Development 140, 965-975 (2013) doi:10.1242/dev.091629

Early cell lineage specification in a marsupial: a case for diverse mechanisms among mammals

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SUMMARY

Early cell lineage specification in eutherian mammals results in the formation of a pluripotent inner cell mass (ICM) and trophoblast. By contrast, marsupials have no ICM. Here, we present the first molecular analysis of mechanisms of early cell lineage specification in a marsupial, the tammar wallaby. There was no overt differential localisation of key lineage-specific transcription factors in cleavage and early unilaminar blastocyst stages. Pluriblast cells (equivalent to the ICM) became distinguishable from trophoblast cells by differential expression of POU5F1 and, to a greater extent, POU2, a paralogue of POU5F1. Unlike in the mouse, pluriblast-trophoblast differentiation coincided with a global nuclear-to-cytoplasmic transition of CDX2 localisation. Also unlike in the mouse, Hippo pathway factors YAP and WWTR1 showed mutually distinct localisation patterns that suggest non-redundant roles. NANOG and GATA6 were conserved as markers of epiblast and hypoblast, respectively, but some differences to the mouse were found in their mode of differentiation. Our results suggest that there is considerable evolutionary plasticity in the mechanisms regulating early lineage specification in mammals.

KEY WORDS: Cell lineage specification, Epiblast, Hypoblast, Marsupial, Pluriblast, Trophoblast

INTRODUCTION

The early development of amniote vertebrates is largely devoted to the establishment of extra-embryonic membranes that contribute to both nourishment and early patterning of the embryo proper. In mammals, the extra-embryonic membranes are further specialised to form a placenta, which transfers nutrients from the mother to the foetus. Gross placental structure varies widely among mammals, but the primary extra-embryonic cell lineages – trophoblast, hypoblast and extra-embryonic mesoderm – are conserved. It should therefore be expected that fundamental, highly conserved mechanisms exist for the establishment of these extra-embryonic lineages, but recent evidence suggests that current models derived mainly from a single species, the mouse, might not be applicable to all mammals.

The current model for trophoblast specification in the mouse (reviewed by Rossant and Tam, 2009) involves positional signals between inner and outer cells of the morula that direct outer cells to polarise and initiate epithelialisation. The early trophoblastic epithelium (trophectoderm) surrounds the remaining inner cells, which constitute the inner cell mass (ICM). ICM-trophoblast segregation occurs independently of any known lineage-specific transcription factors, such as POU5F1 (ICM) and CDX2 (trophoblast), which are only required for subsequent maintenance of these lineages, respectively (Nichols et al., 1998; Strumpf et al., 2005). Lineage-specific gene expression is initiated via the Hippo signalling pathway, converging on the closely related co-activators YAP and WWTR1 (also called TAZ) (Nishioka et al., 2009; Nishioka et al., 2008). In outer cells, YAP and WWTR1 enter the nucleus and cooperate with the transcription factor TEAD4 to activate trophoblast target genes such as CDX2 and GATA3. In inner cells, phosphorylation of YAP and WWTR1 prevents them from entering the nucleus to activate target genes. A mutually inhibitory feedback mechanism between CDX2 and POU5F1 reinforces trophoblast- and ICM-specific developmental programmes, respectively (Niwa et al., 2005).

Mechanisms segregating epiblast and hypoblast within the mouse ICM are relatively less well understood. In the very early blastocyst (~32 cells), expression of the transcription factors NANOG and GATA6 in the ICM is initially heterogeneous but largely overlapping, becoming mutually exclusive by around the 64-cell stage. GATA6-expressing hypoblast precursors segregate from NANOG-expressing epiblast-precursors by a combination of cell sorting, positional signals and selective apoptosis, eventually forming the hypoblast (primitive endoderm) that lines the blastocyst cavity (Chazaud et al., 2006; Plusa et al., 2008). Although the mechanisms that initially specify epiblast and hypoblast precursors are still unclear, receptor tyrosine kinase signalling via the FGF4-FGFR2-ERK (MAPK1) pathway is crucial for fixing hypoblast-versus-epiblast fate (Frankenberg et al., 2011; Nichols et al., 2009; Yamanaka et al., 2010).

Marsupials were popular models for comparative embryology during the late nineteenth and early twentieth centuries and have received renewed attention in recent decades (reviewed by Selwood and Johnson, 2006). Despite this, very little is known of the molecular mechanisms controlling their early development, which is morphologically distinct from that of eutherians. The fertilised marsupial ovum contains a variable quantity of polarised deutoplasm (often misleadingly called 'yolk') that is extruded during early cleavage as one or more deutoplasts ('yolk masses') and a gelatinous extracellular matrix. This reduces cell volume and increases the extracellular space enclosed by the zona pellucida. During cleavage, blastomeres initially do not adhere to each other to form a morula but instead adhere to the zona. After several divisions, blastomeres completely line the zona to form the unilaminar blastocyst; thus, the marsupial conceptus lacks an ICM. After blastocyst expansion, an embryonic disc instead develops within a restricted region of the epithelium. The term 'pluriblast' is

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Accepted 31 December 2012

thus used to refer to the population of cells that gives rise to epiblast and hypoblast (to the exclusion of trophoblast) in all mammals (Johnson and Selwood, 1996). In most marsupials, putative pluriblast precursors are visible morphologically from late cleavage through to overt hypoblast-epiblast segregation, but neither the fate nor the potency of these cells has ever been tested, nor has the expression of key transcription factors that might mark their early specification.

The tammar wallaby (*Macropus eugenii*) has emerged as a standard marsupial model for reproductive and developmental biology (Hickford et al., 2009; Tyndale-Biscoe and Renfree, 1987) and its genome has recently been sequenced (Renfree et al., 2011). Like the mouse, the tammar has embryonic diapause, which occurs when the unilaminar blastocyst has ~80-100 cells that are morphologically indistinguishable from each other. However, diapausing blastocysts of two other marsupials, the feather-tailed glider and the honey possum, continue to expand up to ~2000 cells (Renfree, 1994; Ward and Renfree, 1988). Tammar diapause includes lactational quiescence controlled by the sucking stimulus from late summer to autumn and seasonal quiescence controlled by photoperiod from winter to early summer (Tyndale-Biscoe et al., 1974). During lactational quiescence, removal of pouch young (RPY) reactivates the single blastocyst, which begins to expand again around eight days later (d8 RPY) (Renfree and Tyndale-Biscoe, 1973) (Fig. 1).

As in other marsupials, very little is known of the molecular mechanisms of early tammar development. The tammar thus offers an excellent model for elucidating the evolution of pluripotency and early lineage specification in mammals. This comparative approach is crucial for understanding the degree to which findings in well-studied mammals such as the mouse can be extrapolated to other species. It also aids in identifying mechanisms that are highly conserved, despite morphological differences, and therefore fundamental to mammals or even vertebrates. Here, we present the first molecular analysis of early cell lineage specification in a marsupial, the tammar wallaby. We provide evidence suggesting that, in marsupials: (1) POU5F1 might have a subordinate role to its paralogue, POU2, in early pluriblast-trophoblast differentiation; (2) differential expression or localisation of CDX2 is unlikely to have a role in pluriblast-trophoblast segregation or differentiation; (3) the Hippo pathway paralogues YAP and WWTR1 might have mutually distinct roles in early development; (4) GATA6, but possibly not NANOG, is differentially transcribed during early hypoblast-epiblast segregation; and (5) early hypoblast formation and nuclear-localised YAP mark a putative anterior-posterior axis.

