Localization of transforming growth factor β2 (TGFβ2) and its receptors, TβRI and TβRII, in uteri and blastocysts of the stripe-faced dunnart (Sminthopsis macroura) during gastrulation

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This study describes for the first time the spatial and temporal distribution of a growth factor and its receptors in uteri and conceptuses of a marsupial species during the peri-gastrulation period. Uteri (gravid and non-gravid) and blastocysts from 40 female stripe-faced dunnarts (Sminthopsis macroura) were collected over the peri-gastrula period (days 6.0–8.5) and stained immunohistochemically for transforming growth factor β2 (TGFβ2) and its receptors, TβRI and TβRII, to determine possible roles for TGFβ2 in marsupial embryonic development. The events that occurred during the period examined included the appearance and proliferation of hypoblast and mesoderm, primitive streak and node formation, and early neurulation. Differences in TGFβ2 quantities between gravid and non-gravid uteri reflect differences in uterine morphology, indicating a role for TGFβ2 in endometrial remodelling. In blastocysts, large quantities of all three proteins in the trophectoderm during the node stage coincide with both blastocyst expansion before implantation and the appearance of multinucleated cells, indicating that TGFβ2 plays a role in conceptus elongation and trophectoderm differentiation. In contrast, lack of TβRII in blastocysts during hypoblast formation and proliferation negates any role for TGFβ2 in these processes, as both receptors are required for a response to TGFβ2. High concentrations of TGFβ2 but low concentrations of TβRII in blastocysts during early primitive streak formation indicate that paracrine embryo–maternal signalling may be occurring, as blastocysts cannot respond to TGFβ2 at this stage, but uteri could. A similar situation, but reversed, also occurs during primitive node formation.

Introduction

The transforming growth factor β (TGFβ) superfamily is one of the largest groups of conserved signalling molecules involved in the control of embryonic development. Mammalian members of the immediate TGFβ family – TGFβ1, β2 and β3 – affect cellular interactions, cell differentiation, proliferation and migration, and composition of the extracellular matrix (ECM) (for a review, see Barnard et al., 1990).

Synthesized as a large precursor molecule, TGFβ is usually secreted from cells and stored in the ECM in a latent form, unable to bind to its receptors. When required, TGFβ is released from the ECM and activated via proteolysis, enzymatic deglycosylation or acid treatment, after which it can bind to its receptors (for a review, see Koli et al., 2001). TGFβs signal by binding to two types of transmembrane serine/threonine kinase receptor: TβRI and TβRII. After binding TGFβ, TβRII phosphorylates TβRI, which subsequently phosphorylates Smad proteins, resulting in translocation of the signal to the nucleus (for a review, see Miyazono et al., 2001). TGFβ2 signalling requires a third receptor, TβRIII (beta-glycan), which acts to increase the normally low affinity of TβRII for TGFβ2 (Moustakas et al., 1993).

The TGFβ isoforms have numerous roles in mammalian embryonic development, sometimes overlapping and sometimes distinct. Both TGFβ1 and β2 regulate proliferation of murine visceral and parietal endoderm (Mummery and van den Eijnden-van Raaij, 1990; Roelen et al., 1998), influence mesenchymal–epithelial transformations (Pelton et al., 1989; Millan et al., 1991), and may also affect blastocyst expansion (Doré et al., 1995; Gupta et al., 1996) and implantation (Godkin and Doré, 1998), as well as uterine remodelling (Doré et al., 1996; Godkin and Doré, 1998). In addition, TGFβ1 and β2, respectively, are associated with mesenchymal and epithelial differentiation per se (Heine et al., 1987; Millan et al., 1991). The numerous effects of TGFβ on cell proliferation, differentiation, migration and ECM composition observed both in vivo and in vitro indicate that it would be an excellent candidate for directing the process of gastrulation, although studies on mice do not support such a role.

Mice express TGFβ1, TGFβ2, TβRI and TβRII transcripts in various tissues just before and during gastrulation, but do not express TβRII until early somitogenesis (Roelen et al., 1994); chimaeric embryos that express ectopic TβRII can
initiate, but not successfully complete, gastrulation, due to TGFβ-induced growth inhibition (Zwijsen et al., 1999). Furthermore, mice deficient in functional TGFβ1 or TβRII die at about day 10.5 of gestation, and have defects in yolk sac haematopoiesis and endothelial differentiation (Dickson et al., 1995; Oshima et al., 1997; Goumans et al., 1998), whereas those deficient in TGFβ2 die perinatally, and have defects involving epithelial–mesenchymal interactions, ECM production and tissue remodelling associated with later developmental functions (Sanford et al., 1997).

