Localization of transforming growth factor β 2 (TGF β 2) and its receptors, T β RI and T β RII, 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 immuno-histochemically for transforming growth factor β 2 (TGF β 2) and its receptors, T β RI and T β RII, to determine possible roles for TGF β 2 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 TGF β 2 quantities between gravid and non-gravid uteri reflect differences in uterine morphology, indicating a role for TGF β 2 in endometrial

remodelling. In blastocysts, large quantities of all three proteins in the trophoctoderm during the node stage coincide with both blastocyst expansion before implantation and the appearance of multinucleated cells, indicating that TGF β 2 plays a role in conceptus elongation and trophoctoderm differentiation. In contrast, lack of T β RII in blastocysts during hypoblast formation and proliferation negates any role for TGF β 2 in these processes, as both receptors are required for a response to TGF β 2. High concentrations of TGF β 2 but low concentrations of T β RII in blastocysts during early primitive streak formation indicate that paracrine embryo–maternal signalling may be occurring, as blastocysts cannot respond to TGF β 2 at this stage, but uteri could. A similar situation, but reversed, also occurs during primitive node formation.

Introduction

The transforming growth factor β (TGF β) superfamily is one of the largest groups of conserved signalling molecules involved in the control of embryonic development. Mammalian members of the immediate TGF β family – TGF β 1, β 2 and β 3 – 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, TGF β is usually secreted from cells and stored in the ECM in a latent form, unable to bind to its receptors. When required, TGF β 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). TGF β s signal by binding to two types of transmembrane serine/threonine kinase receptor: T β RI and T β RII. After binding TGF β , T β RII phosphorylates T β RI, which subsequently phosphorylates Smad proteins, resulting in translocation of the signal to the nucleus (for a review, see Miyazono *et al.*, 2001). TGF β 2 signalling requires a third

receptor, T β RIII (beta-glycan), which acts to increase the normally low affinity of T β RII for TGF β 2 (Moustakas *et al.*, 1993).

The TGF β isoforms have numerous roles in mammalian embryonic development, sometimes overlapping and sometimes distinct. Both TGF β 1 and β 2 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, TGF β 1 and β 2, respectively, are associated with mesenchymal and epithelial differentiation *per se* (Heine *et al.*, 1987; Millan *et al.*, 1991). The numerous effects of TGF β 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 TGF β 1, TGF β 2, T β RI and T β RIII transcripts in various tissues just before and during gastrulation, but do not express T β RII until early somitogenesis (Roelen *et al.*, 1994); chimaeric embryos that express ectopic T β RII can

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initiate, but not successfully complete, gastrulation, due to TGF β -induced growth inhibition (Zwijnen *et al.*, 1999). Furthermore, mice deficient in functional TGF β 1 or T β RII 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 TGF β 2 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, TGF β s and their receptors are expressed widely in pig embryos during gastrulation. TGF β 1, TGF β 2, T β RI and T β RII 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). TGF β 1/ β 3 and TGF β 2 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 TGF β and its receptors in the peri-gastrula period in a marsupial species. As conceptus material was in short supply, the distribution of TGF β 2, but not of TGF β 1, 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 an extremely short (11 day) gestation period and also 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 TGF β 2 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 TGF β 2 in marsupial embryonic development. At the time this study was performed, antibodies to T β RIII were not commercially available, so only the distributions of T β RI and T β RII 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 TGF β 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 TGF β 2 antibody (cat. no. sc-90) was raised against a peptide corresponding to amino acid residues 352–377 within the carboxy terminal region of TGF β 2, and recognizes both mature and precursor forms of TGF β 2. Both receptor antibodies were raised against epitopes within their kinase domains: the T β RI antibody (cat. no. sc-398) recognizes amino acids 158–179 of T β RI and the T β RII antibody (cat. no. sc-400) recognizes amino acids 246–266 of T β RII. Each antibody recognizes the appropriate epitope in human, rat and murine tissues.