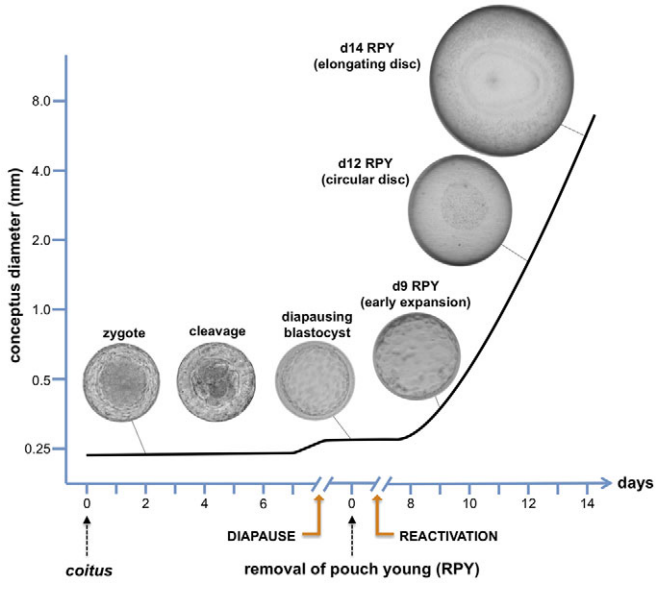

Fig. 1. Early development of the tammar wallaby. A thick mucoid coat and thin shell coat are deposited around the conceptus in the oviduct and uterus. The unilaminar blastocyst forms at around the 32-cell stage. During lactational quiescence, the presence of a sucking pouch young causes the blastocyst to enter diapause when it has 80-100 cells. Reactivation is first evident by renewed expansion 8-9 days after removal of pouch young (d8-9 RPY). With continued expansion, an overt embryonic disc becomes evident at ~d10 RPY.

MATERIALS AND METHODS

Animals and conceptus collection

Tammar conceptuses were obtained as previously described (Hickford et al., 2009; Renfree and Tyndale-Biscoe, 1978), from either wild-shot animals or from the University of Melbourne's captive breeding colony. Mouse conceptuses were derived by natural ovulation from Swiss mice. Conceptuses were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and stored in fixative at 4°C until use. Conceptuses were dissected from their surrounding investments where possible, except for some expanded blastocysts that adhered strongly to the shell coat. All experiments were approved by the University of Melbourne Animal Experimentation Ethics Committees and were in accordance with the National Health and Medical Research Council of Australia (2004) guidelines. Animals were collected under permits from South Australian Department of Environment, Water and Natural Resources and held under permits from the Victorian Department of Sustainability and Environment.

Antibodies

Antibodies with good cross-reactivity to tammar orthologues were available for most proteins, with the exception of POU2, POU5F1 and NANOG. POU2 is a paralogue of POU5F1 and has no eutherian orthologue (Frankenberg et al., 2010; Niwa et al., 2008), and the sequences of POU5F1 and NANOG are highly divergent between marsupials and eutherians. Thus polyclonal antibodies were raised to synthetic peptides with sequences corresponding to tammar POU2, POU5F1 and NANOG (IMVS Veterinary Services Division, SA, Australia). The tammar POU5F1 antibody and a rabbit polyclonal antibody raised to full-length mouse POU5F1 (a kind gift from H. Niwa, RIKEN Center for Developmental Biology, Kobe, Japan) showed comparable staining patterns (data not shown), although the tammar-specific peptide-raised POU5F1 antibody was used for all experiments presented in this study. An antibody raised to full-length opossum POU2 (a kind gift from H. Niwa) showed a similar but much weaker (presumably owing to sequence divergence) staining pattern to our POU2 antibody (data not shown); thus, the latter was used for all experiments presented in this study.

NANOG and POU2 polyclonal antibodies were raised in rabbit to synthetic peptides with the sequences LTHSKPQEDCDYR and KSEYSLEGPSTPDSSQTKC, respectively. A POU5F1 antibody was raised in guinea pig to a synthetic peptide with the sequence ESPSEDSSPESRAC. Peptide-blocking controls for these three antibodies were performed on dissected fragments of a d14 RPY gastrula by pre-incubating antibodies with peptide at a concentration of 1 μg/ml (supplementary material Fig. S1). The expression patterns of POU2, POU5F1 and NANOG were consistent with those shown by *in situ* hybridisation (supplementary material Fig. S1). *In situ* hybridisation was performed as previously described (Frankenberg et al., 2010).

Other primary antibodies used were: rabbit anti-mouse CDX2 (Beck et al., 1995) (a kind gift from Prof. Felix Beck, Leicester University, UK); goat anti-human GATA6 (AF1700, R&D Systems); goat anti-human SOX2 (AF2018, R&D Systems); rabbit anti-human WWTR1 (HPA007415, Sigma Prestige Antibodies); rabbit anti-human YAP1 (ab27981, Abcam); rabbit anti-human YAP1 (sc-15407, Santa Cruz Biotechnology). AlexaFluor fluorophore-conjugated secondary antibodies were all sourced from Molecular Probes (Invitrogen).

Immunohistochemistry

Conceptuses or conceptus fragments were processed for fluorescence immunohistochemistry as follows: two washes in PBS containing 0.1% Triton X-100 (PTX); permeabilisation in 100 mM glycine, 0.5% Triton X-100 in PBS for 5 minutes; heat treatment for 15 minutes at 95°C in 10 mM Tris, 10 mM EDTA (pH 8.9); one wash in PTX; 1 hour in blocking solution of 20% foetal bovine serum (FBS) in PTX; overnight incubation of primary antibodies diluted 1/200 in blocking solution; three washes in PTX; blocking solution for 5 minutes; secondary antibodies diluted 1/200 in blocking solution for 1 hour; three washes in PTX. Secondary antibody incubations were performed sequentially if any were raised in the same species as any of the primary antibodies. Specimens were nuclear counter-stained with DAPI (5 μg/ml in PTX) for 5 minutes, washed in PTX and mounted in Vectashield (Vector Laboratories) under a coverslip on a glass slide.

Confocal imaging

Imaging was performed on a Zeiss LSM 510 Meta confocal microscope. Images were processed and analysed using ImageJ software. Contrast and brightness enhancement was performed for the purpose of clarity in the preparation of figures.

RESULTS

Key lineage-specific transcription factors are not differentially expressed or localised up to and including the diapausing blastocyst stage

To investigate whether transcription factors with key roles in early lineage specification in the mouse have conserved roles in marsupials, we examined their localisation in early tammar conceptuses (Fig. 2). POU5F1 and CDX2 are key transcription factors in the specification and maintenance of pluriblast (ICM) and trophoblast lineages, respectively, in the mouse, but no analogous reciprocal or lineage-specific expression was evident in tammar development up to and including the diapausing unilaminar blastocyst stage (Fig. 2A-F). Other factors, including SOX2, NANOG, GATA6 and the POU5F1 paralogue POU2 also did not mark different cell populations in the diapausing blastocyst or earlier. This suggests either that pluriblast and trophoblast have not become specified by the unilaminar blastocyst stage, despite the presence of morphologically distinct cell populations in unilaminar blastocysts of other marsupial species, or that other transcription factors are important for this process in marsupials.