In contrast to the mouse, TGFβs and their receptors are expressed widely in pig embryos during gastrulation. TGFβ1, TGFβ2, TβRI and TβRII proteins are present in the epiblast before gastrulation and in all three germ layers during early to mid-gastrulation, at which stage down-regulation of some proteins begins (Gupta et al., 1996). TGFβ1/β3 and TGFβ2 proteins are also present in all three germ layers during early and mid-gastrulation, respectively, in chicks (Jakowlew et al., 1994; Sanders et al., 1994). On this basis, it was decided to investigate the distribution of TGFβ and its receptors in the peri-gastrula period in a marsupial species. As conceptus material was in short supply, the distribution of TGFβ2, but not of TGFβ1, was examined.

The morphology of marsupial gastrulation is very similar to that of chicks, and as in pigs, implantation does not occur until gastrulation is complete (Selwood and Hickford, 1999). The stripe-faced dunnart, Sminthopsis macroura, was chosen for this study, as it is polyoestrous, polytocous, has the most detailed normal schedule of development for any marsupial to date (Selwood and Hickford, 1999).

The aim of the present study was to describe the spatial and temporal distribution patterns of TGFβ2 protein and its receptors in the stripe-faced dunnart during the peri-gastrulation period (days 6.0–8.5) and to relate these patterns to possible roles for TGFβ2 in marsupial embryonic development. At the time this study was performed, antibodies to TβRIII were not commercially available, so only the distributions of TβRI and TβRII were analysed. Events that occurred during the period examined include the appearance (day 6.0) and proliferation (day 6.5) of hypoblast, formation of the primitive streak and node (day 8.0), mesoderm formation (day 8.0), and early neurulation (day 8.5) (Selwood and Hickford, 1999). Non-gravid uteri from days 6, 7 and 8 of gestation were also analysed to investigate conceptus influences on TGFβ expression.

Materials and Methods

Collection and preparation of material

The animals used in this study were obtained from a laboratory colony maintained at LaTrobe University by L. Selwood (Selwood and Hickford, 1999). Australian National Health and Medical Council Guidelines for the care and use of experimental animals were followed. Daily monitoring of body weight and examination of urine samples provide an accurate guide to the reproductive status of the animals (Selwood and Woolley, 1991). In brief, the day of ovulation is associated with a decrease in body weight in combination with the presence of cornified epithelial cells and polymorphonuclear leucocytes in the urine (Selwood and Hickford, 1999).

Animals were anaesthetized with 4.5% halothane (Rhone Merieux, Melbourne) delivered in oxygen at 200 ml min⁻¹ by a Cyprane TEC vaporizer. Anaesthesia was maintained using 2.5% halothane. Uteri were removed from the body cavity, rinsed in calcium- and magnesium-free PBS and opened in Dulbecco’s modified Eagle’s medium (Sigma Chemical Co., St Louis, MO). Blastocysts were classified according to their developmental stage, and then blastocysts and uteri were rinsed in PBS and fixed in 4% (w/v) paraformaldehyde or buffered formalin for 24 h. Blastocysts were dissected into embryonic and abembryonic hemispheres and embedded in agar. Where visible, the position of the embryonic axis was marked so that transverse sections could be obtained. Both uteri and the agar blocks containing the blastocysts were dehydrated and embedded in Paraplast + paraffin wax (Oxford Labware, St Louis, MO).

Representative uteri (n = 4–5) and blastocysts (n = 2–4) from each half-day of development from late unilaminar blastocyst stage (day 6.0) to neurula–early somite stage embryos (day 8.5) were obtained. Day 8 material was categorized further as early primitive streak (early stages of streak formation) (E), mid-primitive streak (streak had progressed but not to node stage) (M) or node stage (which included regressing node stages) (L). Sections of uteri from non-gravid females on representative days after ovulation (day 6 (n = 2), day 7 (n = 2) or day 8 (n = 3)) were also stained. Uteri and conceptuses from a total of 40 animals were collected.

