REPRODUCTION
REVIEW

Focus on TGF-β Signalling

TGF-β superfamily expression and actions in the endometrium and placenta

Rebecca L Jones[1,2], Chelsea Stoikos[1], Jock K Findlay[1] and Lois A Salamonsen[1]

[1]Prince Henry's Institute of Medical Research, PO Box 5152, Clayton, VIC 3166, Australia and [2]Academic Unit of Child Health, Division of Human Development, University of Manchester, St Mary's Hospital Research Floor, Hathersage Road, Manchester, M13 0JH, UK

Correspondence should be addressed to R L Jones; Email: rebecca.lee.jones@manchester.ac.uk

Abstract

Transforming growth factor β (TGFβ) superfamily members are closely associated with tissue remodelling events and reproductive processes. This review summarises the current state of knowledge regarding the expression and actions of TGFβ superfamily members in the uterus, during the menstrual cycle and establishment of pregnancy. TGFβs and activin β subunits are abundantly expressed in the endometrium, where roles in preparation events for implantation have been delineated, particularly in promoting decidualisation of endometrial stroma. These growth factors are also expressed by epithelial glands and secreted into uterine fluid, where interactions with preimplantation embryos are anticipated. Knockout models and embryo culture experiments implicate activins, TGFβs, nodal and bone morphogenetic proteins (BMPs) in promoting pre- and post-implantation embryo development. TGFβ superfamily members may therefore be important in the maternal support of embryo development. Following implantation, invasion of the decidua by fetal trophoblasts is tightly modulated. Activin promotes, whilst TGFβ and macrophage inhibitory cytokine-1 (MIC-1) inhibit, trophoblast migration in vitro, suggesting the relative balance of TGFβ superfamily members participate in modulating the extent of decidual invasion. Activins and TGFβs have similar opposing actions in regulating placental hormone production. Inhibins and activins are produced by the placenta throughout pregnancy, and have explored as a potential markers in maternal serum for pregnancy and placental pathologies, including miscarriage, Down's syndrome and pre-eclampsia. Finally, additional roles in immunomodulation at the materno-fetal interface, and in endometrial inflammatory events associated with menstruation and repair, are discussed.

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Introduction

Reproductive organs are unusual in their recurrent proliferative and remodelling activity in adult life. In particular, the human endometrium undergoes remarkable cycles of remodelling, involving proliferation, differentiation, breakdown and repair, every 28 days. This cyclical activity is regulated by the ovarian steroids oestrogen and progesterone, but at a paracrine level by a myriad of growth factors, cytokines and proteases. Unsurprisingly, transforming growth factor (TGF) β superfamily members are abundantly and dynamically expressed in the endometrium, and appear, through their actions associated with cell proliferation, differentiation, apoptosis and tissue remodelling, to have instrumental roles in modulating cellular events involved in menstruation, proliferation, decidualisation and the establishment of pregnancy. Further, the expression of many TGFβ superfamily members have been described in the placenta, another organ undergoing rapid development and remodelling. This review will focus on the expression, production and roles of TGFβ and activins/inhibins in the human endometrium and placenta, and introduce the emerging roles of these and other family members in modulating the events during the preparation for, and establishment of, pregnancy.

Overview of endometrial function

The endometrium is a highly specialised tissue, providing an optimal environment to enable, yet regulate, the implantation of the semi-allogeneic

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embryo. Following oestrogen-induced proliferation, progesterone induces differentiative events within all compartments of the endometrium, creating an environment receptive for blastocyst attachment and invasion (Salamonsen & Jones 2003). Endometrial epithelial glands undergo morphological and functional differentiation, and commence active secretion of a complex nutritive and growth factor-rich media contributing to uterine fluid. This provides support to the pre-implantation embryo, promoting growth and development before endometrial attachment. In some primates, rodents and bats, stromal fibroblasts surrounding the developing spiral arterioles begin to differentiate, or decidualise, eventually producing the decidua of pregnancy. Cells enlarge, become more rounded, and deposit a decidual-specific extracellular matrix (ECM), rich in laminin, collagen IV and fibronectin; they also start to produce a wide range of cytokines, growth factors and immunomodulatory agents that are undoubtedly involved in maternal regulation of trophoblast invasion. In addition, the decidua possesses a unique immune environment, characterised by the presence of large numbers of uterine-specific natural killer cells (uNK) and smaller population of macrophages (Bulmer *et al.* 1988, King *et al.* 1998). These appear to be recruited and acquire their uterine-specific phenotype by chemokines and cytokines (particularly interleukin-15) produced by the decidua (Verma *et al.* 2000, Croy *et al.* 2003). The specific exclusion of inflammatory and cytotoxic lymphocytes, together with the defined interactions between uNKs and foetal trophoblasts (via human leukocyte antigen; HLA-G), combine to create an environment permissive to embryo implantation (King *et al.* 2000).

