Research Article

Expression analysis of *Cdx2* and *Pou5f1* in a marsupial, the stripe-faced dunnart, during early development[†]

Mary Familiar^{1*}, Phil Chi Khang Au¹, Robb U. de Iongh², Yolanda Cruz^{3,4}, Lynne Selwood^{1,4}

¹School of Biosciences, University of Melbourne, Parkville Victoria 3010 Australia

²Department of Anatomy and Neuroscience, University of Melbourne, Parkville Victoria 3010 Australia

³Department of Biology, Oberlin College, Oberlin, Ohio, 44074, USA

⁴Joint senior authors

*Author for correspondence:

Mary Familiar

School of Biosciences

University of Melbourne

Parkville, Victoria 3010

Australia.

Telephone: +613 8344 5069

Facsimile: +613 8344 7909

Email: m.familiari@unimelb.edu.au

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SUMMARY

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The first lineage allocation during mouse development forms the trophectoderm and inner cell mass, in which *Cdx2* and *Pou5f1* display reciprocal expression. Yet *Cdx2* is not required for trophectoderm specification in other mammals, such as the human, cow, pig, or in two marsupials, the tammar and opossum. The role of *Cdx2* and *Pou5f1* in the first lineage allocation of *Sminthopsis macroura*, the stripe-faced dunnart, is unknown. In this study, expression of *Cdx2* and *Pou5f1* during oogenesis, development from cleavage to blastocyst stages, and in the allocation of the first three germ-layer lineages was analyzed for this dunnart. *Cdx2* mRNA was present in late antral-stage oocytes, but was lost until Day 5.5. *Pou5f1* mRNA was present from primary follicles to zygotes, and then accumulated starting at the early unilaminar blastocyst stage. By contrast, all cleavage stages and the pluriblast and trophoblast cells co-expressed CDX2 and POU5F1 proteins, which persisted until early stages of hypoblast formation. Hypoblast cells also possessed both POU5F1 and CDX2 once they were allocated, and this persisted during their division and migration. Our studies thus suggest that CDX2 and POU5F1 are maternal proteins, and that the first lineage to differentiate is the trophoblast, which differentiates to trophectoderm after shell loss one day before implantation. In the stripe-faced dunnart, cleavage cells, as well as trophoblast and pluriblast cells, are polarised, suggesting the continued presence of CDX2 in both lineages until late blastocyst stages may play a role in the formation and maintenance of polarity.

INTRODUCTION

The first lineage allocation in mammalian embryonic development results in the segregation of the trophoblast and pluriblast or inner cell mass lineages (Arnold and Robertson, 2009; Rossant and Tam, 2009; Berg et al., 2011; Schrode et al., 2013). Two transcription factors, CDX2 and POU5F1 (previously known as OCT4; see Frankenberg et al., 2010) play a key role in this process (Cockburn and Rossant, 2010). The term inner cell mass, however, is not applicable to marsupials

because they have no morula (Selwood and Smith, 1990); instead, the term pluriblast is used, as suggested by Johnson and Selwood (1996).

The absence of a morula stage in marsupials is due to zona pellicuda-blastomere adhesion that precedes cell-cell adhesion, resulting in a unilaminar blastocyst (Selwood, 1992; 1994; 1996; Kress and Selwood, 2004; Morrison et al., 2013). In the stripe-faced dunnart (*Sminthopsis macroura*), the unilaminar blastocyst forms on Day 3.0 of gestation (Selwood and Hickford, 1999), and consists of an embryonic area of more-rounded pluriblast cells that are distinguishable from the more-flattened and vesicular trophoblast cells that comprise the remainder of the blastocyst epithelium (Selwood, 1996; Selwood and Johnson, 2006). As the blastocyst expands, the pluriblast and trophoblast cells, which are temporarily indistinguishable on Day 4.5 of gestation – although they can be recognised if separated in vitro (Yousef and Selwood, 1996). Soon after, however, these two lineages become morphologically distinguishable at the bilaminar blastocyst stage (Day 6.0), when the hypoblast arises from the pluriblast, which transforms to the epiblast, and the trophoblast begins its transformation to the trophectoderm (Selwood and Woolley, 1991; Kress and Selwood, 2004). This delay of trophectoderm differentiation and epiblast/hypoblast allocation has also been observed in other marsupial species, including *S. crassicaudata*, the fat-tailed dunnart (Roberts and Breed 1996), *Isodon obesulus*, a bandicoot (Lyne and Hollis 1977), and *Trichosurus vulpecular*, the brushtail possum (Casey and Selwood, 2002).

The relationship between cell lineage changes and the presence of *Pou5f1* and *Cdx2* mRNA and proteins was first outlined in the mouse. *Pou5f1* transcripts are found in the murine oocyte, zygote, and early-cleavage stages, but become progressively restricted to the inner cell mass from the 16-cell morula stage after zygotic genome activation. In the late blastocyst, *Pou5f1* expression is restricted to the epiblast and the hypoblast after precocious implantation at Day 4.5 (Schöler et al., 1989; Wu and Schöler, 2014). The cytoplasm of unfertilized eggs and zygotes contain low levels of

POU5F1 protein, whereas higher levels are found in the nucleus following fertilization and in early-cleavage stages, with a further increase in nuclear abundance at the 8-cell and morula stages. As the blastocyst forms and the trophectoderm differentiates, POU5F1 levels diminish in the trophectoderm but remain high in the nuclei of inner cell mass and late-hypoblast cells (Palmieri et al. 1994). Murine *Cdx2* transcripts, on the other hand, first accumulate in the 8-cell stage, and are subsequently enriched in the trophectoderm cells of the late morula at the 32-cell stage (Dietrich and Hiiragi, 2007; Jedrusik et al., 2008; Ralston and Rossant, 2008). Similarly, CDX2 protein becomes restricted to the trophectoderm cells of the morula and blastocyst, causing the down-regulation of POU5F1 in this lineage by the late-blastocyst stage.

The pattern of *Pou5f1* and *Cdx2* expression in the mouse appears to be at least partly related to its precocious implantation, compared to other species. In species whose implantation is relatively delayed, such as in the pig or the cow, POU5F1 protein is expressed in both the inner cell mass and the trophoblast cells. In the pig, analysis of POU5F1 and CDX2 expression from the oocyte to the expanded-blastocyst stage revealed that CDX2 is restricted to the trophectoderm while POU5F1 is not expressed in morulae, but is present in both the trophectoderm and inner cell mass of blastocysts (Kuijk et al., 2008; Fujii et al., 2013). In the cow, *POU5F1* transcript first appears in the morula (16-cell), and its abundance increases in the compacted morula (64-cell) (Kurosaka et al., 2004). By contrast, *CDX2* expression is first detected in the Day-7, early-expanding bovine blastocysts containing ~100 cells (Berg et al., 2011; Groissis and Cibelli, 2014). Interestingly, whereas murine *Pou5f1* and *Cdx2* mutually inhibit each other's expression to generate the first two lineages (Niwa et al. 2005), *CDX2* does not repress *POU5F1* expression in the bovine trophoblast (Berg et al. 2011). This difference in expression profile may account for the ability of Day-7 trophectoderm cells of bovine blastocysts to contribute to inner-cell-mass-derived lineages, even though they normally remain committed to a trophectoderm fate in the absence of experimental intervention (Berg et al. 2011).

The first molecular analyses of early lineage allocation in marsupial embryos have recently been undertaken. In both the tammar wallaby and gray short-tailed opossum, pluriblast cells can be morphologically distinguished from trophoblast cells prior to the blastocyst stage, yet both cell types coexpress CDX2 and POU5F1; it is only after the unilaminar blastocyst stage that these two cell lineages differentially express POU5F1 (Frankenberg et al. 2013; Morrison et al. 2013). It is unknown whether *Cdx2* and *Pou5f1* are involved in lineage allocation into pluriblast and trophoblast at the 16-cell stage or later during trophectoderm differentiation at the bilaminar blastocyst stage in the dunnart. The aims of this study were thus to clone *Cdx2* and *Pou5f1* from the stripe-faced dunnart, and to analyse their gene and protein expression during lineage allocation in early development.