Antibodies

All three antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) used were isoform-specific, affinity-purified rabbit polyclonal antibodies raised against human proteins. The TGFβ2 antibody (cat. no. sc-90) was raised against a peptide corresponding to amino acid residues 352–377 within the carboxy terminal region of TGFβ2, and recognizes both mature and precursor forms of TGFβ2. Both receptor antibodies were raised against epitopes within their kinase domains: the TβRI antibody (cat. no. sc-398) recognizes amino acids 158–179 of TβRI and the TβRII antibody (cat. no. sc-400) recognizes amino acids 246–266 of TβRII. Each antibody recognizes the appropriate epitope in human, rat and murine tissues.

The TGFβ2 antibody was used at 1.3 μg ml⁻¹ and was diluted in Tris-buffered saline (TBS, 137.0 mmol NaCl l⁻¹, 25.0 mmol Tris base l⁻¹, 2.7 mmol KCl l⁻¹, pH 7.7) containing 0.1% (v/v) BSA (Sigma Chemical Co.). The two receptor antibodies were each used at 4 μg ml⁻¹, with TBS–0.1% BSA–1% (v/v) normal goat serum (Sigma Chemical Co.) as
Table 1. The basis of quantification of the staining present for transforming growth factor β2 (TGFβ2) and its receptors, TβRII and TβRI, in regions of uterine sections from the stripe-faced dunnart (Sminthopsis macroura)

<table>
<thead>
<tr>
<th>Grade</th>
<th>Luminal epithelium</th>
<th>Glandular epithelium</th>
<th>Stroma/myometrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Few scattered +ve</td>
<td>&lt;5% of glands +ve</td>
<td>&lt;5% of area +ve</td>
</tr>
<tr>
<td>2</td>
<td>Small groups +ve</td>
<td>5–25% of glands +ve</td>
<td>5–25% of area +ve</td>
</tr>
<tr>
<td>3</td>
<td>Extensive areas +ve</td>
<td>26–50% of glands +ve</td>
<td>26–50% of area +ve</td>
</tr>
<tr>
<td>4</td>
<td>Most areas +ve</td>
<td>&gt;50% of glands +ve</td>
<td>&gt;50% of glands +ve</td>
</tr>
</tbody>
</table>

*Grading of glandular staining was performed according to the number of positive (+ve) glands, regardless of the proportion of positive cells within the glands.

Immunohistochemistry

Sections (5 μm in thickness) from the mid-regions of uteri from a single stage were arranged on to five slides (one for each of the three antibodies and two negative control slides), each of which also contained a section of a paraformaldehyde-fixed dunnart pouch young as a positive control. Blastocysts were cut into serial sections of 6 μm and the wax ribbon of each blastocyst was divided into equal regions (four for day 6.0–7.5 blastocysts, and eight to ten for later stages). Representative sections of each region were stained with each antibody. Positive controls consisted of sections of a uterus that had demonstrated strong staining for all three antibodies. For each uterus, at least two sets of sections were stained for each antibody to ensure the validity of the results obtained.

Sections were mounted on poly-L-lysine-treated (Sigma Chemical Co.) slides and dried on a hotplate overnight. The basic immunohistochemical staining protocol used followed the Histostain SAP kit (Zymed Laboratories, San Francisco, CA).

After clearing and rehydrating, uterine sections were treated with 0.1% (v/v) Triton-X100 (Sigma Chemical Co.) and TBS for 15 min to increase tissue permeability. Uterine sections were digested with 1 mg hyaluronidase ml⁻¹ (bovine testicular hyaluronidase type I-S; Sigma Chemical Co.) buffered to pH 5.5 with 0.1 mol sodium acetate l⁻¹ in 0.15 mol NaCl l⁻¹ (Heine et al., 1987) at 37°C for 15 min. Enzyme activity was stopped by washing the slides in 0.1% (w/v) BSA in TBS. Digestion of uterine sections with hyaluronidase before staining yielded crisper staining compared with undigested sections. Digestion of blastocyst sections did not make any appreciable difference to the staining but increased background staining in the negative controls, so no enzyme treatment was used for the blastocysts.