In the absence of pregnancy in women and old world monkeys, the endometrium functionalis is shed during menstruation. The occurrence of menstruation is thought to be an evolutionary adaptation related to the highly invasive nature of trophoblast invasion in these species. Significant endometrial preparation (decidualisation, spiral arteriole development, etc.) occurs in anticipation of pregnancy, producing a terminally differentiated endometrium that must be shed ahead of a subsequent new ovulatory cycle. Progesterone withdrawal, due to regression of the corpus luteum, lifts the repressive anti-inflammatory effect of this pregnancy-related steroid hormone, leading to a cascade of events resulting in inflammatory cell influx, production of inflammatory cytokines, prostaglandins, vasomodulatory agents and proteases, and culminating in endometrial breakdown. These events are very focal, occurring simultaneously with endometrial repair, reinforcing the involvement of infiltrating leukocytes and their locally secreted factors in the initiation of endometrial breakdown. Endometrial repair occurs very rapidly, with re-epithelisation complete within 48 h of the initiation of menstrual bleeding (Ferenczy 1976). Importantly, endometrial repair occurs without scarring, similar to foetal repair *in utero* (Samuels & Tan 1999); however, the mechanisms are poorly understood.

Regulation of endometrial function

Many members of the TGFβ superfamily are expressed by human endometrium at different stages of the menstrual cycle, consistent with their general involvement in rapidly proliferating or remodelling tissues. The three TGFβ isoforms are differentially expressed in endometrium, with TGFβ2 predominantly localising to stroma whilst TGFβ1 and TGFβ3 are present in both epithelial and stromal cells (Gold *et al.* 1994, Godkin & Dore 1998). TGFβ1 has been shown to be secreted apically from endometrial glands and is present in uterine fluid (Polli *et al.* 1996). Cyclical changes in expression level are not evident for TGFβ1 and TGFβ2, whilst maximal glandular production of TGFβ3 occurs in the late secretory phase. Activins are also highly abundant in the endometrium. Activin β subunits (βA and βB) are primarily localised to endometrial glands in the non-pregnant endometrium (Leung *et al.* 1998, Otani *et al.* 1998, Petraglia *et al.* 1998, Jones *et al.* 2000), with maximal levels seen in the secretory phase. Expression of inhibin α subunit has been described, again in glandular epithelium, but to a lesser degree than activin β subunits, indicating that activin dimers are preferentially produced. Indeed, isolated epithelial cells in culture secrete activin A at 1000-fold higher concentrations than either inhibin A or B; similarly, activin A is secreted from epithelial glands *in vivo* into uterine fluid (Petraglia *et al.* 1998).

The production and secretion of TGFβ and activins by epithelial glands in the secretory phase suggest roles in either the preparation of the endometrium for implantation, or direct actions on the pre-implantation embryo, facilitating development or differentiation for implantation. In support of the first theory, TGFβ receptors are expressed by oviductal/Fallopian tube and uterine epithelial cells (Zhao *et al.* 1994, Chow *et al.* 2001). Recently, it has been demonstrated that both TGFβ1 and activin A enhance the production of the pro-implantatory cytokine, leukaemia inhibitory factor from endometrial epithelial cells (Perrier d'Hauterive *et al.* 2005) (Fig. 1). Furthermore, retroviral overexpression of the TGFβ antagonist, lefty, in the mouse uterus in the peri-implantation phase reduces the number of implantation sites, possibly by negatively influencing the endometrial environment (Tang *et al.* 2005*a*). This is reinforced by its abnormally elevated expression in human endometrium during the receptive phase in women experiencing infertility (Tang *et al.* 2005*a*).

Regulation of decidualisation

Endometrial decidualisation induces the production of a wide array of growth factors and cytokines, which act in

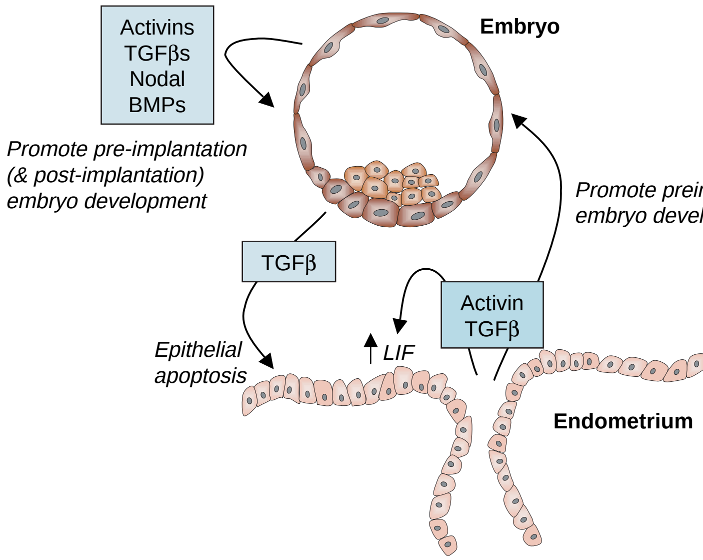

Figure 1 Summary of the proposed actions of transforming growth factor (TGF) β superfamily members during implantation. Activins and TGFβs are produced and secreted by the epithelial lining of the Fallopian tube and uterus. Whilst autocrine actions of activins and TGFβs on the uterus have been described during the preparation for implantation (such as the stimulation of pro-implantatory leukaemia inhibitor factor production), receptors for these factors are also expressed by embryos at varying stages of development. Indeed, *in vitro* studies have demonstrated that activin A and TGFβ promote pre-implantation embryo development suggesting these factors are involved in maternal–foetal communication during the establishment of pregnancy. Continued functions can be extrapolated for these factors in post-implantation development from the spatial and temporal expression patterns of the ligands, receptors and binding proteins. TGFβ secreted from the blastocyst has been proposed to induce apoptosis of endometrial epithelial cells during implantation.