RESULTS

Cdx2 and *Pou5f1* sequence analysis

Nested reverse-transcription PCR was used to isolate partial cDNAs for *Cdx2* (231 base pairs) and *Pou5f1* (350 base pairs). Alignment of this partial *S. macroura Cdx2* (GenBank accession number GQ844867) with published cDNA sequences from two eutherian species, *Mus musculus* (86.7% identity) and *Homo sapiens* (83.9%), and one marsupial, *Monodelphis domestica* (97.8%), showed that *Cdx2* is evolutionarily conserved (Fig. S1).

Given the extensive evolutionary analysis recently undertaken for *Pou5f1* (Frankenberg et al 2010; 2013), it was more appropriate to compare the *S. macroura Pou5f1* cDNA (GenBank accession number KM401577) with sequences available for metatherians and prototherians than with eutherian species. Alignment of the partial *S. macroura Pou5f1* sequence with three marsupial species – *T. vulpecula* (93% identity), *M. domestica* (91%), and *Macropus eugenii* (91%) – and one protherian – *Ornithorhynchus anatinus* (71%) – showed that stripe-faced dunnart *Pou5f1* is also highly conserved (Fig. S2). The deduced amino acid sequence for *S. macroura Pou5f1* (data not

shown) is coded by exons 4 and 5 when compared to the full-length *M. eugenii* sequence (Frankenberg et al., 2010).

***Cdx2* and *Pou5f1* expression in *S. macroura* tissues and early development**

The expression of *Pou5f1* and *Cdx2* was evaluated by reverse-transcription PCR on RNA isolated from various *S. macroura* tissues, from oogenesis through specific stages of development. *Gapdh* is commonly used as a loading control for somatic tissues, but it is inactivated during the meiotic and post-meiotic phases since glycolysis is disabled in germ cells and early embryos (Mori et al., 1992; Welch et al., 1992; Eddy et al., 1994; Aswal et al., 2008). *18S* was therefore used as a loading control for oocytes, follicle cells, and cleavage to blastocyst-stage embryos whereas *Gapdh* was used as a control for somatic tissues.

Cdx2 amplicons of the expected size were detected specifically in the ovary and hindgut, but not in testis, brain, or other somatic tissues (Fig. 1A). While *Cdx2* was not expressed in primary oocytes or follicle cells of secondary follicles (Fig. 1B), it was expressed in ovaries collected from Day-40 pouch young (Fig. 1C) and could also be detected in late-antral oocytes (Fig. S3). Oocyte maturation occurs very quickly in the stripe-faced dunnart, and ovulation is not synchronous within or between ovaries (Menkhorst et al. 2007), therefore individual oocytes would only be positive for *Cdx2* if the oocyte was close to ovulation. *Cdx2* was not expressed in any conceptuses examined from the zygote to the 64-cell stage (Fig. 1D). Distinct, but transient, *Cdx2* expression was, however, detected in Day-5.5 unilaminar blastocysts and Day-6.5 to -7.5 bilaminar blastocysts (Fig. 1E).

Interestingly, *Pou5f1* was expressed in primary and antral follicles and zygotes, but was absent during cleavage from the 2- to 32-cell stage (Fig. 2A). *Pou5f1* was detectable again in the early unilaminar blastocyst at the beginning of expansion (64-cell stage; Day 3 of gestation), presumably following zygotic genome activation, which is thought to occur approximately 12 hours earlier at the 16-cell stage, based on nucleolar appearance (Selwood and Hickford, 1999; Kress and

Selwood, 2003). *Pou5f1* expression persisted throughout the remaining blastocyst stages (Day 4.0 to 7.5) (Fig 2A). As amplification of *Pou5f1* from primordial and primary follicle samples was variable among the reverse-transcription PCR reactions, we confirmed expression at these stages using the nested PCR primers. This approach yielded distinct amplification of *Pou5f1* from the initial amplifications of cDNA from primordial, primary, and antral follicles as well as from 64-cell stage embryos and older – but not from early zygote to 32-cell stages (Fig. 2B).

Co-localization of CDX2 and POU5F1 in cleavage stages (Day 0.5 to 2.5)

The presence or absence of *Cdx2* or *Pou5f1* mRNA in cleavage stages does not guarantee that the corresponding protein is not present in early dunnart conceptuses, so we used immunofluorescence to assess the CDX2 and POU5F1 protein abundance. Both POU5F1 (Fig. 3B) and CDX2 (Fig. 3C) were found to co-localise (Fig. 3D) in the nucleus and yolk mass of the polarised zygote soon after syngamy. Similar co-localisation of POU5F1 with CDX2 was detected in the cytoplasm and nucleus of ovulated oocytes and in blastomeres of all cleavage stages up to the 64-cell as well as at the 256-cell stages (Day 4.5) (Fig. S4). This is best exemplified at the 4-cell stage (Fig. 3E-H), when individual blastomeres are clearly labelled. Hoechst co-staining confirmed the presence of both proteins in the nucleus, and their absence from the nucleolus (Fig. 3H, arrow).

Specificity of the CDX2 immunofluorescence signal was assessed by substituting an equal concentration of normal / non-immune mouse immunoglobulins for the primary mouse anti-CDX2 immunoglobulins, and omitting the primary anti-POU5F1 immunoglobulins; no specific reactivity was detected (Fig. S5B-C), indicating that the original CDX2 signal was reliable. Evaluation of the POU5F1 signal specificity was achieved by pre-absorbing the primary chicken anti-POU5F1 immunoglobulins with a fivefold molar excess of POU5F1 protein to competitively inhibit the binding of specific antibodies to endogenous POU5F1 in the tissues. As expected, this competition step blocked POU5F1 (Fig. S5F) but not CDX2 signal (Fig. S5G).

Co-localisation of CDX2 and POU5F1 in blastocysts

We next examined the spatio-temporal localisation of the proteins in the developing bilaminar blastocyst from Day 6.5 to 8.5 of gestation, a period when *Pou5f1* and *Cdx2* showed distinct mRNA expression. The Day-6.5 stripe-faced dunnart blastocysts were approximately 1.0 to 1.2 mm in diameter, and had just started to become bilaminar, with small numbers of putative hypoblast cells apparent within the pluriblast pavement epithelium. The different lineages can be easily distinguished because the pluriblast cells are more-rounded and smaller than the more-flattened, larger and vesicular trophoblast cells.

In all embryos examined, cells in the pluriblast layer showed variable levels of POU5F1 staining in the cytoplasm and nucleus (Fig. 4B), sometimes aggregated in numerous perinuclear vesicles (Fig. 4B inset, arrowheads). Some cells within the pluriblast layer showed more-intense staining, and some of these were observed to be dividing (Fig. 4B inset, asterisk). These intensely stained cells often contained densely stained granules in the cytoplasm (Fig. 4B inset, open arrowhead). The smaller size and morphology of these cells (Fig. 4B, arrows) indicate that they may be hypoblast cells; many of these intensely POU5F1-positive, putative hypoblast cells also stained intensely with CDX2 (Fig. 4C-D, arrows). On the other hand, many of the cells within the pluriblast layer showed weak CDX2 nuclear staining, and CDX2-negative cytoplasm.

The trophoblast cells in Day-6.5 bilaminar blastocysts (Fig. 4E-H) are larger than the pluriblast cells (Fig. 4A-D), and showed strong nuclear and weaker cytoplasmic POU5F1 staining (Fig. 4F). Trophoblast cells had dark, unstained perinuclear vesicles, but these were smaller and fewer in number than in the pluriblast cells (Fig. 4F insert, arrowhead). Co-staining for CDX2 revealed an intense ring of perinuclear CDX2 (Fig. 4G, arrows). Cytoplasmic distribution of CDX2 at this stage is consistent with the timing of embryonic *Cdx2* expression in Day-5.5 unilaminar blastocysts (Fig. 1E).