YAP and WWTR1 localisation suggests a non-conserved role for Hippo signalling in early lineage segregation in mammals

In the mouse, early differential expression of lineage-specific factors depends on the Hippo signalling pathway, which responds to positional information to prevent nuclear localisation of YAP and WWTR1 specifically in inner cells (Nishioka et al., 2009). Marsupials, by contrast, have no ‘inside’ cells and analogous positional information is thought to be derived instead from early conceptus polarity that results in greater cell density at the embryonic pole (Selwood, 1992). Thus, in many marsupial species, putative pluriblast and trophoblast precursors are morphologically identifiable in the unilaminar blastocyst, although all cells of the tammar diapausing blastocyst appear identical. Despite the lack of differential expression of key transcription factors before tammar blastocyst formation (as described above), we hypothesised that the Hippo pathway may nevertheless be functional and result in differential expression of other unidentified factors.

In live or unstained tammar cleavage stages, detailed morphological features such as cell number and conceptus polarity are difficult to distinguish, unlike in other marsupials such as dasyurids (Selwood and Smith, 1990). However, after confocal imaging, conceptus polarity was evident throughout early cleavage. In a two-cell conceptus, nuclei were localised near an early cleavage furrow at one pole, whereas signs of deutoplasmolysis were evident in the opposite hemisphere (Fig. 3A). In a single 15-cell conceptus, there was some evidence of two cell populations (Fig. 3C) in a mode resembling that described in dasyurids (Selwood and Smith, 1990).

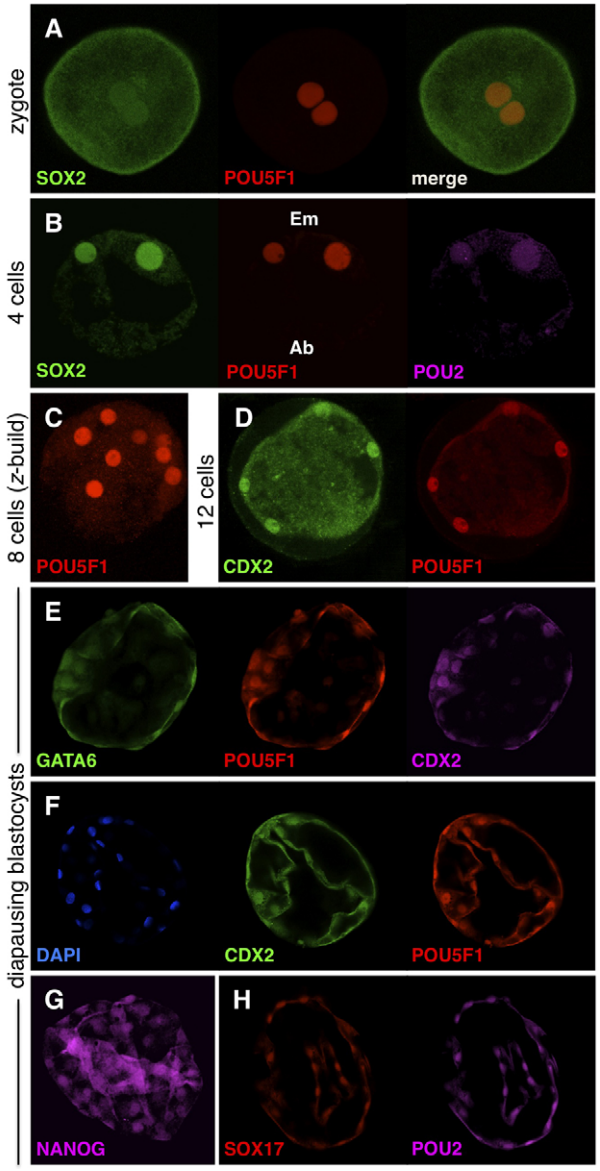

Fig. 2. Immunolocalisation of transcription factors in tammar conceptuses from zygote to diapausing blastocyst. (**A**) Weak SOX2 and strong POU5F1 pronuclear localisation in a zygote. (**B**) SOX2, POU5F1 and POU2 nuclear localisation in a four-cell conceptus. All four blastomeres are positioned close to the embryonic pole (Em). Ab, abembryonic pole. (**C**) POU5F1 nuclear localisation in an eight-cell conceptus. Blastomeres are mostly positioned in one hemisphere. (**D**) CDX2 and POU5F1 nuclear localisation in a 12-cell conceptus. Blastocyst formation is not yet complete. (**E**) GATA6, POU5F1 and CDX2 nuclear localisation in a diapausing blastocyst. (**F**) POU5F1 and CDX2 nuclear localisation in a diapausing blastocyst. (**G**) NANOG nuclear localisation in a diapausing blastocyst. (**H**) SOX17 and POU2 nuclear localisation in a diapausing blastocyst.

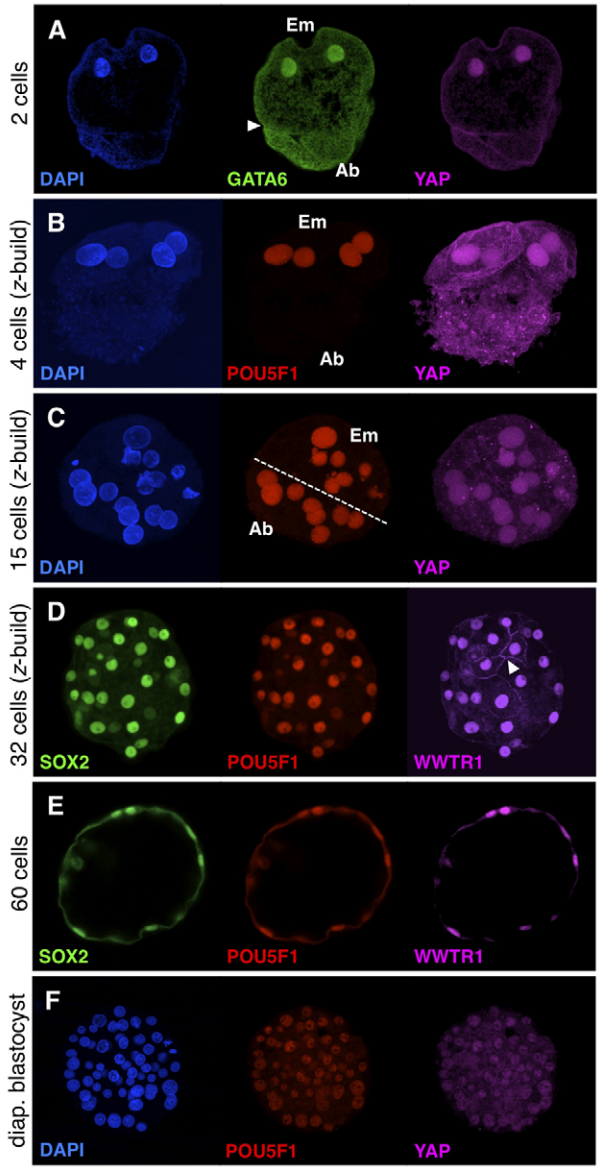

Fig. 3. Immunolocalisation of YAP and WWTR1 in tammar conceptuses from zygote to diapausing blastocyst. (**A**) GATA6 and YAP nuclear localisation in a two-cell conceptus. Although the first mitosis is complete, cleavage is still only partial. Conceptus polarity is evident, associated with deutoplasmolysis (arrowhead) in the abembryonic hemisphere. (**B**) POU5F1 and YAP nuclear localisation in a four-cell conceptus (*z*-build). All four blastomeres are positioned close to the embryonic pole. (**C**) POU5F1 and YAP nuclear localisation in a 15-cell conceptus (*z*-build). In this specimen, differences in DAPI staining and cell cycle progression are discernible between two tiers of blastomeres (demarcated by a dashed line) occupying separate presumptive embryonic and abembryonic hemispheres. (**D**) SOX2, POU5F1 and WWTR1 localisation in a 32-cell conceptus (*z*-build), around the stage of blastocyst formation. WWTR1 also localises to cell-cell junctions (arrowhead). (**E**) SOX2, POU5F1 and WWTR1 nuclear localisation in a pre-diapause 60-cell blastocyst. (**F**) POU5F1 and YAP nuclear localisation in a diapausing blastocyst (collapsed in mounting, thus most cells are in the same optical plane). Ab, abembryonic pole; Em, embryonic pole.