concert to mediate the decidualisation reaction, and/or to create an extracellular and immunological environment conducive to trophoblast invasion. Activin βA and βB subunits are dramatically upregulated during decidualisation (Otani *et al.* 1998, Jones *et al.* 2000) both in *in vivo* and *in vitro* models of decidualisation. Similarly, TGFβ isoforms are present to varying extents in the decidua (particularly TGFβ2) (Simpson *et al.* 2002), and both TGFβ and activins are highly expressed in the extensively decidualised endometrium induced by intrauterine delivery of progestin (Jones *et al.* 2000, Roopa *et al.* 2003). Furthermore, activin A promotes decidualisation *in vitro*, while neutralisation of activin action by treatment with follistatin significantly retards the decidual response (Jones *et al.* 2002*a*, Tierney & Giudice 2004) (Fig. 2). This appears to be due, at least in part, to the stimulation of matrix metalloproteinases (MMPs) by activin in endometrial cells (Jones *et al.* 2006). In our *in vitro* model of decidualisation, we show that MMP-2 secretion is enhanced when decidualisation is accelerated by treatment with activin, whilst its production is ablated by blockade of activin bioactivity by inhibin A, coincident with reduced decidualisation. As MMP activity is critical for decidualisation in the rat and primate, for endometrial remodelling and growth factor processing (Alexander *et al.* 1996, Rechtman *et al.* 1999, Strakova *et al.* 2003), this provides a probable downstream mechanism for activin during decidualisation. Whether TGFβs influence decidualisation is unclear, as studies in the literature report contrasting effects on prolactin production (an established marker of decidualisation) by endometrial stroma (Kubota *et al.* 1997, Kim *et al.* 2006). Other TGFβ superfamily members are likely to be expressed by the decidua, and involved in decidualisation. For example, macrophage inhibitory cytokine (MIC)-1 is upregulated in decidual cells and facilitates decidualisation *in vitro* (Marjono *et al.* 2003). However, its mode of action differs from that of activin, as in this model MIC-1 inhibits activation of MMP-2 and -9 (Fig. 2).

In the rodent uterus, activin is similarly upregulated with the onset of decidualisation, yet its expression is dynamic, and follows a characteristic wave-like pattern of up- and downregulation preceding the wave of decidualisation (Gu *et al.* 1995, Jones and colleagues unpublished observations). With blastocyst attachment, activin βA expression becomes polarised to the primary decidual zone. In the following days, activin expression switches from anti-mesometrial to mesometrial zones with the initiation of secondary decidualisation, but by mid-pregnancy, expression is limited to the decidua basalis. These expression patterns suggest a role for activins in preparation of the endometrium for decidualisation, potentially through regulation of MMP expression, which follow a similar pattern (Alexander *et al.* 1996), or through stimulation of decidual-specific ECM components (e.g. fibronectin) (Caniggia *et al.* 1997*a*). TGFβ1 and TGFβ2, but not TGFβ3, are predominantly expressed by stroma immediately underlying the luminal and glandular epithelium in the rat, and