The trophoblast and pluriblast were more-easily distinguishable in slightly later Day-6.5 bilaminar blastocysts (Fig. 4J-M), which were 2 mm in diameter. Hypoblast cells appeared to be forming a reticulum beneath the pluriblast, with individual hypoblast cells migrating beneath the adjoining trophectoderm. In both embryos examined at this stage, the pavement pluriblast epithelial cells showed intense cytoplasmic but no nuclear POU5F1 staining (Fig. 4K). By contrast, the trophoblast cells showed only weak POU5F1 staining, whereas the hypoblast cells migrating under the trophoblast showed distinct nuclear POU5F1 staining (Fig. 4K, open arrowheads) as well as cytoplasmic staining in extended cell processes of some hypoblast cells (Fig. 4K, arrow). CDX2 staining was intense in the nuclei of pluriblast epithelial cells (Fig. 4L-M). The POU5F1-positive hypoblast cells (Fig. 4L-M, open arrowheads) also showed more-intense CDX2 staining than the surrounding trophoblast cells (Fig. 4L-M, arrowheads).

By Day 8.5 of gestation, the shell-free bilaminar dunnart blastocysts are 3 mm in diameter, and gastrulation has advanced to form a late primitive streak, the node, notochord, and early neural plate. In the embryos examined, the yolk sac was dissected away from the embryonic area, and divided into flattened radial strips to facilitate visualisation of the densely populated bilaminar yolk-sac region near the embryonic area and the less-densely populated unilaminar yolk sac (Fig. 4N) towards the abembryonic pole. The bilaminar yolk sac is almost completely lined by hypoblast cells, which have formed a dense pavement epithelium, whereas the unilaminar yolk sac is formed predominantly by trophoblast cells, with occasional hypoblast cells forming a looser reticulum as they migrate under the abembryonic region. The trophoblast cells in the unilaminar yolk sac, which at higher magnification were observed to be binucleate (Fig. 4Q inset, arrowheads), showed little or no reactivity for POU5F1 in the nucleus and none in the cytoplasm (Fig. 4O). Similarly, the occasional large trophoblast cell visible in the bilaminar yolk sac also showed little or no POU5F1 reactivity (Fig. 4O, arrowheads). By contrast, the hypoblast cells, which have almost completely lined the bilaminar yolk sac, and the occasional hypoblast cell in the unilaminar yolk sac, show

intense nuclear staining for POU5F1 (Fig. 4O, arrows). CDX2 co-localised with POU5F1 in the nuclei of hypoblast cells, but was virtually absent from trophoblast cells (Fig. 4P, arrowheads).

In summary, the abundance and immunolocalisation patterns of POU5F1 and CDX2 during early embryonic development are dynamic in the stripe-faced dunnart. Maternal *Cdx2* and *Pou5f1* mRNA derived from late-antral follicles likely account for the presence of their respective protein products in the unfertilized oocytes, and from the zygote to the Day-5.5 blastocyst. Yet, once embryonic gene expression initiates, at the 64-cell stage for *Pou5f1* and at the early blastocyst stage for *Cdx2*, their expression and localization are dictated by events associated with lineage allocation. Specifically, both CDX2 and POU5F1 are detected within the pluriblast and trophoblast throughout early development, but begin to segregate around Day 5.5, when these lineages start to differentiate.

DISCUSSION

In this study, we partially cloned striped-face dunnart *Cdx2* and *Pou5f1*. Both genes have high sequence homology to their mammalian orthologues, including other marsupials, suggesting a conserved function. The gene-expression profile and distribution of each protein during early development indicate a maternal origin for CDX2 – from the zygote to Day-5.5 unilaminar blastocyst – and POU5F1 – from the zygote stage onwards. The presence of *Pou5f1* mRNA in the zygote, however, hints that zygotic expression of *Pou5f1* may occur even at the earliest stages of development; further experiments using transcriptional inhibitors would allow us to resolve this. Both POU5F1 and CDX2 colocalize in pluriblast and trophoblast layers until early stages of hypoblast or primitive endoderm formation in the bilaminar dunnart blastocyst, but subsequently become more-restricted after trophectoderm differentiation, gastrulation, and shell loss on D8.5, one day before implantation. Intriguingly, newly emerged hypoblast cells stain strongly for CDX2 and POU5F1 – especially when migrating or dividing.

A comparison of the expression of these two factors in mammals (Table 1) suggests that their contribution to lineage allocation varies among species. One event that is conserved among all species studied to date is the initial allocation of a trophoblast cell type – one that is characterised by cellular polarisation in the early stages, the presence of both CDX2 and POU5F1, and a faster cell-doubling time. Prolonged transition to a differentiated trophectoderm occurs in late-implanting blastocysts –such as dunnart, pig, and cow– compared to a fast-transition in the precociously implanting mouse embryo.

The timing of *Pou5f1* expression during stripe-faced dunnart oogenesis is similar to that in the cow (Khan et al., 2012), pig (Kuijk et al., 2008), and mouse (Monti and Redi, 2009). By contrast, *Cdx2* expression occurs late during dunnart oogenesis and CDX2 remains abundant in ovulated oocytes, whereas murine or bovine *CDX2* is expressed during early oogenesis and declines during antral stages, and CDX2 protein is either not detectable (mouse) or is present at low levels (cow). Blij and colleagues demonstrated that maternal *Cdx2* is not required for mouse development (Blij et al., 2012), whereas Jedrusik and colleagues reported a dependence on both maternal and zygotic expression of murine *Cdx2* (Jedrusik et al., 2015). The presence of CDX2 in all stages of stripe-faced dunnart development, from ovulated oocytes to blastocyst stages, suggests that maternal CDX2 is required in early embryogenesis since *Cdx2* expression does not begin again until Day 5.5 in blastocysts, one day before the second lineage allocation.

POU5F1 and *CDX2* are detected at variable levels in all embryonic cell types until restricted during lineage allocation, which occurs around Day 3 to 3.5 in mice or Day 14 in cow and pig blastocysts (Kirchhof et al., 2000). Cleavage stages of marsupial embryos resemble the mouse in that *Pou5f1* transcript is present and POU5F1 and CDX2 protein co-localise for a period, but the duration of these events is longer in the stripe-faced dunnart than in the mouse (Table 1); in most other respects, however, marsupial blastocyst stages are more similar to cow and pig embryos (Kirchhof et al., 2000). CDX2 and POU5F1 proteins are also present during cleavage and blastocyst stages in two

other marsupials, the opossum (Morrison et al., 2013) and the tammar wallaby (Frankenberg et al., 2013). The gene expression profile in dunnart embryos and the overlapping protein distribution in opossum, tammar wallaby, and dunnart together suggest that both CDX2 and POU5F1 are required in a cell during early marsupial development, but not during initial lineage allocation. Indeed, the first sign of differential protein distribution in dunnart blastocysts occurs at the time of hypoblast formation, prior to shell coat loss and implantation, when POU5F1 is absent from the pluriblast nuclei and CDX2 is lost from the puriblast and trophoblast cytoplasm. This differs from the tammar wallaby (Frankenberg et al., 2013), in which changes in protein distribution at similar stages is related to loss of nuclear CDX2 in both puriblast and trophoblast cells, further illustrating the diversity of differentiation programs during lineage allocation in mammals.

The extended co-existence of CDX2 and POU5F1 may be a consequence of different genetic pathways that are responsible for lineage separation in the late-implanting blastocysts – as reported for pig, rabbit, and cow (Kirchhof et al., 2000; Kuijk et al., 2008; Berg et al., 2011) – and/or that the presence of CDX2 has a role in maintaining trophectoderm integrity (Goissis and Cibelli, 2014). Berg and colleagues speculated that animals with later implantation than the mouse – such as cow, human, and rabbit – may not possess the binding sites for the transcription factor AP2, a member of the *Tcfap2* gene family, found in the upstream CR4 enhancer region of murine *Pou5f1* that mediates *Cdx2*-independent repression (Berg et al., 2011). If this is the source of the difference, then AP2 sites should also be absent from marsupial *Pou5f1* enhancers; this remains to be determined. An alternative model involves the direct regulation of lineage allocation by POU5F1, as one study reported a limited capacity of POU5F1 to control the loss of trophoblast cells in early blastocysts of the dasyurid-like dunnart *Antechinus stuartii* (Selwood, 1986).