The TGF β 2 antibody was used at 1.3 μ g 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% (w/v) BSA (Sigma Chemical Co.). The two receptor antibodies were each used at 4 μ g 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 β 2 (TGF β 2) and its receptors, T β R1 and T β R2, in regions of uterine sections from the stripe-faced dunnart (*Sminthopsis macroura*)

Grade	Luminal epithelium	Glandular epithelium ^a	Stroma/myometrium
1	Few scattered +ve cells	< 5% of glands +ve	< 5% of area +ve
2	Small groups of +ve cells	5–25% of glands +ve	5–25% of area +ve
3	Extensive areas +ve	26–50% of glands +ve	26–50% of area +ve
4	Most areas +ve	> 50% of glands +ve	> 50% of glands +ve

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

the diluent. Two types of negative control were used for immunohistochemical staining of uteri. Rabbit IgG (Sigma Chemical Co.) diluted to 4 μ g ml⁻¹ in TBS–0.1% (w/v) BSA and 1% (v/v) normal goat serum was used in place of the primary antibody, or the antibodies were pre-absorbed before use by control peptides (Santa Cruz Biotechnology Inc.). These control peptides are purified forms of the proteins against which the antibodies were raised. Each antibody in its appropriate diluent was incubated with a tenfold excess (by weight) of control peptide for 2 h at room temperature before use. Owing to a shortage of material, for blastocyst sections only the IgG control was used.

Immunohistochemistry

Sections (5 μ m 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 para-formaldehyde-fixed dunnart pouch young as a positive control. Blastocysts were cut into serial sections of 6 μ m 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 l⁻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 Maxicollour 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 ($\times 40$) (Table 1). Results are expressed as mean $\pm$ 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 $\beta 2$ (TGF $\beta 2$) and its receptors, T β RI and T β RII, in various regions of the uterus of the stripe-faced dunnart (*Sminthopsis macroura*) during gastrulation. (a) T β RII 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 T β RI (arrowheads), characteristic of glandular epithelium close to the myometrium. (c) Globular (type 2) T β RII 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 I) staining is also evident. (d) Granular

blastocyst) was recorded. Staining was described as either pale or marked, depending on the prominence of the reaction. By allocating a grade of zero for no staining, one (+) for pale staining and two (++) for dark staining, a grade for each stage of development (an average was taken within stages to account for the different numbers of both blastocysts and tissues present between stages) was calculated. The developmental terminology used in this paper follows that described by Johnson and Selwood (1996). Cells and tissues arising before gastrulation are prefixed or suffixed with the term 'blast', whereas those arising during gastrulation are suffixed with 'derm'.

Results

Immunohistochemical staining for TGF β 2, T β RI and T β RII 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 of the three antibodies showed distinct staining patterns (in both positive controls and test tissues), and staining for 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 T β RI reflected that of TGF β 2 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 TGF β 2, T β RI and T β RII 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 TGF β 2, T β RI and T β RII 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 TGF β 2, T β RI and T β RII 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 TGF β 2, T β RI and T β RII in blastocysts is described (Table 2; Figs 3 and 4). The intensity of T β RII staining in uteri was often similar to that of TGF β 2 and T β RI, but was usually much lower in blastocysts.

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

Glandular staining in non-gravid uteri was more pronounced for T β RI and T β RII on day 6, and less on day 8 for TGF β 2 and T β RI, compared with the gravid uteri at corresponding stages (Fig. 2). At both stages, the types of staining pattern present were similar between gravid and

(type 3) staining for T β RII, with examples of both lined (arrow) and filled (arrowhead) lumina. (e) TGF β 2 staining in the endometrial stroma, showing both a fibrillar pattern and a positive macrophage-type cell (arrow). (f) T β RI-positive cells within the myometrium. L: lumen (of glands). Scale bar represents 50 μ m.