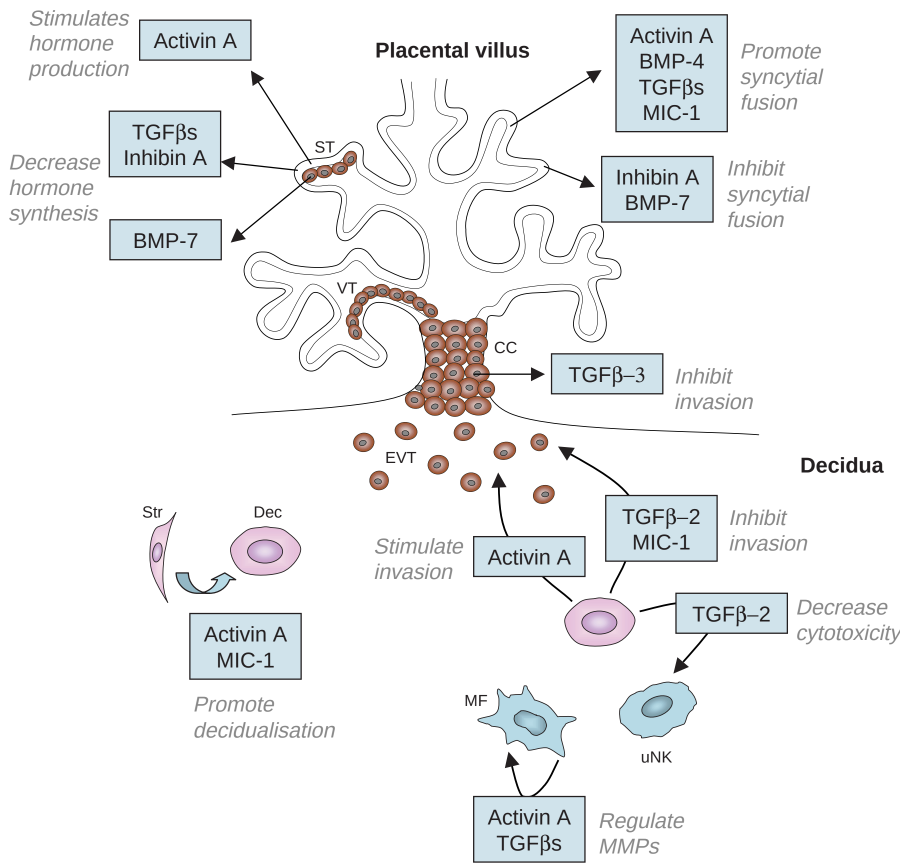

Figure 2 Summary of the proposed actions of TGFβ superfamily members at the maternal–foetal interface during establishment of pregnancy. A number of TGFβ superfamily ligands are expressed by the placental villous, by the either syncytiotrophoblast (ST) or inner villous cytotrophoblast layer (VT). These have been shown to modulate hormone production (oestrogen, progesterone, placental lactogen), and/or syncytial fusion (as assessed by human chorionic gonadotrophin production). Activin A and macrophage inhibitory cytokine (MIC-1) are also expressed by the maternal decidua, and promote the differentiation of stromal cells (Str) into decidualised cells (Dec). The two growth factors, together with transforming growth factor (TGF) β2, play important roles in the decidual regulation of extravillous cytotrophoblast (EVT) invasion, whilst differentiating cytotrophoblast cells in the cell column (CC) also produce TGFβ3 which is inhibitory to invasion. Decidual TGFβ is proposed to act on uterine natural killer cells to downregulate their cytotoxicity producing the uterine-specific phenotype. Tissue macrophages (MΦ) within the implantation site, and in the non-pregnant endometrium, produce a wide range of cytokines, including TGFβ and activins, which have opposing actions to tightly regulate matrix metalloproteinase expression and activity.

are upregulated following implantation at day 5–6 of pregnancy (Shooner *et al.* 2005). Later in pregnancy, both TGFβ (all isoforms) and activin A have been attributed with roles in apoptotic regression of the decidua basalis in the rat, both through their overlapping localisation with apoptotic cells and their pro-apoptotic actions on decidual cells *in vitro* (Moulton 1994, Tessier *et al.* 2003, Shooner *et al.* 2005). Undoubtedly, these actions are tightly regulated in both rodent and human; antagonists of TGFβ and activins, lefty A and follistatin respectively, are expressed in the decidua, generally fluctuating in conjunction with their ‘ligands’ (Jones *et al.* 2002*b*, Tessier *et al.* 2003, Tang *et al.* 2005*b*). Expression and activation of Smads 2 and 4 also correlate with activin expression during the establishment of pregnancy, and later with TGF and activin during decidual regression (Liu *et al.* 2004).

Bone morphogenetic proteins (BMPs) have been detected in the murine uterus during decidualisation and the establishment of pregnancy. In particular, the spatial and temporal expressions of BMP-2 tightly mimics the spread of the decidualisation reaction (Ying & Zhao 2000, Paria *et al.* 2001). Its mRNA is immediately upregulated in the anti-mesometrial stroma underlying the implantating blastocyst, where it appears to be an early event during primary decidualisation. Indeed, the application of embryonic factor heparin binding-epidermal growth factor (EGF) to the uterine epithelium, via coated glass beads, stimulates the expression of stromal BMP-2 (Paria *et al.* 2001). Expression shifts to the secondary decidual zone with the progression of normal pregnancy. Other BMPs are present, but exhibit distinct expression patterns: BMP-7 is expressed throughout the stroma in advance of implantation, and subsequently becomes highly localised to the subepithelial stroma in the mesometrial zone; BMP-8a is upregulated in the anti-mesometrial zone after primary decidual regression; and BMP-4 is predominantly associated with decidual vasculature (Ying & Zhao 2000). In addition, a number of BMP-binding proteins (noggin, twisted gastrulation and dan/dante) and co-receptors (Dragon) have been detected in the implantation site (Paria *et al.* 2001), further supporting important roles for the BMP family in modulating endometrial function. Although the functions of the BMPs in the decidua have not been established, two separate studies indicate roles for BMP-2 and -5 in regulating embryo spacing during implantation (Pfendler *et al.* 2000, Paria *et al.* 2001). To date, there are no reports describing the expression of BMPs by human uterine cells.