When analysing the early lineage allocations in mammals, Johnson and Selwood (1996) pointed out that the initial separation would be between the pluriblast (equivalent to the inner cell mass in the mouse) and trophoblast. In precociously implanting conceptuses, such as the mouse, the

trophoblast rapidly transforms to the trophectoderm over the next two divisions (about one day) when all cells contain CDX2 and POU5F1 (Morrison et al., 2013). Fleming and Johnson (1988) provided a valuable summary of the transition from trophoblast to trophectoderm, and defined an advanced polar state in the outer lineage of the mouse embryo that displays features including an apical cytocortical zone and microvilli, the appearance of definitive lysosomes, completion of the zonular tight junctions, and the appearance of desmosomes. In animals that implant relatively late, however, the transition from trophoblast to trophectoderm is gradual and is associated with the dual presence of CDX2 and POU5F1 (Table 1). This transformation occurs over a minimum of 7 days in the cow (Table 1) (Betteridge and Fléchon, 1988; Barcroft et al., 1998; Maddox-Hyttel et al., 2003), versus 4.5 days in the dunnart and only occurs in larger and more advanced blastocysts. Indeed, perinuclear CDX2 was detected in the dunnart trophoblast at Day 6.5, suggesting that zygotic CDX2 protein translation is linked with the transition to trophectoderm differentiation.

An unusual feature of the marsupial embryo is the intimate relationship between the blastomeres and the zona pellucida as well as the mucoid and shell coats. Ultrastructural features in the dunnart suggest that the first phenotypic allocation occurs between the 16- to 32-cell stage, when all cells are polarised with respect to the zona pellucida (Kress and Selwood 2003; 2004; 2006). The trophoblast, which gradually becomes polarised, does not begin water uptake until Day 4 to Day 5-5.5, when blastocysts of 500–1000 cells show definitive expansion (Selwood and Hickford, 1999) and the zona pellucida and mucoid coats have been absorbed by the blastocyst (Kress and Selwood, 2004). In vitro development following removal of the shell coat from blastocysts in the brushtail possum (Casey and Selwood, 2003) or dunnart (Selwood, 1989) resulted in the loss of trophoblast integrity, demonstrating that the shell coats are required to maintain epithelial integrity in marsupials. Considering these observations, it is worth noting that changes to CDX2 distribution can be detected after shell loss in Day-8.5 dunnart blastocysts, implying a correlation between CDX2 function and trophoblast differentiation. Alternatively, CDX2 may contribute to the proliferation of blastocyst

epithelium – similar to its role in the intestine (Bae et al., 2015) – and could be related to trophoctoderm function during implantation. Indeed, changes in CDX2 and POU5F1 distribution consistently occur just prior to implantation in the mouse (0.5 days), cow (4 days), and stripe-faced dunnart (1.5 days).

Our studies suggest that CDX2 and POU5F1 are required in both the trophoblast and pluriblast during early development of late-implanting species, and their reciprocal expression is probably not involved in the first lineage allocation in these species. The expression of these is not mutually exclusive until just before implantation, when the trophoblast is transformed into trophoctoderm and displays an advanced polarised state. Of particular interest are the high levels of POU5F1 in the nucleus and cytoplasm, and of the high levels of nuclear CDX2 in hypoblast cells. The presence of POU5F1 has already been noted in mouse primitive endoderm cells (Cockburn and Rossant, 2010). Our results suggest that POU5F1 – which is expressed in hypoblast cells in mouse, tammar wallaby, and dunnart – may also play a conserved role in the formation of the trophoctoderm. On the other hand, this is the first report of CDX2 immunoreactivity in hypoblast cells, which suggests an expanded role for CDX2 in the stripe-faced dunnart. Indeed, as cleavage-stage as well as trophoblast and pluriblast cells are polarised, the presence of CDX2 in both lineages until the late blastocyst stage suggests that this transcription factor plays contributes to the formation and maintenance of polarity.

MATERIALS AND METHODS

Animals and tissue collection

The stripe-faced dunnarts (*S. macroura*) used in this study were from a laboratory colony maintained by Lynne Selwood at the University of Melbourne, School of Biosciences, following National Health and Medical Research Council Australian Code for the Care and Use of Animals for Scientific Purposes, and held under permits issued by the Victorian Department of Sustainability and Environment. Samples collected were either snap-frozen and stored at -80°C, or fixed in

paraformaldehyde as described below. All animals were humanely killed by inhalation of halothane (Rhone Merieux), followed by cervical dislocation. Somatic tissues (brain, heart, hindgut kidney, liver, lung, ovary, spleen, and testis) were snap frozen and stored at -80°C.

Collection of secondary oocytes and follicle cells

S. macroura follicles were dissected free from isolated ovaries using two insulin syringe needles (Merry et al. 1995) in Dulbecco's Modified Eagle's Medium with L-Glutamine (Invitrogen) on a 35C plate under a dissecting microscope (Zeiss, North Ryde, NSW, Australia). Follicles surrounded by a single layer of squamous or cuboidal cells were identified as primordial or primary follicles, respectively. Follicles containing two layers of cuboidal follicle cells and measuring between 228 to 285 µm on the long axis and 217 to 277 µm on the short axis, as measured by a calibrated ocular micrometer, were identified as secondary follicles (Kress et al. 2001). Larger follicles containing an early or late antrum were identified as antral follicles; polar bodies were not evident in these follicles, so it is unlikely that the oocytes had been released from prophase-I arrest. Very-late antral follicles, which represent the stage immediately before ovulation, are much more transparent than other follicular stages.

Secondary follicles were digested in 0.05% trypsin (Sigma-Aldrich, NSW, Australia)/0.02% EDTA with vigorous pipetting using a 10-µl tip in order to facilitate the separation of follicle cells from their oocytes. The addition of a half volume of 1% bovine serum albumin (BSA)/phosphate-buffered saline without magnesium or calcium (PBS⁻) (Sigma-Aldrich) was used to stop digestion. Final separation of the oocyte and follicle cells was achieved mechanically using two insulin syringe needles. Cells were washed in PBS⁻ at 35°C, collected in a minimal volume of PBS⁻, and either snap frozen and stored at -80°C or fixed in paraformaldehyde, as described below.

Conceptus collection

The stage of the oestrous period (7 days) was determined by the presence of high numbers of cornified epithelial cells in the urine, and a rise and then fall in weight on the day of ovulation (Selwood and Woolley, 1991). Females were killed late on the day of ovulation and on subsequent days of gestation to collect stage-specific, ovulated follicles, or conceptuses. Early-cleavage stages (zygote to 64-cell unilaminar blastocyst stage) were collected between Day 0.5 to Day 3.5 of gestation, following the most recent *S. macroura* embryonic development timetable (Selwood and Hickford, 1999). Some cleavage stages were collected from females induced to ovulate using equine serum gonadotrophin between Day 7 to Day 11 after the initial injection (Day 1 to Day 4 of gestation), following an induced ovulation timetable (Au et al. 2010). Female reproductive tracts were removed, washed in PBS⁻ at 35°C, and examined under the dissecting microscope to determine oestrous cycle stage by the presence of antral follicles or corpora lutea, which were counted to give an estimate of the number of conceptuses/parthenotes in the uteri. Each uterus was transferred to warmed Dulbecco's Modified Eagle's Medium with L-Glutamine (Invitrogen), slit longitudinally along the midline, and carefully everted to roll conceptuses into the medium. Conceptuses were analysed for evidence of fertilization, stage, and normal development; washed in PBS⁻; collected in a minimal volume of PBS⁻; and stored at -80°C.

RNA extraction

RNA was extracted from 50-100 mg of homogenized tissues using an acid-phenol extraction (Trizol, Invitrogen, Victoria, Australia), followed by column purification using an RNAeasy Mini Kit (Qiagen, Victoria, Australia), according to the manufacturers' protocols. RNA was analysed on a 1.5% (w/v) agarose/ 1X 3-morpholinopropanesulfonic acid (MOPS)/ 3% formaldehyde gel against 2.0 µg RNA markers (Promega, Victoria, Australia). Concentration was determined spectrophotometrically.

