situ analyses performed on stage 10.25 embryos. All embryos are positioned with the vegetal hemisphere facing upwards and the dorsal lip towards the top, apart from Bmp4 embryos that are positioned animal hemisphere upwards.
different POU proteins to rescue the MO phenotype was detected, but addition of all three XlpouV NC proteins fully rescued the MO-induced phenotype (Table 3). Mouse Oct4 and AmOct4 also efficiently rescued the defect in Xbra expression and could partially rescue Mixer expression, indicating a degree of functional conservation between the amphibian and mammalian members of the PouV family. By contrast, DrPou2 showed very little rescue ability (Table 3).

Depletion of PouV proteins alters germ layer induction by activin in animal cap explants

Our data suggested that the depletion of PouV proteins causes multipotent, non-committed marginal zone cells to differentiate much more readily when exposed to differentiation signals. To investigate this further we used animal cap explants, which can be used to reproduce the in vivo induction of both marginal zone and endoderm or organizer derivatives (Okabayashi and Asashima, 2003).

The molecular effects of XlpouV knockdown in animal caps was monitored by the expression of specific marker genes. Bmp4 was the only marker analysed that is expressed in un-induced (no activin) animal caps, and we observed a significant decrease in Bmp4 expression in uninduced animal caps following XlpouV protein depletion (Fig. 7A). Although differences could be observed between the control MO and XlpouV MO-treated explants that had not been induced with activin, we do not consider these to be significant because of their very low expression levels (Fig. 7A).

Treatment of animal caps with 8 U/ml of activin induces the expression of Xbra in control MO explants, but this induction was reduced in XlpouV-depleted explants (Fig. 7A). Using 16 U/ml of activin, we also observed elevated expression of the organizer and endodermal associated markers Gsc, chordin, cerberus, Mixer, Sox17α and endodermin in XlpouV-depleted explants. Moreover, Gsc and cerberus expression can only be induced at 16 U/ml activin in control MO-treated animal caps whereas the lower dose of 8 U/ml activin was sufficient to induce these organizer markers in the XlpouV depleted animal caps (Fig. 7B). Thus, depletion of XlpouV proteins sensitizes animal cap cells to activin treatment, while reducing expression of marginal zone markers such as Xbra and Bmp4. We also observed that overexpression of XlpouV proteins in activin-treated animal caps dramatically reduced the induction of Gsc (data not shown).

DISCUSSION

Maintenance of cells in a non-committed state prior to gastrulation is a fundamental aspect of triploblastic organisms. Here, we have shown that Xenopus PouV proteins have the ability to maintain ES cells in an undifferentiated state.

Table 3. Percentage rescue of the XlpouV protein depletion phenotype by PouV proteins

<table>
<thead>
<tr>
<th>RNA</th>
<th>Xbra expression</th>
<th>Bmp4 expression</th>
<th>Mixer expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complete</td>
<td>Moderate</td>
<td>Complete</td>
</tr>
<tr>
<td>Xlpou25</td>
<td>10</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Xlpou60</td>
<td>20</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>Xlpou91</td>
<td>30</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>Xlpou25/60/91</td>
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<td>50</td>
<td>70</td>
</tr>
<tr>
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<td>50</td>
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</tr>
<tr>
<td>AmOct4</td>
<td>40</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>DrPou2</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

mRNA (250 pg) was injected into each blastomere of a two-cell stage embryo. For Xlpou25/60/91, 100 pg of each mRNA was injected into each blastomere of a two-cell stage embryo.

Fig. 7. Depletion of PouV proteins produces a heightened activin response in animal cap explants. (A) Real-time RT-PCR analysis of XlpouV protein-depleted animal cap explants. Embryos were injected with control MO or XlpouV MO as above. Animal cap explants were isolated at stage 8 and cultured with or without activin until sibling embryos reached stage 10.25. Relative expression represents values normalized to Odc. 16 U/ml activin was used for induction apart from the Xbra and Bmp4 analysis that used 8 U/ml activin. Experiments were carried out in triplicate. (B) Embryos were injected with control MO or XlpouV MO as above and animal caps explants isolated and induced with either 8 U/ml or 16 U/ml activin. Relative expression represents the Gsc and cerberus values normalized to Odc. Experiments were carried out in triplicate.
state and that their role during Xenopus embryogenesis is to maintain cells as multipotent and non-committed during gastrulation.