Regulation of early embryo development

Pre-implantation embryos express receptors for activin (ActRs) and TGFβ (TGFβR) and hence would be responsive to growth factors secreted by the endometrium. ActRs are expressed by human pre-implantation embryos and are upregulated at the blastocyst stage (He *et al.* 1999). In the mouse, the differential expression of type II receptors has been identified, with ActRIIA limited to the trophectoderm (TE), and ActRIIB present in both inner cell mass (ICM) and TE cells (Debieve *et al.* 2006). Maternally derived TGFβRI and TGFβRII mRNA transcripts are detectable in oocytes and one cell embryos (Osterlund & Fried 2000). With the activation of embryonic genome, TGFβRI is upregulated, whilst TGFβRII mRNA is only detectable at the blastocyst stage (Roelen *et al.* 1998, Chow *et al.* 2001), where the protein becomes limited to the TE. Interestingly, oocyte-derived TGFβRII appears to be critical for embryonic development, as interfering with signalling through this receptor during *in vitro* embryo maturation leads to a block in development at the two-cell stage (Roelen *et al.* 1998). Both mRNA and protein for Smads 2 and 3 are present in human embryos throughout pre-implantation development (Osterlund & Fried 2000), demonstrating that all elements of the signalling pathways for activin and TGFβ are present. Co-expression of inhibitory Smad 7 indicates the potential for tight regulation of activin/TGFβ action during cleavage and blastocyst development (Zwijsen *et al.* 2000).

Exogenous TGFβ facilitates embryonic development *in vitro*, promoting blastocyst proliferation and development and increasing blastocyst cell number (Paria & Dey 1990, Lim *et al.* 1993, Nowak *et al.* 1999) (Fig. 1). Activin A has similar actions: treatment of cultured rodent or bovine embryos with recombinant activin A promotes embryo development, by increasing blastocyst cell number, reducing time taken to reach blastocyst stage and improving hatching rates (Orimo *et al.* 1996, Yoshioka *et al.* 1998, Mtango *et al.* 2003). In addition, activin A treatment of rat blastocysts *in vitro* induces apoptosis, suggesting a role for activin in physiological apoptosis of blastomeres during embryo development (Debieve *et al.* 2006). Pre-implantation embryos also express activin subunits and TGFβ: in both mouse and human embryos activin βA and βB are maximally expressed at the blastocyst stage and become localised to the ICM (Albano *et al.* 1993, Lu *et al.* 1993, He *et al.* 1999), whilst TGFβ expression appears to peak between the eight cell stage and morula in both ICM and TE, and thereafter declines (Chow *et al.* 2001). Interestingly, TGFβ secreted by the blastocyst induces apoptosis of uterine epithelial cells, suggesting that it plays an important role in embryonic signalling to the endometrium during implantation (Kamijo *et al.* 1998). Many TGFβ superfamily members (e.g. activins, TGFβs, nodal and BMPs), their receptors and Smads are expressed in later stage embryos, and have been attributed with modulatory roles during gastrulation and organogenesis (Iannaccone *et al.* 1992, Winnier *et al.* 1995, Zhang & Bradley 1996, Gu *et al.* 1998, Song *et al.* 1999, Zwijsen *et al.* 1999, Zwijsen *et al.* 2000). The phenotypes associated with gene knockout of TGFβ superfamily member ligands, receptors and Smads (including embryonic and perinatal lethality) are summarized in Table 1. The detailed description of embryonic-TGFβ superfamily members expression patterns and actions are outside the realms of this review.

Placental development and function

Placental development is equally dynamic. TE differentiation into invasive syncytial trophoblast is initiated upon attachment to the endometrial epithelium, and this trophoblast forms a protective layer surrounding the blastocyst. The inner lining of cytotrophoblast cells forms the trophoblast shell, from which columns of cytotrophoblast project, forming anchoring columns upon contact with the decidua. During the first few weeks of pregnancy, the villous structure of the placenta develops; villous projections lined with syncytiotrophoblast (ST) generated by fusion of villous cytotrophoblast cells, enclose foetal capillaries, and from the second trimester of pregnancy become the major route of gaseous and nutrient exchange between foetus and mother (Hamilton & Boyd 1960). The ST is also the site of hormone synthesis, including progesterone and human chorionic gonadotrophin (hCG), critical for maintaining pregnancy. Maximal decidual invasion by trophoblast cells occurs between 5 and 12 weeks of pregnancy. Extravillous cytotrophoblast (EVT) cells differentiate from cytotrophoblast cells in the anchoring columns; contact with decidual-derived factors stimulates their differentiation and acquisition of invasive potential. EVTs invade the decidual stroma as interstitial (iEVTs), and

Table 1 Overview of mouse knockout phenotypes for transforming growth factor (TGF) β superfamily ligands, receptors and Smads. Genes have been classified according to the broad phenotype, with a brief description for genes of interest (in bold). Modified from Chang *et al.* (2002).