Cloning dunnart *Cdx2*, from the adult ovary, and *Pou5f1*, from Day-6 pouch young

Two sets of primers (outer and nested) were designed in the most highly conserved regions based on sequence alignment of the *M. domestica* (gi126327412), *M. musculus* (gi145301600), and *H. sapiens* (gi169636434) *CDX2* orthologs, or an alignment of a deduced *M. domestica* (generously provided by Dr. Stephen Frankenberg, University of Melbourne, which can now be found in GenBank (gi611995275)) and *T. vulpecula* (gi33638262) *Pou5f1* sequences. Alignments were undertaken using EMBL-EBI multiple sequence alignment tools, ClustalW2 and Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/>). Outer and nested forward and reverse primers can be found in Supplemental material (Figs. S1 and S2).

Two micrograms of total RNA from dunnart adult ovary Day-6 pouch young genital ridge, isolated as described above, was reverse transcribed using M-MLV Reverse Transcriptase/RNase H Minus/Point Mutant and Oligo dT (Promega), according to the manufacturer's protocols. Nested-PCR was performed to clone a partial cDNAs of *Cdx2* from the ovary and *Pou5f1* from the genital ridge in two sequential PCR reactions, using Promega reagents and protocols. In the first PCR reaction, first-strand cDNA (4 µl) was used as template with outer forward and reverse primers (Figs. S1 and S2). PCR conditions were as follows: 94°C for 2 min; 35 cycles of 94°C for 30 s, 50°C annealing temperature for 30 s, 72°C extension for 30 s; and a final extension at 72°C for 5 min. In the second PCR reaction, 1 µl of the first-round PCR reaction was amplified using nested forward and reverse primers, with similar cycling conditions (annealing temperature of 60) (Figs. S1 and S2). Following analysis on 1.5% agarose gel, PCR products were subcloned into pGEM-T Easy (Promega) according to the manufacturer's protocols.

***Cdx2* and *Pou5f1* sequence analysis**

The *S. macroura* *Cdx2* sequence were aligned with the full-length *Cdx2* sequences of *M. musculus*, *H. sapiens*, and *M. domestica* using Clustal X (Thompson et al. 1997). Clustal Omega

(Goujon et al. 2010) was used to align the partial *S. macroura Pou5f1* nucleotide sequence with full-length *Pou5f1* *M. domestica*, *M. eugenii*, and *O. anatinus*.

***Cdx2* expression in adult gonadal and somatic tissues**

The expression of *Cdx2* in adult gonadal and somatic tissues was determined by reverse-transcription PCR. Two micrograms of total RNA from stripe-faced dunnart ovary, testis, brain, heart, liver, kidney, lung, or hindgut were reverse transcribed, as described above. Five microliters of first-strand cDNA was amplified using *Cdx2* nested forward and reverse primers. *Gapdh* primers were used to control for loading in each PCR reaction, using 1 mM (final concentration) of *Gapdh* forward (5'-ACCCACGGCAAGTTCAACGG) and reverse (5'-GCATGTCAGATCCACAACGG) primers for 35 cycles, with an annealing temperature of 60°C. No -template negative controls contained the *Cdx2* nested primers but no cDNA; positive controls contained *Cdx2* nested primers and *Cdx2* plasmid cDNA

***Cdx2* and *Pou5f1* expression analysis in oocytes, follicle cells and conceptuses**

Follicles, secondary follicles separated into primary oocytes and follicle cells, and conceptuses were lysed using 10 µl lysis buffer (Cells-to-cDNA kit; Ambion) for every two oocytes or conceptuses and 50 µl per set of follicle cells from each follicle. Ten microliters of each lysate was reverse transcribed according to the manufacturer's instructions. First-strand cDNA (2.5 to 5 µl) was used as template in a 50-µl PCR reaction with appropriate outer or nested forward and reverse primers, using *GoTaq* master mix (Promega), according to the manufacturer's instructions. Thirty cycles, with annealing temperatures of 55°C to 60°C, were used for PCR. *18S* (ribosomal subunit *18S* gene) was included to control for loading in each PCR reaction, using 1 mM (final concentration) of *18S* forward (5'- CGGCTACCACATCCAAGG) and reverse (5'-GCTGGAATTACCGCGGCT) primers.

Pou5f1 expression in different follicular stages was confirmed by nested reverse-transcription PCR. The first PCR products obtained using outer forward and reverse primers were purified using QIAquick PCR purification kit (Promega), according to manufacturer's instructions. The purified samples were then used as templates for a subsequent PCR amplification using *Pou5f1* nested primers, with similar conditions as described above.

All experiments were replicated (n=2 to 4) using samples obtained from different animals, except for studies reported in Figure 4, in which primordial and primary follicles, 2-, 4- (arrested), and 16-cell conceptuses, and Day-7 blastocysts (n=2) refers to different samples taken from the same animal.

Immunofluorescence

The conceptuses used in this study were part of a collection of cryopreserved dunnart tissues that were stored at -80°C or had been previously fixed in 4% paraformaldehyde and stored in 70% ethanol. In preliminary immunofluorescence experiments, the mucoid and shell coats of early embryos appeared artefactually positive for CDX2 and POU5F1, and obscured reactivity of the blastomeres. Attempts to remove these extracellular coats were unsuccessful as this invariably caused dispersal of the enclosed blastomeres, which adhere to the zona pellucida. To facilitate better penetration of antibodies, each embryo was punctured to ensure antibody permeation into the perivitelline space and both mucoid layers and shell coats were left in place.

Frozen conceptuses were thawed by careful addition of an equal volume of 8% paraformaldehyde in PBS (pH 7.0). After 5 hrs of fixation, conceptuses were washed five times in PBS; permeabilized for 1 hr in 0.1% Triton (Sigma Chemical Co., St. Louis, MO, USA) in PBS; washed three times in PBS; blocked for 1 h with PBS containing 3% goat serum; washed three times in PBS; and incubated overnight in a 1:100 solution of primary antibody in PBS containing 2% bovine serum albumin (BSA) (Sigma-Aldrich). Following five washes in PBS, conceptuses were

incubated overnight with the appropriate secondary antibody diluted 1:1000 in PBS with 2% BSA. All incubations were performed at room temperature on a slow shaking platform. Immunostained conceptuses were rinsed five times in 1X PBS, and stored under refrigeration in 0.1% NaN_3 in 1X PBS. Control-blastocyst samples were incubated with normal mouse IgG at similar concentrations as the primary antibody, followed by incubations with the appropriate secondary antibody. To control for specificity of the antibody reactivity, the primary POU5F1 antibodies were pre-absorbed overnight at 4°C with a fivefold molar excess of POU5F1 protein (amino acids 1-212; ab95845, Abcam) prior to incubation with embryos. Conceptuses were incubated with 1 µg/ml Hoechst dye (Bisbenzimidazole H33258, Millipore) to visualise cell nuclei.

A similar immunofluorescence protocol was undertaken on flat-mounted blastocysts that had been previously fixed and stored in ethanol. The methods for flat-mounting of blastocysts before staining followed that of Kress and Selwood (2006), with the exception that the flat mounts of the blastocyst epithelium dissected from the blastocysts were dried overnight without heat. Smaller blastocysts were dissected into two halves, each containing pluriblast and trophoblast, using the presence of the yolk mass to confirm the pluriblast and embryonic pole. In later-stage blastocysts, where the epiblast was established and embryogenesis was in progress, only sections of unilaminar and bilaminar yolk sac were dissected for mounting. These fixed samples were gradually rehydrated to distilled water over 24 hours.

All antibodies were obtained from Abcam (Cambridge, MA, USA). The primary antibodies used were chicken anti-OCT4/POU5F1 polyclonal antibody (ab134218, Synthetic peptide within Human POU5F1, aa 1-100 conjugated to keyhole limpet haemocyanin), which reacts with mouse POU5F1 and is predicted to react against rat, rabbit, human, pig, chimpanzee, and Chinese hamster POU5F1. Mouse anti-CDX2 monoclonal antibody was raised against a prokaryotic recombinant protein corresponding to the 180 amino acid N-terminal region of the human CDX2 molecule

(ab15258), and is known to react with human CDX2. Secondary antibodies used were goat anti-chicken IgG-Alexa Fluor 488 (ab150169) and sheep anti-mouse IgG-Texas Red 589 (ab6806).