Fig. 2. Comparison of the quantity of immunohistochemical staining for transforming growth factor $\beta 2$ (TGF $\beta 2$) and its receptors, T β RI and T β RII, in various regions of the uterus, and of staining patterns that occurred within the glandular epithelia of the stripe-faced dunnart (*Sminthopsis macroura*) from day 6.0 to day 8.5 of gestation. (a) Summary of the staining distribution patterns observed for each protein within the glandular epithelia. (■): Vesicular; (▨): globular; (□): granular. The three graphs below show mean quantitative values for staining TGF $\beta 2$ (■), type 1 receptor (T β RI) (□) and type 2 receptor (T β RII) (▨) within the (b) glandular and (c) luminal epithelia, and (d) endometrial stroma. Grading for each region of the gravid uterus ($n = 4$ or 5) was performed according to the number of positive glands, regardless of the proportion of positive cells within the glands, using a relative scale (1 = infrequent, 2 = more extensive, 3 = widespread). Non-gravid (NG) uterine samples on day 6 ($n = 2$), day 7 ($n = 2$) and day 8 ($n = 3$) are also included. Both diagrams are aligned with each other with respect to the day of gestation. (E): early primitive streak (early stages of streak formation) in day 8 blastocysts; (M): mid-primitive streak (streak had progressed but not to the node stage) in day 8 blastocysts; (L): node stage (which included regressing node stages) in day 8 blastocysts.

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 TGF $\beta 2$ and T β RI in the apical membranes of the epiblast of day 8 (L) node-stage

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

Stage (day)	n	Type of cell	TGFβ2	TβRI	TβRII
6.0	4	Pluriblast	0	0	0
		Trophoblast	+ (3)	+ (2)	0
6.5	4	Epiblast	+ (3)	+ (3)	0
		Trophoblast	+ (3)	+ (3)	0
		Hypoblast	0	0	0
7.0	3	Epiblast	+ (2)	+ (2)	0
		Hypoblast (under epiblast)	0	++ (2)	0
		Trophoblast	+	+	0
		Hypoblast (under trophoblast)	0	+ (2)	0
7.5	4	Epiblast	+ (2)	+ (3)	0
		Hypoblast (under epiblast)	++	++	+ (2)
		Trophoblast	0	0	0
		Hypoblast (under trophoblast)	++ (2)	+ (3)	+ (2)
8.0 (E)	3	Epiblast	++	+ (2)	0
		Hypoblast (under epiblast)	++ (2)	+ (2)	+ (2)
		Trophectoderm	++	++	+ (2)
		Hypoblast (under trophoblast)	++	++	+ (2)
8.0 (L)	4	Ectoderm	++	++	+ (2)
		Mesoderm	+ (2)	+ (2)	++ (3)
		Endoderm	+ (3)	++ (3)	++ (2)
		Trophectoderm	++	++	++
		Hypoblast	+ (3)	++	++ (3)
8.5	2	Ectoderm	++	++	0
		Mesoderm	++	++	0
		Endoderm	++	++	++
		Neuroectoderm	++	++	+ (1)
		Trophectoderm	++	++	++
		Hypoblast	++	++	++

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).

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

Fig. 3. Mean quantitative values for immunohistochemical staining for transforming growth factor $\beta 2$ (TGF $\beta 2$) (■) and its receptors, T β RI (□) and T β RII (▨), in cell lineages of blastocysts from the stripe-faced dunnart (*Sminthopsis macroura*) from day 6.0 to day 8.5 of gestation. Grading for each region (a) epiblast, (b) trophoblast–trophectoderm and (c) hypoblast of the gravid uterus ($n = 4$ or 5) was performed according to the number of positive glands, regardless of the proportion of positive cells within the glands, using a relative scale (1 = infrequent, 2 = more extensive, 3 = widespread). No blastocysts from day 8 mid-primitive streak blastocysts were stained. (E): early primitive streak (early stages of streak formation) in day 8 blastocysts; (M): mid-primitive streak (streak had progressed but not to the node stage) in day 8 blastocysts; (L): node stage (which included regressing node stages) in day 8 blastocysts.

in the stripe-faced dunnart uterus have not been described for any mammalian species and appeared to represent either production of protein by the glands or uptake of protein from the stromal ECM (type 1), passage of proteins out of the glands (type 2) and recent protein secretion (type 3). Further support for this idea was the observation that type 2 staining was usually followed by type 3 staining during the next half-day of development.