Eight cells constituting a tier in one hemisphere were more similar to each other in the quality of nuclear DAPI staining than to the seven cells constituting a tier in the opposite hemisphere. Cells of the latter tier appeared to be later-dividing, with one pair of recently divided cells and one large cell yet to divide. DAPI staining in the latter tier was notably weaker and more restricted to the nuclear envelope. Conceptus polarity was no longer distinguishable by confocal imaging from the 32-cell stage, when the unilaminar blastocyst appeared to be completely formed, through to the diapausing blastocyst (Fig. 3D-F).

Despite clear evidence for conceptus polarity, immunostaining for YAP and WWTR1 in cleavage and unilaminar blastocyst stages showed no evidence for a role of Hippo signalling in lineage specification, with both factors localised to the nucleus in all cells. WWTR1 additionally localised to regions of cell-cell contact in a 32-cell conceptus (Fig. 3D), but not at earlier stages examined. These data suggest that, in the tammar, differential cell density before blastocyst formation does not specify later cell fate via the Hippo pathway.

YAP and WWTR1 localisation after pluriblast-trophoblast segregation suggests distinct non-redundant roles in tammar early lineage maintenance and/or differentiation

The first significant expansion of the blastocyst after reactivation from diapause (diameter ~250 μm) is normally evident on day 8 (300 μm) after removal of pouch young (d8 RPY), when it is still unilaminar, and continues to expand over subsequent days (Renfree and Tyndale-Biscoe, 1973). We therefore focused our attention on blastocysts collected on day 9. One of the earliest-stage and more informative blastocysts with respect to lineage segregation had a diameter of 420 μm. This was bisected into two hemispheres, each containing a portion of a putative embryonic disc, and stained for GATA6, POU5F1 and YAP (Fig. 4). POU5F1 nuclear localisation was restricted more to cells of the embryonic disc than to the putative trophoblast, although lower cytoplasmic levels of POU5F1 were evident in all cells. GATA6 showed three types of staining: weak nuclear and cytoplasmic in central cells of the disc; moderately weak nuclear in trophoblast cells; and strong nuclear in putative hypoblast precursors that were irregularly clustered in peripheral parts of the disc. GATA6-positive hypoblast precursors still lay largely within the unilaminar epithelium, although some showed early signs of ingression. YAP localised strongly to nuclei in the central disc but only weakly to nuclei of hypoblast precursors and trophoblast. Notably, this strong nuclear staining was only found in one of the two bisected blastocyst halves, corresponding to one part of the disc, although differential POU5F1 staining was also discernible in the other half, putatively demarcating the other part of the disc. The majority of GATA6-positive hypoblast precursors were also located near the strongly YAP-positive disc cells. We speculate that this staining pattern indicates a prospective anteroposterior axis. Specific nuclear localisation of YAP in the epiblast was also maintained through to later stages (Fig. 4E; data not shown).

A slightly more advanced 540-μm blastocyst was similarly bisected and one half stained for GATA6, POU5F1 and WWTR1. GATA6 staining was similar to that in earlier stages, whereas POU5F1 staining was unexpectedly nuclear in trophoblast cells as well as in cells of the disc (Fig. 5A). WWTR1 was conspicuously absent in nuclei of all cells of the embryonic disc, including hypoblast precursors, but was present in nuclei of trophoblast cells (Fig. 5A). Intriguingly, this is the opposite pattern to that observed for YAP (described above), but more consistent with mechanisms of trophoblast differentiation described in the mouse (Nishioka et al., 2009). Membrane localisation of WWTR1 in regions of cell-cell

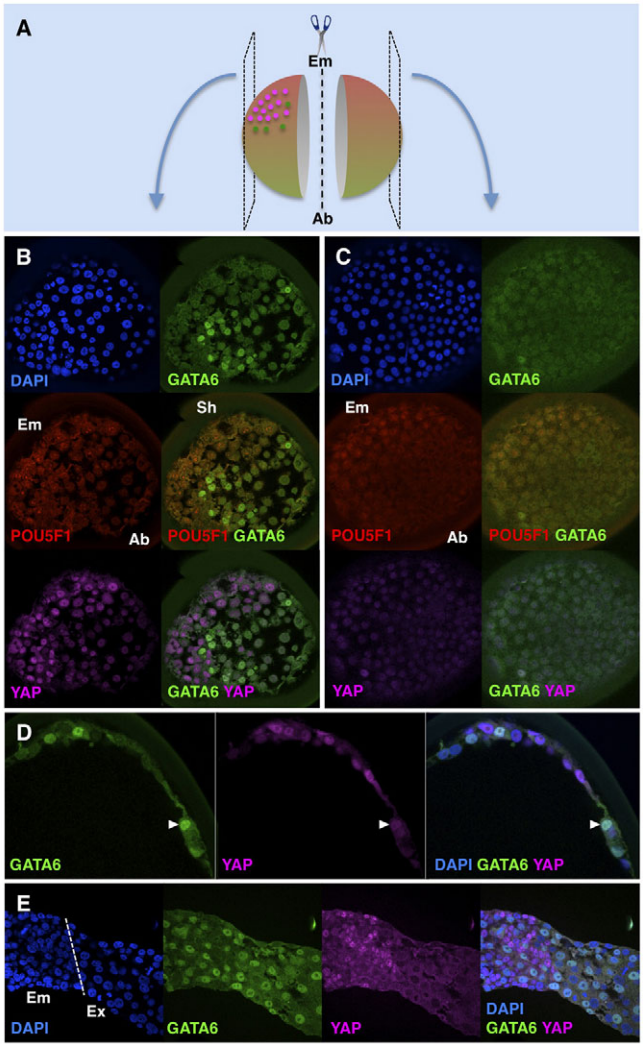

Fig. 4. Immunolocalisation of POU5F1, GATA6 and YAP in an early post-diapause blastocyst (420 µm diameter). (**A**) Schematic of the bisection of the blastocyst into two halves that are represented in panels B and C. (**B**,**C**) Tangential confocal sections in two bisected halves (B and C, respectively) of the blastocyst. Stronger POU5F1 staining marks presumptive pluriblast in a part of both halves, whereas strong YAP signal is restricted to the presumptive pluriblast in only one half (B). Strongly GATA6-positive presumptive hypoblast precursors are distributed in the perimeter of the presumptive pluriblast. (**D**) Transverse optical section through part of the presumptive pluriblast. Some GATA6-positive putative hypoblast precursors (arrowhead) are still clearly within the unilaminar epithelium. (**E**) Dissected fragment of a d12 RPY conceptus (early primitive streak stage), showing nuclear localisation of YAP within the epiblast and GATA6-positive hypoblast cells within both the embryonic and extra-embryonic regions, demarcated by a broken line. The conceptus fragments were obtained by a similar method to that of the d14 RPY specimen described in supplementary material Fig. S1. Ab, abembryonic pole; Em, embryonic pole; Ex, extra-embryonic; Sh, shell coat.