Microscopy

Images of immunostained conceptuses were captured using either a Zeiss Axioskop II microscope, equipped with epifluorescence and a Zeiss HC camera linked to Axiovision imaging software (Carl Zeiss), or a Leica SP2 or SP5 laser scanning confocal microscope at the Department of Biochemistry & Molecular Biology at the Bio21 Institute or an LSM510 Meta confocal microscope (Carl Zeiss) at the Biological Optical Microscopy Platform, University of Melbourne.

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FIGURE LEGENDS

Figure 1. *Cdx2* expression in *S. macroura* tissues, oocytes, follicle cells, and conceptuses

Reverse-transcription PCR for *Cdx2* expression in (A) adult gonadal and somatic tissues; (B) secondary follicles separated into primary oocytes and follicle cells; (C) Day-40 pouch young (PY) ovary; (D) cleavage from zygote to 64-cell blastocyst stages; and (E) blastocysts on Day 4.5 to 7.5 of gestation. -ve, no template negative control; +ve, *Cdx2* cDNA template positive control; b, brain; h, heart; hg, hindgut; k, kidney; li, liver; lu, lungs; o, ovary; s, spleen; t, testis; 4A, arrested 4-cell stage; 4E, early 4-cell stage; 4L, late 4-cell stage; EBB, early bilaminar blastocyst; EUB, Early unilaminar blastocyst; LBB, late bilaminar blastocyst; LUB, Late unilaminar blastocyst.

Figure 2. *Pou5f1* expression in *S. macroura* follicles and conceptuses

Reverse-transcription PCR showing *Pou5f1* expression in primary and antral, but not primordial, follicles and in conceptuses from 64-cell to Day-7.5 bilaminar blastocyst stages using (A) outer *Pou5f1* primers and *18S* primers for loading control and (B) nested *Pou5f1* primers. 4A, arrested 4-cell stage; AO, antral oocyte; PDF, primordial follicle; PF, primary follicle; EBB, early bilaminar blastocyst; EUB, early unilaminar blastocyst; LBB, late bilaminar blastocysts; LUB, late unilaminar blastocyst; M, 1 Kb+ ladder (Invitrogen).

Figure 3. Co-localization of POU5F1 and CDX2 in Day 0.5 to 2.5 cleavage stages in *S. macroura*.

Representative confocal images showing Hoechst nuclear stain (blue) (A, E, J), immunoreactivity for POU5F1 (green) (B, F, K) and CDX2 (red) (C, G, L), and merged images (D, H, M). Scale bar, 40 μ m.

A-D: A polarised zygote (Day 0.5) soon after syngamy. The nucleus (n) lies in the rim of cytoplasm opposite the yolk mass (ym). Non-specific Hoechst staining occurs in the shell (sc), cytoplasm, and yolk mass (ym). The zygote has collapsed slightly, and folds of the coats (f) lie across parts of the centre of the zygote. Intense POU5F1 and CDX2 immunoreactivity co-localise in the nucleus (n),

with less-intense staining in the yolk mass (ym). Intensely POU5F1 stained granules can be found in the yolk mass (asterisks).

E-H: A 4-cell stage embryo (Day 1.5) in which two pairs of blastomeres (b1, b2) and the yolk mass (ym) are visible. Intense Hoechst staining is seen in the nuclei (n) of the b2 blastomeres, and there is less intense non-specific Hoechst staining in the shell coat (sc), cytoplasm, and yolk mass (ym). POU5F1 and CDX2 co-localise in the blastomere cytoplasm and nucleus, but the nucleolus is not stained (arrow).

J-M: A 16-cell stage embryo (Day 2.5), viewed from the embryonic pole, in which two tiers of 8 blastomeres have formed. The upper embryonic tier (dashed circle) is comprised of pluriblast (pb) cells that lie closer to the surface and some trophoblast (tb) cells of the lower abembryonic tier that are visible around it. The conceptus has collapsed, and folds (f) obscure some of the cells. The extensive pseudopodial-like processes that attach the cells to the coats have retracted so that each cell has an irregular outline. Hoechst stains 5 pluriblast cell nuclei; the others are obscured by the folds of the shell and mucoid coats. Nuclei of trophoblast cells and sperm nuclei (asterisks) trapped in the mucoid coat also stain with Hoechst dye. The nucleus and cytoplasm of both pluriblast and trophoblast cells stain strongly for POU5F1. CDX2 staining in the nucleus and cytoplasm of the pluriblast and trophoblast cells mimics that of the POU5F1. The merged image shows co-localisation of POU5F1 and CDX2 in the nucleus and cytoplasm of both cells types.

Figure 4. Co-localisation of CDX2 and POU5F1 in flat-mounted *S. macroura* blastocysts.

Representative confocal images of blastocysts at early Day 6.5 (A-H), late Day 6.5 (J-M), and Day 8.5 (N-Q). Conceptuses were mounted with the inner surface or hypoblast on top. Image series show Hoechst nuclear stain (blue) (A, E, J, N), POU5F1 immunoreactivity (green) (B, F, K, O), CDX2 (red) (C, G, L, P) immunoreactivity. and merged images (D, H, M, Q). D#, age of conceptus, in days. Scale bars, 50 μ m (A-H), 200 μ m (J-Q).

A-D: Pluriblast (pb) region in Day-6.5 very-early bilaminar blastocyst. Pluriblast cells show predominately nuclear and cytoplasmic POU5F1 staining, while more-intense staining is associated with the putative hypoblast cells (arrows), some of which are dividing (inset, asterisk). Higher magnification of POU5F1 stained cells (insert) revealed dark, unstained vesicles (closed arrowheads). Moderate CDX2 staining occurs in the cytoplasm and nuclei of pluriblast cells, although some nuclei do not stain.

E-H: Trophoblast (tb) region of a Day-6.5 very-early bilaminar blastocyst, which is folded in some regions (f). The trophoblast cell nuclei are larger than nuclei of pluriblast cells shown in 'A'. POU5F1 staining is strong in the nucleus and perinuclear cytoplasm of the trophoblast cells. At higher magnification, variably sized, unstained vesicles (inset, arrowhead) are visible. CDX2 staining is more intense in a distinct perinuclear region in the trophoblast cells (arrows); this pattern was detectable at all confocal planes, and is absent in some nuclei.

J-M: Late Day-6.5 bilaminar blastocyst, mounted with the hypoblast on top. Hypoblast cells have formed a dense reticulum under the pluriblast, which is converting to epiblast, and individual hypoblast cells have migrated under the trophoblast. The pluriblast lies along the bottom of the images, and contains a triangular tear (t); the trophoblast adjoins the pluriblast, and is folded (f) along the top of the images. Pluriblast cell cytoplasm stains strongly for POU5F1, but the nuclei are negative. The adjoining trophoblast cells stain more in the nuclei than in the perinuclear cytoplasm (solid arrowhead). Hypoblast cells stain more strongly in the nucleus (arrow, arrowheads) and cytoplasmic processes than either pluriblast or trophoblast cells. Nuclear staining is more intense in the hypoblast cells (inset, arrow and open arrowheads) than trophoblast cells (inset, solid arrowheads) that show no nucleolar or cytoplasmic staining. The merged image shows co-localised staining in the nuclei of all cell types, although CDX2 staining is strongest in hypoblast cells (inset, open arrowheads) compared to trophoblast cells (closed arrowheads).