Outcome of gene knockout	Gene ablated	References
Reproductive abnormalities		
Ligands	**Inhibin α:** elevated FSH and activin levels, gonadal stromal tumours, cachexia and death prior to reproductive maturity	Matzuk *et al.* (1992)
	Activin βB: elevated activin βA expression, increased gestation length, perinatal lethality of offspring	Vassalli *et al.* (1994)
	BMP-8B: male infertility due to germ cell depletion; defects in PGC and allantois development	Ying *et al.* (2000)
	BMP-15: female subfertility; impaired ovulation, cumulus cell expansion and fertilisation	Yan *et al.* (2001)
	GDF-9: female infertility; folliculogenesis arrested at primary follicle stage	Dong *et al.* (1996)
	MIS: females fertile but experience early recruitment and depletion of primordial follicles, males develop uteri	Behringer *et al.* (1994), Durlinger *et al.* (2001)
Receptors	**ActRII:** female infertility, thin uteri and small ovaries, normal folliculogenesis, but no ovulation	Matzuk *et al.* (1995*b*)
	ALK6: female infertility due to defects in oestrous cycle regularity, cumulus cell expansion, oestradiol biosynthesis and endometrial gland formation	Yi *et al.* (2001)
Smads	**Smad 3**: reduction in body size; reduced litter size from homozygous matings	Zhu *et al.* (1998)
Viable and fertile		
Ligands	**Activin βB knockin (BK):** βB gene knocked into βA locus, rescues neonatal lethality of βA knockout, decreased fertility due to reduced preovulatory follicles. Activin βC, Activin βE, BMP-3, BMP-5, BMP-6, BMP-8A, GDF-5, GDF-7, GDF-8, GDF-10, GDF-11/BMP-11, GDF-15	Storm *et al.* (1994), McPherron *et al.* (1997, 1999), Solloway *et al.* (1998), Zhao *et al.* (1998, 1999), Solloway & Robertson (1999), Brown *et al.* (2000), Lau *et al.* (2000), Hsiao *et al.* (2000), Daluiski *et al.* (2001), Settle *et al.* (2001)
Receptors	ALK 7	(Jornvall *et al.* 2004)
Embryonic lethal		
Ligands	**BMP-2:** abnormal amniochorion development	Zhang & Bradley (1996)
	Nodal: abnormal placentation due to increased numbers of invasive giant cells	Iannaccone *et al.* (1992), Conlon *et al.* (1994)
	BMP-4, GDF-1, lefty-1, lefty-2	Winnier *et al.* (1995), Meno *et al.* (1998, 1999), and Rankin *et al.* (2000)
Receptors	ALK-1, ALK-2, ALK-3, ALK-4, ALK-5, BMPR-2, β-glycan (TGFβRIII)	Mishina *et al.* (1995), Gu *et al.* (1998, 1999), Beppu *et al.* (2000), Oh *et al.* (2000), Larsson *et al.* (2001), Stenvers *et al.* (2003)
Smads	**Smad 1:** failure of chorioallantoic fusion to produce the umbilical connection to the placenta – due to impaired allantois development and chorion overgrowth	Tremblay *et al.* (2001)
	Smad 2, Smad 4, Smad 5	Weinstein *et al.* (1998), Yang *et al.* (1998), Chang *et al.* (1999)
Perinatal lethal		
Ligands	**TGFβ1**: $>$50% die *in utero*, remainder perinatal	Shull *et al.* (1992)
	Activin βA, TGFβ2, TGFβ3, BMP-7	Dudley *et al.* (1995), Kaartinen *et al.* (1995), Matzuk *et al.* (1995*b*), Proetzel *et al.* (1995), Sanford *et al.* (1997)
Receptors	**ActRIIB**: minority survive and are fertile	Oh & Li (1997)
	TGFβRII, follistatin	Matzuk *et al.* (1995*a*), Oshima *et al.* (1996)

BMP, bone morphogenetic protein; GDF, growth differentiation factor; MIS, Müllerian inhibitory substance; R, receptor; ALK, activin-receptor like kinase.

appear to accumulate around spiral arteries. A further population target and enter maternal spiral arterioles as endovascular (vEVTs) (Zhou *et al.* 1997). These form plugs in the arteries, blocking maternal blood flow and subsequently transform the maternal vessels into low-resistance vascular sinuses, capable of handling the high flow rate needed for placental perfusion throughout the second and third trimesters of pregnancy (Pijnenborg *et al.* 1983).

Regulation of trophoblast invasion

Trophoblast invasion of the decidua and maternal vasculature is regulated at least in part by decidual factors. Invading cytotrophoblasts express a repertoire of adhesion molecules, chemotactic receptors and proteases, which enable migration through the decidual matrix. Amongst these, MMPs have been highlighted as a family of proteases necessary for EVT invasion; in humans, MMP-2 is most closely linked to invasive potential in first trimester implantation sites and in primary trophoblast culture studies (Isaka *et al.* 2003, Bai *et al.* 2005, Jones *et al.* 2006). Decidual conditioned medium contains factors that have an overall stimulatory effect on cytotrophoblast cell motility (Bischof *et al.* 1994); a number of constituents have been individually examined and demonstrated to regulate the balance of invasion. As discussed earlier, activin A is abundantly secreted by decidual cells (Jones *et al.* 2002*a*) and exogenous activin A stimulates MMP-2 production by cytotrophoblast cells and promotes their outgrowth from villous tips in an *in vitro* model of EVT invasion (Caniggia *et al.* 1997*a*). This has been confirmed using primary cytotrophoblast cells in an *in vitro* invasion assay. Activin A stimulates the invasive potential of cytotrophoblasts isolated from placentae up to 10 weeks of gestation, whereas follistatin is inhibitory in late stage first trimester cytotrophoblasts (Bearfield *et al.* 2005). Activin expression by invasive cytotrophoblast cells is low *in vivo* (Jones *et al.* 2006), suggesting that maternally derived activin promotes trophoblast invasion (Fig. 2).