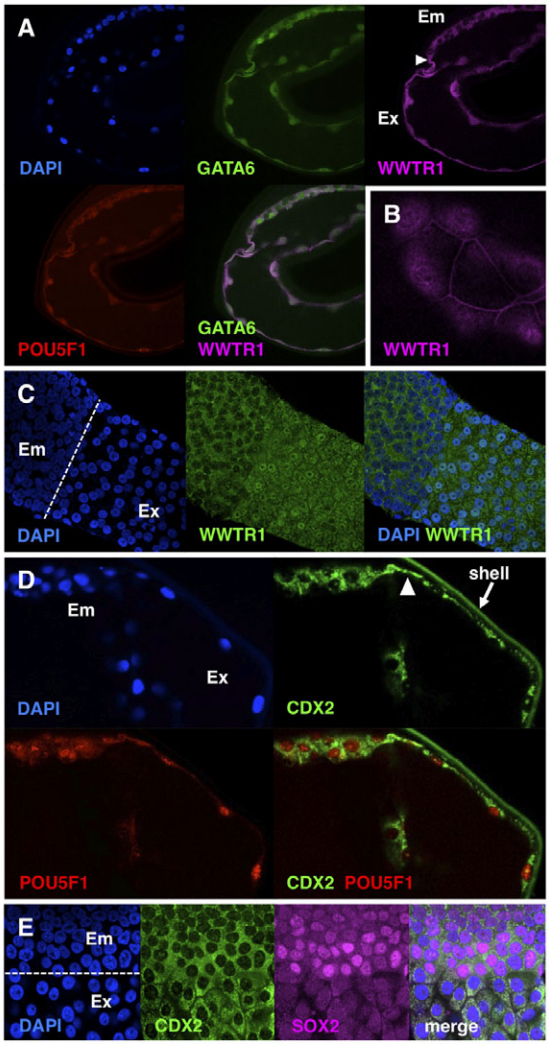

Fig. 5. Immunostaining of WWTR1 and CDX2 in bisected halves of an early post-diapause blastocyst (540 µm diameter). (**A**) WWTR1 is excluded from the nucleus in all cells of the embryonic disc but not the trophoblast, in which it is also localised strongly to cell-cell junctions. An arrowhead marks the boundary between the embryonic and extra-embryonic regions. (**B**) Higher magnification of a tangential optical section through part of the trophoblast of the above specimen, showing WWTR1 localisation to cell-cell junctions. (**C**) Dissected fragment of a d12 RPY conceptus (early primitive streak stage), showing that nuclear localisation of WWTR1 is specifically in the trophoblast and is also maintained at later stages. The dashed line represents the boundary between embryonic and extra-embryonic regions. (**D**) CDX2 is excluded from the nucleus in all cells of both trophoblast and the embryonic disc. An arrowhead marks the boundary between the embryonic and extra-embryonic regions. (**E**) Dissected fragment of a d12 RPY conceptus (early primitive streak stage), showing that global nuclear exclusion of CDX2 is also maintained at later stages. The epiblast is marked by nuclear-localised SOX2. The dashed line represents the boundary between embryonic and extra-embryonic regions. Em, embryonic; Ex, extra-embryonic.

contact, similar to that observed in the 32-cell conceptus, was also specific to the trophoblast (Fig. 5B). This pattern of WWTR1 localisation was maintained in later, early-gastrula stages (Fig. 5C). Thus, WWTR1, but not YAP, appears to have a conserved role in trophoblast maintenance in mammals, although not in its initial specification.

The contrasting localisation of YAP (Fig. 4) and WWTR1 (Fig. 5) during early tammar development was unexpected, because in the mouse both factors were reported to act redundantly and are similarly excluded from the nucleus of ICM cells (Nishioka et al., 2009). We therefore re-examined their localisation in early mouse conceptuses (Fig. 6). Strong nuclear localisation of WWTR1 (using

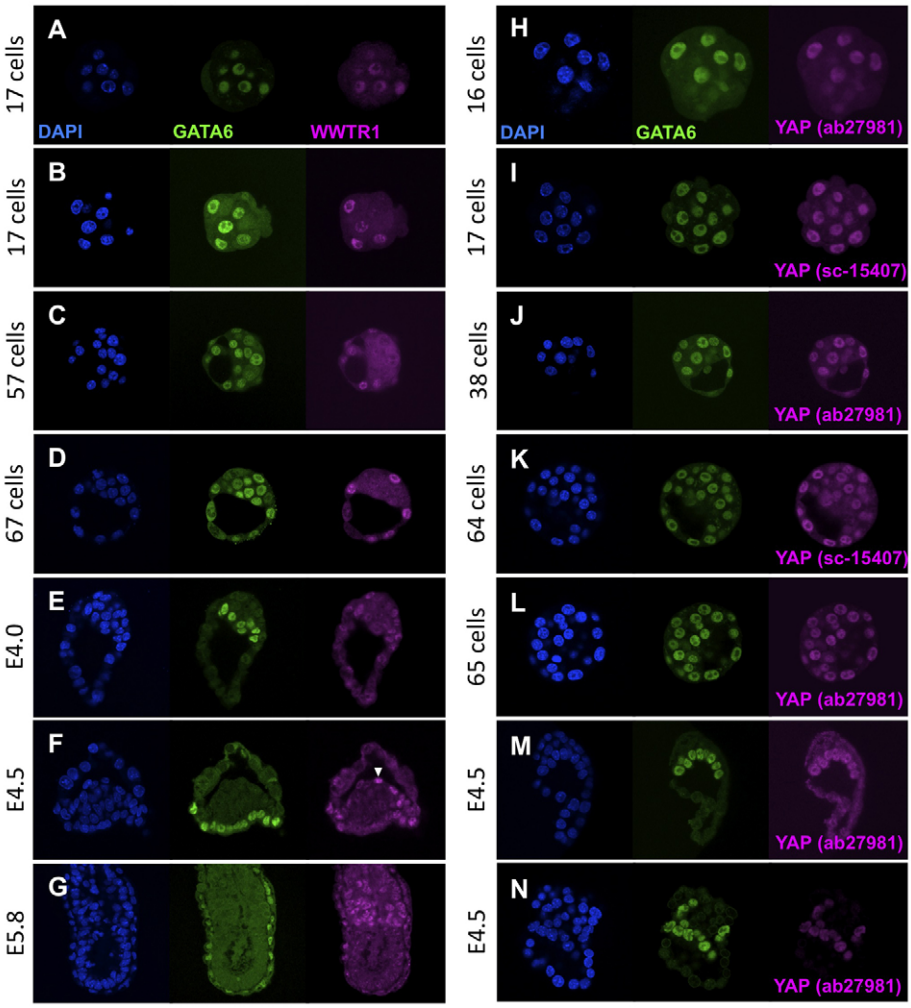

Fig. 6. Immunostaining of mouse pre- and peri-implantation conceptuses for WWTR1 and YAP. (**A-G**) WWTR1 and GATA6 immunostaining. Note that in F, the trophoblast has ruptured during processing and the conceptus everted, such that the hypoblast appears on the outside. (**H-N**) YAP and GATA6 immunostaining. Two different YAP antibodies were used, indicated in parentheses (see Materials and methods). E, embryonic day.

a different antibody to that used by Nishioka et al.) was clearly specific to trophoblast cells from early blastocyst stages (Fig. 6A-G), consistent with Nishioka et al. (Nishioka et al., 2008). However, exclusion from inner cells of morulae was less evident (Fig. 6A,B), which might be due to differences in mouse strains used. In addition, we observed previously unreported nuclear-localised WWTR1 in hypoblast and polar trophoblast cells of peri-implantation stages, a pattern that was maintained through to egg cylinder stages (Fig. 6E-G), suggesting a role in proliferation of these two lineages. By contrast, we failed to detect overt nuclear exclusion of YAP in inner morula cells or early ICM cells using two different antibodies (Fig. 6H-N). Similarly to WWTR1, YAP nuclear localisation in late blastocyst and peri-implantation stages was specific to hypoblast cells but, unlike WWTR1, was not detected in polar trophoblast cells. Notably, GATA6 was widely expressed (Fig. 6D,K,L) until much later than previously reported for conceptuses that were similarly staged by cell number (Plusa et al., 2008), again possibly owing to differences in the mouse strains used.