Blocking for endogenous avidin–biotin was unnecessary, as neither uteri nor blastocysts showed any evidence of endogenous activity when staining was in the absence of both the primary and secondary antibodies. After blocking the sections against non-specific binding with 5% normal goat serum and 0.5% BSA in TBS, primary antibody incubation was performed overnight at 4°C. Bound antibody was detected using the Histostain SAP kit (Zymed Laboratories). This kit uses a biotinylated anti-mouse, rabbit, rat and guinea-pig secondary antibody to detect the rabbit primary antibodies, and an avidin–biotin–streptavidin conjugate to detect bound secondary antibody. Positive reactions were visualized with 5-bromo-4-chloro-3-indoxyl phosphate/nitro blue tetrazolium (Sigma Chemical Co.), to which 2 mmol levamisole 1⁻¹ (Sigma Chemical Co.) was added to block endogenous alkaline phosphatase activity. Positive reactions were marked by the presence of a deep purple stain. Sections were counterstained with 0.1% (w/v) nuclear fast red, dehydrated and mounted in DePeX.

Analysis of results

Photography was performed with an Olympus camera mounted on a Nikon inverted microscope, using 400 ASA Kodak Maxicolour film.

The abundance of staining in the four main regions of the uterus, the luminal epithelium, glandular epithelium, stroma and myometrium, was quantified under low magnification (∗×40) (Table 1). Results are expressed as mean ± SE. As the initial grades were based on subjective observations, no further statistical calculations were performed.

For each blastocyst, the presence or absence of staining in each of the main types of cell (pluriblast, trophoblast, hypoblast, epiblast, trophectoderm, ectoderm, primary endoderm and mesoderm, depending on the stage of the
Fig. 1. Examples of staining patterns for transforming growth factor β2 (TGFβ2) and its receptors, TβRI and TβRII, in various regions of the uterus of the stripe-faced dunnart (Sminthopsis macroura) during gastrulation. (a) TβRII staining in the luminal epithelium (le), showing both vesicular staining in basal regions of the epithelium (arrow) and a thin, positive line along the apical membrane of the epithelium (arrowhead). (b) Vesicular (type 1) staining for TβRI (arrowheads), characteristic of glandular epithelium close to the myometrium. (c) Globular (type 2) TβRII staining in glands close to the luminal epithelium (not in view). Note the varying positions of the positive globules (arrowheads) within the glands. Some vesicular (type 1) staining is also evident. (d) Granular
Results

Immunohistochemical staining for TGFβ2, TBRI and TBRII was performed successfully on uteri and blastocysts of 40 stripe-faced dunnarts from day 6.0 to day 8.5 of gestation, and also on day 6.0, day 7.0 and day 8.0 non-gravid uteri. Although western blot analyses were not undertaken, each antibody was abolished after pre-absorption with the appropriate control peptide. In instances in which IgG was used as a negative control, some background staining in the ECM and connective tissue of uteri was observed occasionally. In these instances, the uteri were stained again; if background staining persisted, then only staining above the background in the negative controls was recorded.

Analysis of staining

Immunohistochemical staining of uteri revealed that usually the different uterine tissues showed one of several main staining patterns, and in many instances, the distribution of TBRI reflected that of TGFβ2 quite closely.

Luminal epithelium. The most common staining pattern for all three antibodies was around the perimeter of numerous vesicles, usually located basally (Fig. 1a), within the epithelial cells. Occasionally staining was also observed either on the apical membranes (Fig. 1a) or within the cytoplasm of these cells.

Glandular epithelium. The distribution of TGFβ2, TBRI and TBRII usually followed one of three main patterns, although the patterns were not mutually exclusive. The most common pattern (type 1, vesicular) was observed in the glandular epithelium closest to the myometrium, and staining occurred around the perimeter of vesicles located within the basal half of the epithelium (Fig. 1b). Generally, glands closest to the myometrium had positive staining in the majority of cells, and there was a decrease in the number of positive cells per gland as the distance from the myometrium increased.

The second main pattern (type 2, globular) was characterized by a much crisper, globular form of staining that usually occurred in glands closest to the luminal epithelium. These glands showed wide variation in the number of positive cells per gland, and the location of staining within the epithelium varied markedly, even within a single gland (Fig. 1c). The final main pattern of staining (type 3, granular) was observed in some glands near the luminal epithelium. In these instances, staining was present either in the apical membrane of glandular epithelial cells or it filled the glandular lumina (Fig. 1d). The temporal distribution of these three patterns is shown (Fig. 2).