Conversely, TGFβ is a major repressor of cytotrophoblast outgrowth (Fig. 2). Unlike activin, TGFβs are expressed by cytotrophoblast cells, co-expressed with TGFβRs (Schilling & Yeh 2000). Whilst early publications reported a similar protein localisation for the different TGFβ isoforms at the maternal–foetal interface (Graham *et al.* 1992, Selick *et al.* 1994, Lysiak *et al.* 1995, Schilling & Yeh 2000), the use of highly specific antibodies against the individual isoforms reveal cell-specific expression (Simpson *et al.* 2002), consistent with differential mRNA expression patterns (Ando *et al.* 1998). TGFβ1 and TGFβ2 are the most abundant isoforms in cytotrophoblast cell columns, but TGFβ1 is downregulated in invasive EVTs. TGFβ2 and TGFβ3 are present in the maternal tissues, with strong expression of TGFβ2 in decidual cells, whilst TGFβ3 is present only in immune cells. This is in marked contrast to findings from Caniggia *et al.* (1999), describing TGFβ3 production by first trimester trophoblast cells, and its selective upregulation in pre-eclamptic placentae. These inconsistencies may be methodological, due to differing antibody affinities or specificities. Alternatively, TGFβ3 may only be expressed in significant quantities in placental pathologies, an explanation that is supported by the fact that TGFβ3 mRNA is downregulated in normal first trimester decidua compared to non-pregnant endometrium (Ando *et al.* 1998).

In addition to their differential expression, *in vitro* studies suggest differential actions for the TGFβ isoforms in the implantation site (Fig. 2). TGFβ1 inhibits cytotrophoblast cell migration and invasiveness at least in part through the upregulation of the endogenous tissue inhibitors of MMPs (TIMPs)-1 and -2 (Graham & Lala 1992, Karmakar & Das 2002, Tse *et al.* 2002). In addition, TGFβ1 inhibits the invasion-promoting effects of hepatocyte growth factor (Caniggia *et al.* 1999). TGFβ3 also potently inhibits trophoblast outgrowth, and inhibition of TGFβ3 expression or activity results in increased outgrowth, elevated MMP production/activity and fibronectin deposition (Caniggia *et al.* 1997*b*). Importantly, blockade of the excessive expression of TGFβ3 in pre-eclamptic placentae restores their invasive potential, supporting a role for TGFβ3 in the pathogenesis of this disorder. There are no reports in the literature describing an inhibitory effect of TGFβ2 on cytotrophoblast outgrowth. One potential explanation for the differential actions of the isoforms is that endoglin, an accessory receptor protein for TGFβ1 and TGFβ3, but not TGFβ2, has been shown to be necessary for the inhibitory effect of TGFβ on trophoblast differentiation (St-Jacques *et al.* 1994). Endoglin is specifically expressed by cytotrophoblast cells in the anchoring cell column that are undergoing differentiation, and is lost in the fully differentiated EVTs invading the decidua (Graham *et al.* 1992, Xu *et al.* 2002), suggesting an active participation in the differentiation process. However, TGFβ2, along with TGFβ1, exerts anti-proliferative effects on extravillous cells (Li & Zhuang 1997). Subsequent experiments suggest this effect may be via inhibition of EGF-stimulated proliferation (Graham *et al.* 1994). Importantly, this growth inhibitory effect is lost in choriocarcinoma cells (e.g. JAR, JEG-3 cell lines), partially due to downregulation of endogenous Smad 3. This can be overcome by transfection with Smad 3, as can the regulation of TIMP-1, however, these effects are insufficient to restore anti-invasive actions of TGFβ (Xu *et al.* 2003), indicating the importance of multiple downstream pathways for TGFβ in regulating trophoblast outgrowth. Interestingly, most of the immunoreactivity for TGFβ appears to be extracellular, suggesting it is sequestered in the matrix. This is consistent with TGFβ being bound to the proteoglycan decorin, which acts as a storage pool or negative regulator of TGFβ action. Indeed decorin is expressed in the decidua, and itself can attenuate cytotrophoblast outgrowth, in the presence of endogenous and exogenous TGFβ (Lysiak *et al.* 1995, Xu *et al.* 2002).

MIC-1 is also abundant at the implantation site, both in decidual cells as described earlier, and in the developing placenta (Moore *et al.* 2000, Marjono *et al.* 2003). *In vitro* studies using EVT cells indicate that MIC-1 has overall inhibitory actions on trophoblast invasion, through growth inhibition and stimulation of apoptosis (Morrish *et al.* 2001). The signalling pathway for MIC-1 has not been delineated, thus the degree of overlap with, or compensation for, TGFβ actions is unclear.