N-Q: Day-8.5 bilaminar yolk sac outside the embryonic area. On the right hand side, hypoblast cell migration has almost completely lined the bilaminar yolk sac (bys), which lies nearest the embryonic area, and fewer hypoblast cells have migrated under the unilaminar yolk sac (uys), making it appear less dense. A few lightly stained nuclei of trophoblast cells remain visible on the right-hand side (solid arrowheads), with many more on the left-hand side, which also shows some POU5F1-negative nuclei. Arrows point to positive-staining hypoblast cells lying in both parts of the yolk sac. The hypoblast cells positive for POU5F1 in 'O' are also positive for CDX2 in 'P' (arrows). Trophoblast cells (solid arrowheads) in both regions of the yolk sac stain faintly for CDX2 in the cytoplasm but not the nucleus. Higher magnification of trophoblast cells reveal that many of these are binucleate (inset, arrowheads).

SUPPLEMENTARY FIGURE LEGENDS

Figure S1: Nucleotide sequence alignment of *S. macroura* *Cdx2* with other mammalian species.

Alignment of *Cdx2* cDNA from two marsupial species, *S. macroura* and opossum (*M. domestica*), and two eutherian species, mouse (*M. musculus*) and human (*H. sapiens*). Nucleotides that are fully conserved are indicated (*). Sequences of the forward (for) and reverse (rev) primers used in cloning the *S. macroura* *Cdx2* sequence are also highlighted (bold, underlined).

Figure S2: Nucleotide sequence alignment of *S. macroura* *Pou5f1* with other marsupial species and platypus.

Alignment of *S. macroura* *Pou5f1* cDNA with three metatherian (marsupial) species – brushtail possum (*T. vulpecula*), opossum (*M. domestica*), and tammar wallaby (*M. eugenii*) – and one prototherian species (monotreme) – platypus (*O. anatinus*). Nucleotides that are fully conserved are indicated (*). Sequences of the forward (for) and reverse (rev) primers used in cloning the *S. macroura* *Cdx2* sequence are also highlighted (bold, underlined).

Figure S3: *Cdx2* expression in *S. macroura* oocytes and zygote

Reverse-transcription PCR data showing *Cdx2* is expressed in late antral oocytes, but not in primary oocytes or zygote. 1-cell, zygote; 1°O, primary oocyte; AO, antral oocyte; -ve, negative control, no template added; +ve, positive control (D6.5, Day-6.5 early bilaminar blastocyst with *S. macroura* first-strand cDNA added).

Figure S4: Staining of CDX2 and POU5F1 in *S. macroura* unfertilized oocytes and cleavage-to-early unilaminar blastocysts.

Representative confocal images of POU5F1 (green) (A, C, E, G, J) and CDX2 (red) (B, D, F, H, K) staining.

A-B: Both proteins are expressed in the unfertilized egg, but not in the yolk mass (ym).

C-D: Following fertilization, when the egg is undergoing its first cell division on Day 1, both cells show intense cytoplasmic staining for POU5F1 and CDX2. The nucleus is not visible in these cells, which have the typical appearance of a “shaggy bowtie”. Arrowhead points to the connecting midpiece of dividing cells.

E-F: 4-cell stage on Day 2, showing intense staining for POU5F1 and CDX2 in all cells that are associated with the zona pellucida. Portions of 3 cells are visible. The view is from the embryonic pole, so that the mucoid coat (mc) and shell coat (sc) are sectioned obliquely and appear wider.

G-H: The 16-cell stage, viewed from the side with the embryonic pole at the top, where 4 pluriblast cells (arrowheads) lie. One trophoblast cell lies in the abembryonic hemisphere (arrow). Both cell types contain POU5F1 and CDX2.

J-K: Unilaminar blastocyst with around 256 cells, D4.5. Pluriblast cells (pb) occupy about one-quarter of the blastocyst at the embryonic pole (within dotted line), and more sparsely distributed trophoblast cells occupy the remainder. All blastomeres stain for both proteins (J, POU5F1; K, CDX2). Scale bar = 100 µm (A-H) and 200 µm (J-K).

Figure S5: Immunofluorescence Control Experiments.

A-D: Hoechst staining of the cytoplasm of parts of two cells (b) and the shell coat. Incubation of 4-cell stage conceptus with mouse non-immune serum (NIS) resulted in no specific reactivity for POU5F1 (B) nor CDX2 (C). The large triangular mass visible outside the shell on the left hand side is an artefact.

E-H: Control for POU5F1 antibody specificity in a 4-cell stage conceptus. (E) Non-specific Hoechst staining is present in the cytoplasm of three of the four blastomeres (b) that are being released through a cut in shell and mucoid coats, which also stain positive for Hoechst. (F) POU5F1 protein blocks POU5F1 antibody staining of blastomeres. (G) Positive CDX2 reactivity is seen in the cytoplasm of the blastomeres in the presence of POU5F1 protein. (H) The merged image shows strong CDX2 staining, but no POU5F1 staining, confirming the specificity of the POU5F1 antibody.

J-M: Day-6.5 early bilaminar blastocyst wall showing Hoescht staining in pluriblast (pb) and trophoblast (tb) regions. Incubation of blastocysts without primary antibodies, but in the presence of secondary antibodies and mouse non-immune serum (NIS), resulted in no specific reactivity for POU5F1 (K) and CDX2 (L). Only Hoechst staining is evident in the merged image.

Scale bar, 40 μ m.

Table 1.

Relevant electron microscopy phenotypic characters, and gene expression changes for *POU5F1* and *CDX2* from the first to the beginning of the second lineage allocation in early concepti in the mouse, cow, gray short-tailed opossum and the striped-faced dunnart. Only mammals in which relevant details of phenotypic characters, transcript, and protein abundance details are available were included.

STAGE/ PHENOTYPE	Mouse (Days post-coitum) ¹⁻¹⁷	Cow[†] (Days post-insemination) ¹⁸⁻³²	Gray short-tailed opossum (Days post-coitum) ³³⁻³⁷	Striped-faced dunnart (Days post-ovulation) ³⁸⁻⁴⁵
<i>Pre-first lineage allocation</i>	<i>early 8-cell (D2.5)</i>	<i>8-cell (D3)</i>	<i>late 8-cell (D3- 3.2)</i>	<i>late 8-cell (D2.5)</i>
Cell phenotype Cell-cell contacts	Apolar Gap junctions	Apolar Electrically coupled	Early polar. Apical microvilli and cell-zona pellucida adhesion via microvilli Exocytosis of extracellular matrix basally No cell-cell contacts	Early polar. Apical microvilli and cell-zona pellucida adhesion via microvilli Exocytosis of extracellular matrix basally. No cell-cell contacts
<i>POU5F1</i> <i>CDX2</i>	+ve +ve, variable levels	RT-PCR +ve, ISH -ve	ND ND	-ve -ve
POU5F1 CDX2	+ve, mainly nuclei -ve nuclei	+ve, mainly nuclei -ve	+ve, mainly nuclei +ve, mainly nuclei	+ve cytoplasm +ve cytoplasm
zygote genome activation	1-to 2-cell (0.5-1.5D)	8- to 16-cell (3-4D)	16- to 32-cell (5.0 D)	8- to 16-cell (2.5-3.0 D)
<i>Initiation of first lineage allocation</i>	<i>Compacted morula, late 8- cell (D2.5)</i>	<i>Morula (D4-6) D4. 16-cell early morula D5. 32-cell compact morula D6. 64-cell late compaction</i>	<i>D4. 16-cells as two tiers of ~8 (pluriblast upper embryonic tier +trophoblast lower abembryonic tier) polar adherent cells form a bowl open at the abembryonic pole</i>	<i>D2.5. 16-cells as two tiers of 8 (pluriblast upper embryonic tier +trophoblast lower abembryonic tier) polar adherent cells forming a bowl open at the abembryonic pole</i>
Phenotype	Early polar	From D4. Outer trophoblasts early polar. Early inner inner cell mass apolar	All cells early polar. Pluriblast in embryonic tier contacted first, trophoblasts in abembryonic tier	All cells early polar. Pluriblast contact first, trophoblasts later. Within tiers, cell division order determines cell-cell contact order.