Stroma. The presence of TGFβ2, TBRI and TBRII in the endometrial stroma was represented by both diffuse and fibrillar (Fig. 1e) staining in the stromal ground substance, and by either cytoplasmic or pericellular staining or both of some stromal cells resembling fibroblasts, monocytes or macrophages.

Myometrium. All three proteins were present in the myometrium at various stages. Staining varied from pale, diffuse and cytoplasmic to dark and peri-nuclear (Fig. 1f).

Spatial and temporal distribution of TGFβ2, TBRI and TBRII in uteri and blastocysts

The quantitative results for immunohistochemical staining of the luminal epithelium, glandular epithelium and stroma, and the temporal distribution of types of staining within the glandular epithelium are shown (Fig. 2). The spatial distribution of TGFβ2, TBRI and TBRII in blastocysts is described (Table 2; Figs 3 and 4). The intensity of TBRII staining in uteri was often similar to that of TGFβ2 and TBRI, but was usually much lower in blastocysts.

In gravid uteri, the glandular epithelial TGFβ2 content reached a peak on day 8 (E) and day 8 (M) (Fig. 2) immediately before and during formation of the primitive streak. TBRI was also abundant on day 8 (M), as were both proteins within the luminal epithelium. Glandular epithelial TGFβ2 concentration decreased on day 8 (M), when it was also absent from the luminal epithelium. A general trend was that when TGFβ2 was abundant in the glandular epithelium, the stromal concentration of TGFβ2 was usually lower (Fig. 2), and vice versa.

Glandular staining in non-gravid uteri was more pronounced for TBRI and TBRII on day 6, and less on day 8 for TGFβ2 and TBRI, compared with the gravid uteri at corresponding stages (Fig. 2). At both stages, the types of staining pattern present were similar between gravid and
non-gravid uteri, unlike on day 7, on which the quantity of staining was similar but types of staining pattern were more restricted in the non-gravid uteri than in the gravid uteri. Consistent, dark staining of blastocysts first occurred on day 7.5 (Table 2); the most marked was for TGFβ2 and TβRI in the apical membranes of the epiblast of day 8 (L) node-stage.
blastocysts, although this staining was generally absent around the nodal region. There was very little TβRII present in blastocysts before day 8 (L). Occasionally, some staining for TGFβ2, TβRI or TβRII was observed in the ECM between the shell coat and the underlying cells. In all instances, staining within the trophoblast and trophectoderm was very irregular (patchy) for all three proteins, both within and between blastocysts.

### Discussion

The type of staining for TGFβ2 varied both within and between types of cells and developmental stages. Cytoplasmic, pericellular and perinuclear staining was observed, as was staining of the ECM. Cytoplasmic staining would represent protein production by the cells, and secreted TGFβ is stored in the ECM (Koli et al., 2001). The pericellular staining may represent TGFβ2 bound to the transmembrane receptors and perinuclear staining may represent TGFβ2 in the process of nuclear translocation via Smad proteins (Miyazono et al., 2001). Most receptor staining occurred either on cell membranes, as expected for transmembrane receptors, or in the cytoplasm. Cytoplasmic staining was not expected, but has also been observed for TβRII in pigs (Gupta et al., 1996), mice (Roelen et al., 1994) and hamsters (Roy and Kole, 1995). In hamsters, in addition to the membrane-associated TβRII form, a cytosolic form was also detected (Roy and Kole, 1995), and was recognized by the same antibody used in the present study.