Conservation and diversification of PouV proteins
Our data indicate a role for PouV proteins in the maintenance of non-committed cell populations in Xenopus (Fig. 8A). In mouse, PouV function is a known requirement for both ES cell self-renewal and primordial germ cell development. The similarities between these roles suggest PouV proteins have a conserved role in maintaining multipotent, non-committed cells. Thus, Xenopus PouV proteins can sustain ES cell self-renewal and mouse Oct4 can rescue PouV depletion phenotypes in Xenopus. By contrast, the zebrafish PouV protein, DrPou2, showed no ability to substitute for Oct4 in ES cells or rescue the Xenopus PouV depletion phenotype. Consistent with these observations, DrPou2 was shown to have a distinct function during gastrulation, as a mediator of endoderm induction and differentiation in zebrafish (Lunde et al., 2004; Reim and Brand, 2002; Reim et al., 2004).

Although the zebrafish protein DrPou2 was unable to rescue Oct4 null ES cells or the gastrulation stage XlPouV depletion phenotype, its role in neural patterning shows some conservation with Xenopus. XlPou25 and XlPou91 were found to be required for maintenance of gene expression in the MHB and the anterior hindbrain. A similar role has been reported for DrPou2 and this aspect of the XlPouV depletion phenotype is similar to the spiel-ohne-grenzen (spg) (DrPou2 mutant) phenotype (Belting et al., 2001; Hauptmann et al., 2002; Reim and Brand, 2002). Consistent with the conservation of function during neural development, overexpression of mouse Oct4 mRNA in spg mutant zebrafish can rescue a defect in Pax2.1 expression in the MHB. However, although mouse Oct4 rescues this aspect of the zebrafish spg phenotype, there is currently no evidence for a requirement for Oct4 in neural development. In addition, despite the rescue of Pax2.1 expression in neural patterning there is no evidence that mouse Oct4 can rescue the zygotic or maternal/zygotic defects in endoderm induction. Moreover, DrPou2 induces endoderm cooperatively with the SRY box protein, Sox32 (also known as Casanova) (Reim et al., 2004). As PouV proteins usually bind DNA coordinately with a member of the Sox family (Dailey and Basilio, 2001) and no Sox32-like protein has been identified in other vertebrates, the Sox32-Pou2 endoderm induction mechanism may be unique to teleosts. It is possible that the genes encoding Sox32 and Pou2 arose from a duplication event in which an ancient PouV-Sox gene set retained original function and the other acquired a novel endoderm inducing activity. However, despite the large number of POU genes present in the sequenced teleost genomes, we have been unable to find an obvious candidate. This is particularly surprising as sequences resembling both Pou2 and Oct4 have been identified in sturgeon, a species that occupies a more basal phylogenetic position than teleosts (Andrew Johnson, personal communication). The divergent function of DrPou2 in endoderm induction may explain why it was unable to rescue ES cell self-renewal or the gastrulation stage XlPouV depletion phenotype.

As Xenopus lacks trophectrodem and the XlPouV depletion does not affect germ cell development (data not shown), the conserved PouV function in Xenopus and ES cells argues against Oct4 function being a unique innovation of mammals for these specific lineages. In support of this, the relationship between Caudal (Cdx) and PouV proteins does not appear to be exclusive to trophoblast versus ICM cell fate decisions in mammals. Xenopus lacks trophectoderm tissue, yet an orthologue of Cdx2, Xcad3, exists (Pownall et al., 1996). Loss of PouV proteins in both ES cells and Xenopus embryos results in ectopic Cdx2 (Nichols et al., 1998; Niwa et al., 2000) and Xcad3 expression (Fig. 5D), respectively. Moreover we have found similar sets of genes to be regulated by PouV proteins in ES cells and during Xenopus gastrulation. Thus, we conclude that mouse Oct4 is not a mammalian-specific protein, but rather the function of PouV proteins in suppressing differentiation and commitment has been adopted by mammals to maintain pluripotency in ES cells, in part by blocking commitment to the trophoblast lineage.