Pluriblast-trophoblast segregation correlates with a global nuclear-to-cytoplasmic transition of CDX2 localisation

In the mouse, CDX2 expression is specifically upregulated in outer cells and is required cell-autonomously for maintenance and differentiation of the trophoblast lineage (Ralston and Rossant, 2008; Strumpf et al., 2005). To determine whether it might have a similar role in the tammar, the other half of the 540-μm blastocyst was stained for POU5F1, GATA6 and CDX2. In contrast to the global nuclear localisation observed in diapausing blastocysts and earlier (see above), CDX2 was still present in all cells but was exclusively cytoplasmic (Fig. 5D). This pattern was also maintained in later-stage conceptuses (Fig. 5E). Thus, the role of CDX2 in early tammar development appears to differ markedly from that in the mouse.

POU2 marks pluriblast and epiblast more specifically than does POU5F1

POU2 is a paralogue of POU5F1 that has orthologues in most other vertebrate lineages, such as teleost fishes, birds and amphibians (Frankenberg et al., 2010; Niwa et al., 2008). To investigate whether POU2 might have a role similar to that of POU5F1 in specifying the pluripotent lineage, one half of a 440-μm blastocyst (containing both embryonic and extra-embryonic parts) was stained for GATA6, POU5F1 and POU2 (Fig. 7). POU2 was strongly nuclear-localised in GATA6-negative pluriblast cells (within unilaminar parts of the disc) and epiblast cells (within bilaminar parts), but not in trophoblast cells. GATA6-positive cells were found either in unilaminar, mostly peripheral regions of the disc, or as nascent hypoblast cells scattered in more central parts of the disc. Notably, POU2 appeared to be overtly downregulated in all GATA6-positive cells (including non-ingressed cells), in contrast to mouse POU5F1, which is downregulated only after the hypoblast is fully formed

(Batlle-Morera et al., 2008; Grabarek et al., 2012; Palmieri et al., 1994). In contrast to POU2, tammar POU5F1 expression was a comparatively poor marker of both pluriblast and epiblast in the stages presented here (Figs 4, 5, 7, 8), but was more consistently specific to the epiblast at later stages (supplementary material Fig. S1).

Differential nuclear localisation of NANOG marks early tammar pluriblast and epiblast

In the mouse, NANOG is essential for the acquisition of pluripotency and is expressed in the ICM and early naive epiblast (Chambers et al., 2003; Chambers et al., 2007; Mitsui et al., 2003; Nichols et al., 2009; Plusa et al., 2008). In mid-blastocyst stages, NANOG and GATA6 become mutually exclusive in their expression and mark epiblast and hypoblast precursors, respectively, which then sort to their respective layers by the late blastocyst stage (Chazaud et al., 2006; Plusa et al., 2008). To investigate whether mechanisms of epiblast-hypoblast segregation might be conserved in mammals, the other half of the 440-µm blastocyst described above was stained for GATA6, POU5F1 and NANOG. GATA6-positive hypoblast precursors within unilaminar parts of the epithelium were mainly found in the periphery of the disc but also interspersed among NANOG-positive cells more centrally; ingressed GATA6-positive hypoblast cells were found more centrally (Fig. 8A). Within the central disc, NANOG was specifically localised to nuclei of unilaminar parts as well as the epiblast layer of bilaminar parts. GATA6 and NANOG nuclear localisation was largely mutually exclusive (Fig. 8A). However, in some strongly GATA6-positive mitotic hypoblast precursors that were dividing perpendicularly to the epithelial plane, cytoplasmic NANOG levels appeared similar to those in GATA6-negative epiblast precursors that were dividing parallel to the epithelial plane (Fig. 8B). This suggests that NANOG expression is not initially downregulated in hypoblast precursors, but its exclusion from the nucleus depends instead on regulation of nuclear transport. Epiblast-specific NANOG nuclear localisation was maintained at later stages (Fig. 8C). No differences in POU5F1 expression and localisation among cells were observable in the 440-µm blastocyst (Fig. 7; Fig. 8A,B).

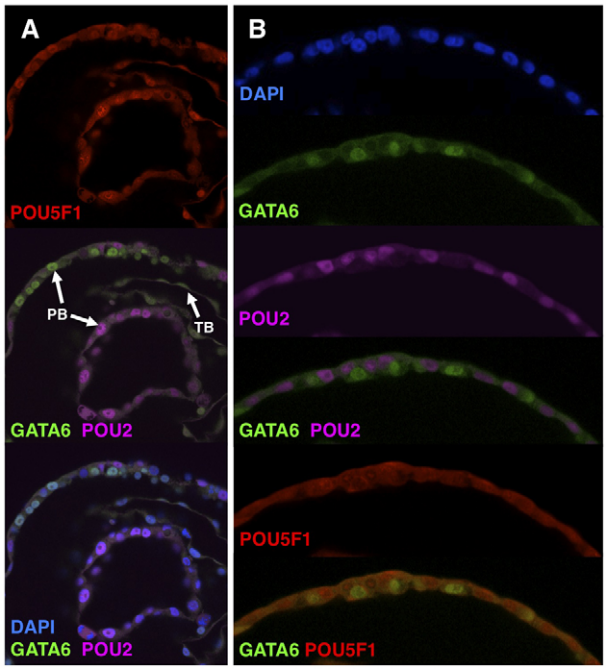

Fig. 7. Immunostaining of POU5F1, GATA6 and POU2 in a bisected half of an early post-diapause blastocyst (440 µm diameter). (**A**) Transverse optical section (note that the conceptus is highly folded in mounting) that includes GATA6-positive cells within the unilaminar, peripheral part of disc; POU2-positive cells within the unilaminar, more central part of the disc; and bilaminar parts of the disc and trophoblast. Strong POU5F1 nuclear localisation is still evident in many trophoblast cells. PB indicates GATA6- and POU2-positive regions of cuboidal pluriblast cells. TB indicates a region of POU2-negative/GATA6-weak squamous trophoblast cells. (**B**) Transverse optical section through unilaminar and bilaminar parts of the nascent embryonic disc. The unilaminar pluriblast contains both presumptive epiblast precursors (POU2 positive) and presumptive hypoblast precursors (GATA6 positive).

DISCUSSION

In marsupials, the absence of a morula stage containing inner cells precludes the 'inside-outside' model (Tarkowski and Wróblewska, 1967) as a mechanism for segregating pluriblast and trophoblast. An alternative model proposes that early conceptus polarity establishes an embryonic-abembryonic axis before blastocyst formation, with prospective pluriblast cells concentrated at the embryonic pole and surrounded by prospective trophoblast cells (Frankenberg and Selwood, 1998; Selwood, 1992). This study confirms that early conceptus polarity, previously noted in the zygote (Renfree and Lewis, 1996), is present in the tammar throughout early cleavage and potentially establishes distinct cell populations. However, we could find no evidence for early differential nuclear localisation of key transcription factors that might discriminate these putative populations. There are several possible explanations for this. First, differences in relevant transcription factor levels might have been too small to detect by immunohistochemistry. This is unlikely because small differences in transcription factor levels during cleavage should translate into more robust differences in later blastocyst stages. Alternatively, lineage segregation in later blastocyst stages might be driven by other unknown and later-acting mechanisms. This is also unlikely, because in other marsupials morphologically distinct cell types are distinguishable continuously from cleavage through to embryogenesis. We consider it more likely that early conceptus polarity causes asymmetric segregation of maternal determinants that results in either early (during cleavage) or late (after unilaminar blastocyst formation) differential expression of transcription factors that drive pluriblast-versus-trophoblast specification. If the former, they may be unidentified transcription factors not examined in this study.