Regulation of endometrial TGF β expression differs among species, indicating different roles for TGF β within this tissue. In ewes, TGF β may control remodelling of the endometrium between cycles and between pregnancies (Doré *et al.*, 1996). In humans, TGF β 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 TGF $\beta 2$, T β RI and T β RII 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 TGF $\beta 2$, T β RI and T β RII 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 TGF $\beta 2$, T β RI and T β RII 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 TGF $\beta 2$ may be involved in at least some aspects of endometrial remodelling in the stripe-faced dunnart.

Observations on differences in TGF β expression between gravid and non-gravid uteri in sheep (Doré *et al.*, 1996) and pigs (Gupta *et al.*, 1998) indicate embryonic down-regulation of TGF β expression in sheep, but upregulation in pigs. No similar conclusions can be made in the stripe-faced dunnart, as differences in the quantities of TGF β between gravid and non-gravid uteri varied depending on the tissue examined. However, high concentrations of TGF $\beta 2$ but low concentrations of T β RII in blastocysts during early primitive streak formation indicate that paracrine embryo–maternal signalling may be occurring, as blastocysts cannot respond to TGF $\beta 2$ at this stage, but uteri could. A similar situation, but reversed, also occurs during primitive node formation.

Quantities of uterine glandular TGF $\beta 2$ were very high on day 8 (M), and it is possible that the marked TGF $\beta 2$ present on the outer layer of the epiblast on day 8 represents a maternally secreted source. However, the paucity of T β RII in the epiblast, thus confounding any means of binding TGF $\beta 2$ by the epiblast, indicates that the TGF $\beta 2$ 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 TGF $\beta 2$, it may have been a latent form of TGF $\beta 2$.

TGF $\beta 2$ 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, TGF $\beta 2$ is expressed only in the embryo proper and not in pre-embryonic stages. Similarly, murine embryonal carcinoma and embryonic stem cells do not express TGF $\beta 2$ 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, TGF $\beta 2$ 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 TGF $\beta 2$ was detected using the same antibody as in the present study, and this antibody does not

Fig. 4. Examples of staining for transforming growth factor β 2 (TGF β 2) and its receptors, T β RI and T β RII, in blastocysts of the stripe-faced dunnart (*Sminthopsis macroura*). (a) T β RI 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) TGF β 2 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 T β RII in all three cell lineages from a day 8.0 node stage blastocyst. (d) An adjacent section to (c), stained for TGF β 2. Again, staining is present in all three cell lineages. epi: epiblast; hypo: hypoblast; meso: mesoderm; s: shell. Scale bar represents 50 μ m.

distinguish between active and latent TGF β 2, the TGF β 2 in the epiblast of both species during gastrulation may be latent.

The distribution of TGF β 2 and its receptors in the trophoblast–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). TGF β 1 induces the formation of multinucleated cells from human trophoblast cells *in vitro* (Graham *et al.*, 1992), and it is possible that TGF β 2 has a similar effect. From day 8 (E), TGF β 2 and T β RI quantities increased in the trophectoderm, as did T β RII from day 8 (L), coincident with blastocyst expansion before implantation (Selwood and Hickford, 1999). TGF β 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 TGF β 2 and its receptors *in vivo* in the hypoblast at this time (days 6.0–7.0) confutes any direct role for

TGF β 2 in this process. TGF β 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 TGF β binds to the ECM (Koli *et al.*, 2001), so it is possible that the TGF β 2 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 TGF β 2 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 T β RIII, to which no antibodies were commercially available when this study was performed, and determina-

tion of the location of phosphorylated Smad2 and Smad3, which would provide a more accurate indication of when active TGF β 2 signalling is occurring.

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