The three different glandular staining patterns observed

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**Table 2.** Summary of the distribution of transforming growth factor β2 (TGFβ2) and its receptors, TβRI and TβRII, in blastocyst cell lineages of the stripe-faced dunnart (Sminthopsis macroura) from day 6.0 to day 8.5 of gestation

<table>
<thead>
<tr>
<th>Stage (day)</th>
<th>n</th>
<th>Type of cell</th>
<th>TGFβ2</th>
<th>TβRI</th>
<th>TβRII</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>4</td>
<td>Pluriblast</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trophoblast</td>
<td>+ (3)</td>
<td>+ (2)</td>
<td>0</td>
</tr>
<tr>
<td>6.5</td>
<td>4</td>
<td>Epiblast</td>
<td>+ (3)</td>
<td>+ (3)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trophoblast</td>
<td>+ (3)</td>
<td>+ (3)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypoblast</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.0</td>
<td>3</td>
<td>Epiblast</td>
<td>+ (2)</td>
<td>+ (2)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypoblast (under epiblast)</td>
<td>0</td>
<td>++ (2)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trophoblast</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypoblast (under trophoblast)</td>
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<td>+ (2)</td>
<td>0</td>
</tr>
<tr>
<td>7.5</td>
<td>4</td>
<td>Epiblast</td>
<td>+ (2)</td>
<td>+ (3)</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
<td>Hypoblast (under epiblast)</td>
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<td></td>
<td></td>
<td>Hypoblast (under trophoblast)</td>
<td>++ (2)</td>
<td>+ (3)</td>
<td>+ (2)</td>
</tr>
<tr>
<td>8.0 (E)</td>
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<td>++</td>
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<td>+ (2)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Trophoderm</td>
<td>++</td>
<td>++</td>
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<td>++</td>
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<tr>
<td></td>
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<td>+ (2)</td>
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<td>Endoderm</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Neuroectoderm</td>
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<td></td>
<td>Trophoderm</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypoblast</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

0: absence of staining; +: some pale staining; ++: dark staining.
Numbers in brackets represent the number of blastocysts with staining. In cases in which there are no values in brackets, all of the blastocysts stained. If less than two blastocysts of a particular stage stained, it was recorded as –ve.
Day 8 (E): early primitive streak (early stages of streak formation); day 8 (L): node stage (which included regressing node stages).
Regulation of endometrial TGFβ expression differs among species, indicating different roles for TGFβ within this tissue. In ewes, TGFβ may control remodelling of the endometrium between cycles and between pregnancies (Doré et al., 1996). In humans, TGFβ may restrict cellular proliferation while promoting differentiation and inhibiting degradation of the ECM (Tang et al., 1994). In the stripe-faced dunnart, differences in the quantities of TGFβ2, TβRI and TβRII between gravid and non-gravid uteri often reflected differences in uterine morphology. The concentrations of both receptors in the luminal epithelium were markedly higher in non-gravid than in gravid uteri on day 6, when the height of the luminal epithelium in non-gravid uteri is significantly lower than that in gravid uteri (Cruz and Selwood, 1997). Glandular concentrations of TGFβ2, TβRI and TβRII were much higher in gravid compared with non-gravid uteri on day 8, when gland density is significantly lower in the non-gravid uteri compared with the gravid uteri (Cruz and Selwood, 1997). In contrast, stromal concentrations of TGFβ2, TβRI and TβRII at this time were similar between gravid and non-gravid uteri, although the endometrial stroma is significantly thinner in non-gravid uteri on day 8 (Cruz and Selwood, 1997). These observations indicate that TGFβ2 may be involved in at least some aspects of endometrial remodelling in the stripe-faced dunnart.

Observations on differences in TGFβ expression between gravid and non-gravid uterus in sheep (Doré et al., 1996) and pigs (Gupta et al., 1998) indicate embryonic down-regulation of TGFβ expression in sheep, but upregulation in pigs. No similar conclusions can be made in the stripe-faced dunnart, as differences in the quantities of TGFβ between gravid and non-gravid uteri varied depending on the tissue examined. However, high concentrations of TGFβ2 but low concentrations of TβRII in blastocysts during early primitive streak formation indicate that paracrine embryo-maternal signalling may be occurring, as blastocysts cannot respond to TGFβ2 at this stage, but uteri could. A similar situation, but reversed, also occurs during primitive node formation.

Quantities of uterine glandular TGFβ2 were very high on day 8 (M), and it is possible that the marked TGFβ2 present on the outer layer of the epiblast on day 8 represents a maternally secreted source. However, the paucity of TβRII in the epiblast, thus confounding any means of binding TGFβ2 by the epiblast, indicates that the TGFβ2 that was present was of embryonic origin, and if acting on the blastocysts, was acting in a paracrine manner, either on the mesoderm, endoderm or extraembryonic tissues. Alternatively, as the detecting antibody does not distinguish between active and latent TGFβ2, it may have been a latent form of TGFβ2.