The Hippo pathway is important for suppressing tumourigenesis and limiting organ size in animals ranging from *Drosophila* to mammals (reviewed by Pan, 2010). In the early mouse conceptus, cell-cell contacts activate the Hippo signalling pathway specifically in inner cells, causing phosphorylation of YAP and WWTR and targeting them for degradation. In outer cells, YAP and WWTR1 enter the nucleus and drive expression of trophoblast-specific genes in cooperation with the transcription factor TEAD4 (Nishioka et al., 2009; Nishioka et al., 2008). This new model provided a mechanism for the 'inside-outside' model some four decades after its inception (Tarkowski and Wróblewska, 1967). A more recent extension of the inside-outside model suggested that cell-cell contacts could play a similar role in marsupial pluriblast-trophoblast segregation, because blastomeres are initially more densely populated in the embryonic hemisphere, although this polarity is more overt in some species than others. Thus, prospective pluriblast cells closest to the

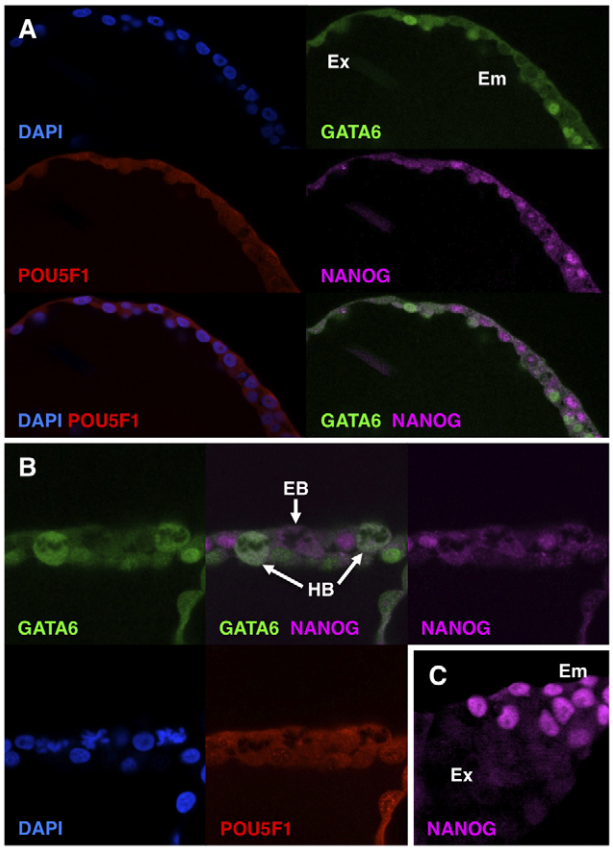

Fig. 8. Immunostaining of POU5F1, GATA6 and NANOG in a bisected half of an early post-diapause blastocyst (440 μm diameter). (**A**) Transverse optical section through part of the nascent embryonic disc. Both unilaminar and bilaminar parts are visible. GATA6-positive cells are found both scattered within the unilaminar part and constituting the nascent hypoblast layer of the bilaminar part of the disc. (**B**) Mitotic presumptive hypoblast (HB) and epiblast (EB) precursors within a bilaminar part of the embryonic disc, which clearly differ in their level of cytoplasmic GATA6, but not of cytoplasmic NANOG. (**C**) Dissected fragment of a d12 RPY conceptus (early primitive streak stage), showing that nuclear-localised NANOG is also maintained at later stages. Em, embryonic region. Ex, extra-embryonic region.

embryonic pole would have more cell-cell contacts than the more peripherally located prospective trophoblast cells prior to completion of the unilaminar blastocyst epithelium (Selwood, 1992; Selwood and Johnson, 2006).

Our examination of YAP and WWTR1 localisation in the early tammar conceptus failed to provide clear evidence of a role for Hippo signalling in the context described above. However, nuclear localisation of WWTR1 specifically in the differentiated trophoblast suggests an evolutionarily conserved role for this pathway. Because Hippo signalling generally suppresses proliferation, this role can be considered as having evolved to enhance extra-embryonic tissue formation prior to embryogenesis, especially in organisms with telolecithal eggs in which extra-embryonic membranes must proliferate extensively to encompass the yolk, such as in monotremes, birds and reptiles.

The contrasting pattern of YAP nuclear localisation observed in this study is more difficult to reconcile. In the early mouse conceptus, *Yap* and *Wwtr1* are redundant: only double knockouts fail to form blastocysts (Nishioka et al., 2009), whereas their respective single knockouts exhibit relatively late phenotypes (Hossain et al., 2007; Makita et al., 2008; Morin-Kensicki et al., 2006). Nuclear localisation of YAP but not WWTR1 in the tammar pluriblast and epiblast suggests that these two closely related co-activators have distinct early roles in this species. Our finding that YAP is not excluded from nuclei of inner cells of mouse morulae and early blastocysts contradicts earlier reports (Nishioka et al., 2009; Varelas et al., 2010) but is consistent with another more recent study (Home et al., 2012), which might reflect differences between mouse strains and/or antibodies used.

YAP and WWTR1 also have distinct roles in mouse and human embryonic stem (ES) cells, respectively, which might be due to the different developmental stages they are thought to represent (Tesar et al., 2007). In mouse ES cells, YAP binds to the promoters of many genes implicated in stem cell self-renewal and *YAP* knockdown causes a loss of pluripotency (Lian et al., 2010). This role for YAP is mediated by leukemia inhibitory factor (LIF) signalling via the Src kinase family member YES (Tamm et al., 2011). Thus, the role of YAP in mouse ES cell self-renewal may be homologous to its putative role in the tammar epiblast. By contrast, WWTR1, but not YAP, is necessary for human ES cell self-renewal by interacting with SMAD signalling. A recent study (Qin et al., 2012) showed that the Hippo pathway kinase LATS2, which can phosphorylate both YAP and WWTR1 (Zhao et al., 2010), is important for suppressing tumourigenesis in pluripotent cell types by interacting specifically with WWTR1.