			later.	
Cell doubling		Early trophoblast, fast Early inner cell mass, slow	trophoblast (abembryonic tier) fast pluriblast (embryonic tier) slow	trophoblast (abembryonic tier) fast pluriblast (embryonic tier) slow
<i>POU5F1</i> <i>CDX2</i>	+ve +ve variable levels	low +ve early morula, > +ve, compacted morula -ve	ND ND	-ve -ve
<i>POU5F1</i> <i>CDX2</i>	+ve mainly nuclei +ve some nuclei	+ve, mainly nuclei -ve	All cells +ve, mainly nuclei All cells +ve	All cells +ve nuclei; +ve, cytoplasm All cells -ve, nuclei; +ve, cytoplasm
<i>First lineage allocation</i>	<i>16-cell stage (D3.0)</i> <i>Compacted morula.</i> <i>Allocation during fourth and fifth division</i>	<i>Blastocyst (D7.0)</i> <i>Allocation trophoblast and inner cell mass at early 100-cell blastocyst, early expansion</i>	<i>16-32-cell, unilaminar blastocyst completed ~32 - cells (D4.4)</i> Yolk mass consolidate in embryonic pole. <i>Allocation during forth or fifth division</i>	<i>16-64-cell, unilaminar blastocyst completed ~32-cells (D3.0-3.5).</i> Yolk mass held near pluriblast in the embryonic pole. <i>Allocation during 4th division.</i>
Expansion	None	Early expansion	Preliminary expansion* begins	Preliminary expansion* begins

trophoblast / trophectoderm

Position Phenotype	Peripheral Late polar	Peripheral Late polar, but primitive desmosomes	Peripheral, lines abembryonic pole [No TEM]	Peripheral, lines abembryonic pole, gaps Early polar but with apical primitive desmosomes.
Cell doubling	Fast	Fast	Fast since 8-16 cell stage	Fast
<i>POU5F1</i> <i>CDX2</i>	+ve +ve	RT-PCR +ve, ISH -ve +ve, trophoblasts > pluriblasts	ND ND	-ve (32-cell), +ve (64-cell) -ve (32-cell), -ve (64-cell)
<i>POU5F1</i> <i>CDX2</i>	+ve +ve	+ve, diffuse +ve, nuclei	+ve, mainly nuclei +ve	+ve cytoplasm (16-cell, 32-cell) -ve cytoplasm (16-cell, 32-cell)

pluriblast / inner cell mass

Position Phenotype	Inner Apolar	Inner Apolar	Peripheral, in embryonic hemisphere in centre of the developing unilaminar epithelium [No TEM]	Peripheral, in embryonic hemisphere in centre of the developing unilaminar epithelium Early polar
Cell doubling time	Slow	Slow	Slow	Slow
<i>POU5F1</i> <i>CDX2</i>	+ve -ve	RT-PCR+ve, ISH +ve, high +ve, pluriblast > trophoblast + ve, faint	ND ND	-ve (32-cell); +ve (64-cell) -ve (32-cell); -ve (64-cell)
POU5F1 CDX2	+ve +ve, nuclei	+ve, faint +ve	+ve mainly nuclei +ve	+ve cytoplasm (16- cell, 32-cell) +ve cytoplasm (16- cell, 32-cell)
<i>Blastocyst, cavitation, second lineage allocation, gastrulation implantation</i>	<i>Blastocyst (D3.5-5) D3.5. 32-cell forms blastocoel D4.0. 64-cells D4.5. Second Lineage D5. Implantation D7. Gastrulation</i>	<i>Blastocyst (D8-18). D8. Late blastocyst~160 cells, second lineage D10. Hatched blastocyst. D12. Loss of Rauber's layer, epiblast D14. Gastrulation D18. Implantation</i>	<i>Blastocyst (D6-12) D6. 64-cell unilaminar blastocyst, Defin Exp. D6-12. Expansion of the outer diameter D6-7. >128-cell, second lineage D10. Gastrulation D11-12. Shell loss, implantation at somite stage</i>	<i>Blastocyst (D4-9) D4. 128-cell unilaminar blastocyst, Defin Exp begins D4-9. Expansion of the outer diameter D6-6.5. Second Lineage D8. Gastrulation D8-9. Shell loss, implantation at somite stage</i>
trophoblast / trophectoderm				
Phenotype	D3.5-4.5. Advanced polar	D8-10. Mural TE late polar and some desmosomes, Polar trophectoderm is less polar. D~14. Advanced polar	Comprises 3/4 of the unilaminar blastocyst wall at abembryonic hemisphere/ polarised with respect to coats. D6. Early polar.	Comprises 3/4 of the unilaminar blastocyst wall at abembryonic hemisphere/ polarised re coats. D4-5.5. Late polar D6-6.5. Early to advanced polar D7.5. > advanced polar
Cell doubling time	Fast	Fast (examined to D10)	Fast (examined to D7, 128 cells)	Fast until D5.5.
<i>POU5F1</i> <i>CDX2</i>	D3.5-4.5. -ve D3.5-4.5. very	D8-10. +ve, D14-16. -ve	ND ND	RT-PCR. D4.0, 5.5, 6.5, 7.0. +ve

	+ve	E9-11. very +ve		D4.5. -ve, D5.5, 6.5, 7.5. +ve
POU5F1 CDX2	D3.5-4.5. +ve, nuclei D3.5. +ve	E11. -ve +ve, nuclei	+ve,,weak +ve	D4.0-4.5 +ve; strong +ve nuclei D6.5 +ve nuclei; faint +ve cytoplasm D8.0 -ve nuclei; -ve cytoplasm D4.0-4.5 +ve, D6.5 faint +ve nuclei; -ve cytoplasm D8.0 faint +ve nuclei; -ve cytoplasm
<i>pluriblast / epiblast</i>				
Position Phenotype	Inner Apolar	D12. Become peripheral as Rauber's layer is lost Early polar	Peripheral, in embryonic hemisphere in centre of the developing unilaminar epithelium [No TEM]	Peripheral. Lie in embryonic hemisphere in the centre of the developing unilaminar epithelium , Early polar
Cell doubling time	Slow	Slow (examined to D10)	Slow (examined to D7)	Slow until D5.5
<i>POU5F1</i> <i>CDX2</i>	D3.5-4.5. +ve D3.5-4.5. -ve	E11. RT-PCR & ISH +ve ND	ND ND	RT-PCR. D4.0, 5.5,6.5, 7.0. +ve D4.5. -ve; D5.5, 6.5, 7.5. +ve
POU5F1 CDX2	D3.5-4.5. +ve D3.5-4.0. +ve, nuclei D4.5. -ve	+ve ND	+ve, mainly nuclei +ve	D4.0-4.5 ++ve nuclei D6.5 -ve nuclei; strong +ve cytoplasm D4.0-4.5 +ve D6.5 +ve nuclei; -ve cytoplasm
<i>primitive endoderm / hypoblast</i>				
Position Phenotype	Scattered , then line blastocoel Becomes polar	Scattered, then lines the blastocoel Become early polar	ND [No TEM]	Few in restricted marginal location, then line blastocoel. Migratory, then early polar
<i>POU5F1</i> <i>CDX2</i>	D4.5. +ve D4.5. -ve	E11. +ve E11. -ve	ND ND	D4.0, 5.5, 6.5, 7.0. +ve D4.5. -ve, D5.5, 6.5, 7.5. +ve
POU5F1 CDX2	D4.5. +ve D4.5. -ve	ND ND	ND ND	D6.5 strong +ve nuclei & cytoplasm

				D8.5 weak +ve nuclei & cytoplasm D6.5 strong +ve nuclei & cytoplasm D8.5 weaker +ve nuclei & cytoplasm
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Abbreviations: D, Day; Defin Exp, Definitive expansion, when inner egg coats are lost in marsupials and the outer diameter increases; ISH, in situ hybridization; ND, not determined; RT-PCR, reverse-transcription PCR; TEM, transmission electron microscopy; >, greater than; -ve, negative; +ve, positive.

† Day of development used for cattle follows the in vivo timetable of Betteridge & Fléchon (1988); “E” was used to represent the embryonic day when the exact Day in vivo was not clear.

* Preliminary expansion is within inner egg coats of marsupials, without affecting its outer diameter.

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Fig 1 .

Fig 3 RGB .

Fig 4 RGB .

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Author/s:

Familar, M;Au, PCK;De longh, RU;Cruz, Y;Selwood, L

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