A function for PouV proteins during gastrulation
It is likely that the pivotal role of Oct4 in the maintenance of ES cell pluripotency depends on the ability of this protein to suppress cell fate commitment in multiple lineages and is therefore derived from an ancient role in gastrulation. In Xenopus, the fate of germ layer domains are specified early by the asymmetrical localization of maternal mRNAs such as Vg1, VegT, Wnt11 and ectoderminder (Dupont et al., 2005; Joseph and Melton, 1998; Tao et al., 2005; Xanthos et al., 2001). Although this process regionalizes the embryo into broad domains, cells become committed to specific lineages only as they begin involution in response to these signals. Cells continue to be committed to their particular cell fate throughout gastrulation, but the embryo requires a pool of multipotent precursors to enable the formation of more posterior lineages later during this process. A similar pool of cells is required in mammalian development and the expression of Oct4, initially throughout the epiblast and later in the region of the primitive streak, may indicate the location of these populations (Snape et al., 1987; Wylie et al., 1987). ES cell derivation represents the capture of these epiblast, non-committed populations in vitro. However, in Xenopus, reductive cell division would make it difficult to capture and sustain these populations (Frederick and Andrews, 1994). Regardless of the feasibility of creating ES cell lines, all vertebrates must posses the
ability to prevent premature commitment. We propose here that PouV proteins possess this function, which we have found to be conserved in a number of vertebrate species.

A model illustrating how precocious differentiation in the absence of PouV affects cells fate is shown in Fig. 8. In *Xenopus*, the organizer tissue is the first to gastrulate and become committed, giving rise to dorsal anterior structures while cells from the remaining marginal zone gastrulate later and populate progressively posterior regions of the embryo (Constance Lane et al., 2004; Lane and Smith, 1999). In the PouV-depleted embryos we observe a decrease in the expression of markers associated with non-committed cell types (*Bmp4*, *Xom* and brachyury), whereas the expression domains of markers associated with early commitment (organizer and anterior endoderm) were expanded. The concurrent loss and gain of uncommitted and committed cells, respectively, in the absence of increased cell death leads us to conclude that there is a premature progression of uncommitted marginal zone cells to a more committed cell fate early during gastrulation.

Following PouV depletion in *Xenopus*, there is a striking down-regulation in the expression of *Bmp4*. This extrinsic factor has been associated with maintaining cells in a non-committed state in both ES cell lines and in *Xenopus* embryos where expression of a constitutively active BMP receptor blocks the onset of germ layer commitment (Constance Lane et al., 2004; Ying et al., 2003). We found the expression of both *Bmp4* and its downstream target *Xom* was dependent on PouV in *Xenopus*. Interestingly, *Xom* belongs to the same class of transcription factors as the ES cell marker, Nanog.

We also observed a reduction in the expression of *Xbra* in the PouV-depleted embryos. There is an accumulating body of evidence in mouse that brachyury is a transient marker of both future mesoderm and endodermal cells (Kubo et al., 2004; Wilson et al., 1995). In addition *Xbra* interacts directly with components of the BMP signalling pathway to induce *Xom*, a direct antagonist of the organizer associated gene goosecoid (Messinger et al., 2005). Thus, PouV proteins may block commitment in bipotent precursors of the mesoderm and endoderm in part by maintaining brachyury expression.

Loss of XipouV expression also leads to an expansion of endodermal markers not associated with the organizer. Interestingly, mouse Oct4 has been shown to have a role in inhibiting the expression of endodermal associated genes through an interaction with Foxd3 (Guo et al., 2002). An increase in endodermal gene expression was also observed in Oct4-depleted mouse and human ES cells (Hay et al., 2004).

As the depletion of PouV function in *Xenopus* results in a premature progression to a committed cell type and XIPouV proteins can sustain undifferentiated ES cells, we propose that there is a conserved role for these proteins in maintaining cell multipotency. This would strongly imply that the ability of mouse Oct4 to maintain ES cells as self-renewing and pluripotent is derived from this ancient PouV function.

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References
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