Unlike in eutherians, the genomes of marsupials and monotremes contain genes for both POU5F1 and POU2 – two closely related class V POU domain transcription factors (Frankenberg et al., 2010; Niwa et al., 2008). In the mouse, POU5F1 is expressed in all cells until blastocyst formation, after which it is progressively downregulated in the trophoblast. In the ICM, POU5F1 is expressed in both epiblast and hypoblast precursors until after they have segregated to their respective layers, and is then downregulated in the hypoblast (Batlle-Morera et al., 2008; Grabarek et al., 2012; Palmieri et al., 1994). POU5F1 continues to be expressed in the epiblast and in many epiblast-derived tissues until the eight-somite stage, but its cellular localisation at all stages is exclusively nuclear (Downs, 2008). Thus, the transition from nuclear to nuclear/cytoplasmic POU5F1 staining observed in tammar conceptuses around the time of pluriblast differentiation was unexpected. Unlike POU5F1, POU2 localisation remained strongly nuclear in all cells in which it was expressed. Moreover, differential expression of POU2 between pluriblast/epiblast and trophoblast was more overt and consistent compared with POU5F1, suggesting that it might have a more important role than POU5F1 in this process. Although POU5F1 is rapidly downregulated in the mouse trophoblast soon after blastocyst formation, in other eutherians, such as cow (Berg et al., 2011; van Eijk et al., 1999) and pig (Kuijk et al., 2008), it continues to be detected for several days after blastocyst formation. The relatively early trophoblast-specific downregulation of *Pou5f1* in the mouse compared with the cow depends on mouse-specific cytoplasmic factors as well as TCFAP2 binding sites within the CR4 upstream enhancer region (Berg et al., 2011). These authors speculated that the early downregulation of *Pou5f1* in rodent trophoblast is a more recently evolved mechanism linked to this group's relatively early stage of implantation. They showed that development of bovine trophoblast cells (still expressing *POU5F1*) aggregated with morulae until the gastrula stage contributed to embryonic lineages, suggesting that bovine trophoblast cells might remain totipotent for longer than those of the mouse. Like the cow and pig, the marsupial conceptus has a long period before placental

attachment (reviewed by Renfree, 2010). Considering the uniform expression we observed for a number of key transcription factors (including POU5F1) in the tammar diapausing blastocyst, it is likely that all cells of this stage are totipotent, although they could be biased towards particular fates.

It is unclear whether the variation in tammar POU5F1 differential expression between trophoblast and epiblast reflects conceptus variation or temporal changes during development. In the mouse, POU5F1 is downregulated in nascent hypoblast cells but upregulated again in some migrating parietal endoderm cells (Grabarek et al., 2012). Thus, downregulation of POU5F1 is not necessarily an irreversible process during development, but might temporarily serve to mediate certain developmental transitions.

One of the more unexpected findings from this study was the ubiquitous exclusion of CDX2 from nuclei at around the onset of overt pluriblast-trophoblast differentiation. Aside from its role in trophoblast differentiation, mouse CDX2 is involved in maintenance of the intestinal epithelial lining (Gao and Kaestner, 2010), in which it is normally nuclear localised (James et al., 1994). Nuclear exclusion of CDX2 is associated with some colon adenocarcinomas, suggesting that it might be involved in suppressing epithelial tumourigenicity (Hinoi et al., 2003). A possible explanation for the observed localisation in the tammar might be that loss of global nuclear-localised CDX2 has a permissive role, being a prerequisite for a transition to a state in which cells lose epithelial character and are poised for differentiation. In the mouse, a more instructive role for CDX2 might have evolved that involves the specific downregulation of *Cdx2* in inner cells, whereas in the tammar, CDX2 nuclear exclusion occurs globally and an unidentified opposing mechanism maintains a squamous epithelial phenotype specifically in the trophoblast. A 13-kb fragment (including the first intron and upstream) of the *Cdx2* locus was insufficient to direct trophoblast-specific expression in the mouse, suggesting that essential regulatory elements lie outside this region (Benahmed et al., 2008). NANOG- and POU5F1-binding elements that may be necessary but insufficient for trophoblast-specific expression were nevertheless identified within the promoter and first intron of mouse *Cdx2* (Chen et al., 2009), but these are mostly not conserved in marsupials (data not shown). Among mammals, downregulation of *CDX2* in the pluriblast appears to be a mechanism specific to eutherians. However, in the chick, *CDX2* is also specifically expressed in the extra-embryonic region (area opaca) of pre-gastrulation stages (Pernaute et al., 2010).

NANOG and GATA6 have similar expression patterns in tammar and mouse, indicating that they are clearly conserved as early markers of epiblast and hypoblast, respectively, although in the tammar this may initially be due to differential nuclear transport of NANOG. In the mouse, heterogeneous expression of NANOG and GATA6 is apparently a stochastic process (Chazaud et al., 2006; Dietrich and Hiiragi, 2007; Plusa et al., 2008), although earlier events might have an influence (Morris et al., 2010). Hypoblast precursors then position themselves adjacent to the blastocyst cavity by a process that involves a combination of active migration, positional signals and selective apoptosis (Meilhac et al., 2009; Plusa et al., 2008). The prevalence of GATA6-positive cells within the unilaminar epithelium in the periphery of the early embryonic disc of the tammar suggests that positional signals are important in this species, at least during early stages of hypoblast formation, but evidence of hypoblast precursors arising more centrally also suggests a role for stochastic processes. We propose a model for hypoblast formation in the tammar that involves both mechanisms (Fig. 9). It should be noted that in the chick, extra-embryonic endoderm also arises from two sources: the primary hypoblast, which is derived from isolated 'islands' dispersed throughout the area pellucida (embryonic region), and the junctional endoblast, which lies peripherally but is more prolific in the posterior where it contributes to Koller's sickle (reviewed by Stern and Downs, 2012). Thus, the GATA6-positive cells observed adjacent to YAP-expressing cells in the early tammar disc might similarly mark the future posterior. It would be interesting to determine whether these putative two populations of GATA6-expressing cells differ in expression of markers such as Cerberus, a NODAL antagonist that in the chick is specifically expressed in the hypoblast and not endoblast and thus restricts primitive streak formation to the posterior (Bertocchini and Stern, 2002).

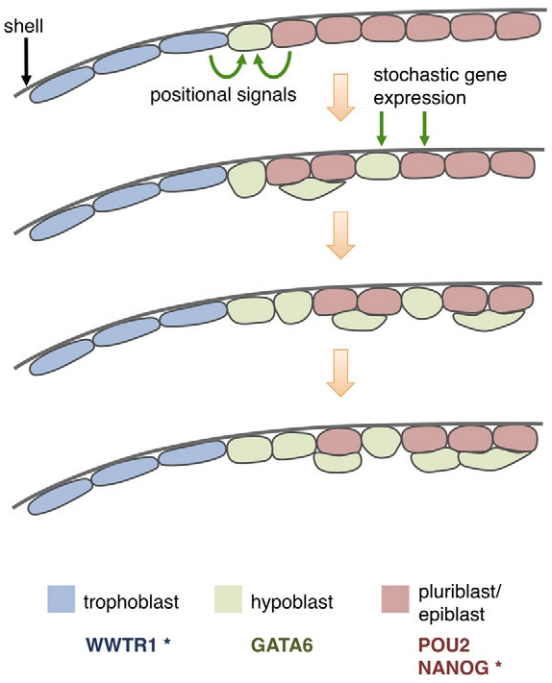

Fig. 9. Model for hypoblast differentiation in the tammar. GATA6-positive hypoblast precursors are initially found in the periphery of the emerging unilaminar pluriblast, suggesting that positional signals arising from trophoblast-pluriblast interactions initially specify hypoblast fate. In more advanced stages, GATA6-positive cells are found within more central unilaminar parts of the embryonic disc, suggesting that stochastic mechanisms similar to those proposed in the mouse ICM direct some pluriblast cells to form hypoblast. Asterisks denote that differential nuclear transport might be responsible for differential nuclear localisation, at least at early stages of specification.

Conclusion

Early lineage specification in this marsupial suggests mechanisms that differ from and challenge mammalian paradigms established from studies on mouse development. These new data, together with emerging information on cow, pig and even human show that early developmental mechanisms in mammals are more evolutionarily plastic than was previously recognised.

Acknowledgements

We thank members of the wallaby research group for assistance with animals.

Funding

This study was supported by a grant from the Australian Research Council to M.B.R. and A.J.P.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.091629/-/DC1

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