TGFβ2 protein is also expressed within the epiblast-ectoderm of chick (Jakowlew et al., 1994; Sanders et al., 1994) and pig (Gupta et al., 1996) node-stage embryos. In contrast, in mice, TGFβ2 is expressed only in the embryo proper and not in pre-embryonic stages. Similarly, murine embryonal carcinoma and embryonic stem cells do not express TGFβ2 until after they differentiate (Mummery et al., 1990; Goumans et al., 1998). In contrast to mouse embryos, stripe-faced dunnart and pig embryos do not implant until after gastrulation. It is possible that in the last two species, TGFβ2 present in the epiblast interacts with one or more other growth factors secreted by the uterus, influencing epiblast development. The mouse may obtain similar signals/factors directly, via the maternal circulation. Alternatively, as the pig TGFβ2 was detected using the same antibody as in the present study, and this antibody does not
distinguish between active and latent TGFβ2, the TGFβ2 in the epiblast of both species during gastrulation may be latent.

The distribution of TGFβ2 and its receptors in the trophectoderm of the stripe-faced dunnart blastocysts was very patchy, both spatially and temporally. This patchiness resembles the patchy appearance of multinucleated cells (L. Selwood and D. Hickford, unpublished).

TGFβ1 induces the formation of multinucleated cells from human trophoblast cells in vitro (Graham et al., 1992), and it is possible that TGFβ2 has a similar effect. From day 8 (E), TGFβ2 and TβRI quantities increased in the trophectoderm, as did TβRII from day 8 (L), coincident with blastocyst expansion before implantation (Selwood and Hickford, 1999). TGFβ expression is also coincident with conceptus elongation in sheep (Doré et al., 1995) and pigs (Gupta et al., 1996).

In the stripe-faced dunnart, studies of dissociated early bilaminar blastocysts in vitro indicate that a uterine signal may be required for the formation and proliferation of hypoblast cells (Yousef and Selwood, 1996), but negligible staining for TGFβ2 and its receptors in vivo in the hypoblast at this time (days 6.0–7.0) confutes any direct role for TGFβ2 in this process. TGFβ is not present during the initial stages of hypoblast formation in the mouse (Slager et al., 1991) or chick (Sanders et al., 1994), but it has been implicated in regulating the proliferation of visceral and parietal endoderm in the mouse (Roelen et al., 1998).

The occasional staining between the shell and the underlying cells observed for both receptors may have been an artefact, as the receptors are transmembrane proteins and as such would not be expected to be present within the ECM. However, secreted TGFβ binds to the ECM (Koli et al., 2001), so it is possible that the TGFβ2 staining was real. In the horse conceptus, insulin-like growth factor and its binding protein are present within the ECM of the conceptus capsule (Herrler et al., 2000). The presence of TGFβ2 within the embryo coats may play a role in modifying embryo-maternal signals.

This report describes for the first time the spatial and temporal distribution of a growth factor and its receptors in uteri and conceptuses of a marsupial species during the peri-gastrulation period. Further analysis when more conceptuses become available could include localization of TβRIII, to which no antibodies were commercially available when this study was performed, and determina-

Fig. 4. Examples of staining for transforming growth factor β2 (TGFβ2) and its receptors, TβRI and TβRII, in blastocysts of the stripe-faced dunnart (Sminthopsis macroura). (a) TβRI staining in hypoblast cells (arrowhead) underlying the epiblast of a day 7.0 blastocyst. The shell (arrow) has detached from the surface of the epiblast. (b) TGFβ2 staining (arrowhead) in the apical membrane of epiblast cells from a day 8.0 node stage (which included regressing node stages) blastocyst. (c) Staining for TβRII in all three cell lineages from a day 8.0 node stage blastocyst. (d) An adjacent section to (c), stained for TGFβ2. Again, staining is present in all three cell lineages. epi: epiblast; hypo: hypoblast; meso: mesoderm; s: shell. Scale bar represents 50 μm.
tion of the location of phosphorylated Smad2 and Smad3, which would provide a more accurate indication of when active TGFβ2 signalling is occurring.

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