A role for nodal during placentation has been indicated by the abnormal placental development observed in the nodal null homozygous mouse (Iannaccone *et al.* 1992). In the mid-gestation placenta, an excess of invasive giant cells are present, and overexpression of nodal *in vitro* is inhibitory to giant cell differentiation (Ma *et al.* 2001). In a human trophoblast cell line, overexpression of nodal decreases proliferation and increases apoptosis (Munir *et al.* 2004). The signalling pathways involved are unclear; nodal can signal via activin receptors (ALK-4/ActRIIB) or via ALK-7/ActRIIB. Although both pathways result in activation of Smad 2/3, the former signalling pathway requires cripto as a co-receptor. Cripto is abundantly expressed in the developing embryo (Dono *et al.* 1993, Baldassarre *et al.* 2001) in concert with Nodal, however, its expression by the placenta has not been fully elucidated (Baldassarre *et al.* 2001). Conversely, ALK-7 is expressed by the placenta, and is specifically upregulated after the first trimester, following similar expression dynamics as nodal (Roberts *et al.* 2003). A soluble form of ALK-7 is also abundant from mid-gestation, implying that nodal signalling is tightly regulated in the latter half of pregnancy; its actions throughout pregnancy are currently under investigation.

Regulation of placental development and function

Activin, TGFβ and MIC-1 are abundantly expressed by ST cells of the placental villi (Qu & Thomas 1995, Simpson *et al.* 2002, Marjono *et al.* 2003). Activin A and inhibin A are detectable in newly fusing syncytium, *in vivo* and *in vitro* (Debieve *et al.* 2000, Jones *et al.* 2006), indicating a potential involvement in cytotrophoblast fusion and syncytialisation, whilst TGFβ is a potent inhibitor of this process (Morrish *et al.* 1991). In contrast, low concentrations of MIC-1 induce syncytialisation of cytotrophoblast cells (Morrish *et al.* 2001). Furthermore, treatment of human embryonic stem cells with BMP-4 upregulates hCG production, indicating the formation of ST (Xu 2006, Fig. 2).

TGFβ superfamily members also have roles in regulating placental hormone production (Fig. 2): activin A stimulates, whilst TGFβ inhibits the production and/or secretion of hCG, human placental lactogen, progesterone and oestradiol (Petraglia *et al.* 1989, Song *et al.* 1996, Luo *et al.* 2002, Morrish *et al.* 1991). BMP-7/osteogenic protein-1 is expressed by cytotrophoblast cells rather than by the ST, but appears to play a negative paracrine role in steroidogenesis (Martinovic *et al.* 1996). In contrast to its low expression in the uterus, inhibin A is abundantly produced by the placenta (Qu & Thomas 1995). This is predominantly due to syncytial expression, and its co-expression with beta-glycan (Jones *et al.* 2002*b*, Ciarmela *et al.* 2003) is consistent with inhibin acting as a functional antagonist for activin in the placenta. Indeed, inhibin A potently inhibits steroidogenesis and production of hCG by the ST (Petraglia *et al.* 1989). Other roles for inhibin and activin during pregnancy are indicated by their high concentrations in circulating maternal blood, increasing with gestational age (Woodruff *et al.* 1997). Follistatin levels also rise, but to a lesser degree in the third trimester, suggesting that activin A may be biologically active as an endocrine factor in late pregnancy. Further increases in inhibin and activin A levels are detectable around the onset of parturition, suggesting potential roles in the cascade of events during labour (Muttukrishna *et al.* 1995).

Inhibins and activins in placental pathologies

As their major source is the placenta, both inhibins and activins have been explored as potential candidates for screening or diagnostic tests for pregnancy disorders. Inhibin A is a particularly specific marker of early placental development, and is detectable from 14 days postembryo transfer following IVF, indicating its potential as an early marker of IVF success (Birdsall *et al.* 1997). Conversely, a low level of inhibin A in early pregnancy is indicative of pregnancy failure, and several studies have shown a clear correlation between low inhibin A levels and subsequent miscarriage (Wallace *et al.* 2004, Prakash *et al.* 2005). Analysis of inhibin α mRNA and protein in placental tissue demonstrates that expression levels are unaltered; instead low levels are likely to be representative of low placental mass (Muttukrishna *et al.* 2004). For this reason, it has also been suggested that presence of elevated inhibin A levels may be employed to assess retention of trophoblast cells following molar pregnancies (Florio *et al.* 2002). MIC-1 has also proven to be successful in terms of identifying failing pregnancies: maternal serum levels are significantly lower in those women who subsequently miscarry, suggesting MIC-1 may be a biochemical and predictive marker of placental malfunction (Tong *et al.* 2004).

An elevated maternal serum level of inhibin A in the second trimester of pregnancy is indicative of foetal Down's syndrome, and has been adopted in some centres in combination with other markers (e.g. hCG, α feto-protein (AFP)) as an adjunct screening test (Aitken *et al.* 1996, Wallace *et al.* 1996, Malone *et al.* 2005) to assess risk prior to amniocentesis or chorionic villus sampling. High serum levels of inhibin A and activin A have also been reported in women with pre-eclampsia (PET) (Bersinger *et al.* 2003), a serious pregnancy complication, characterized by severe maternal hypertension and systemic inflammation and endothelial dysfunction, that remains the leading cause of foetal and maternal morbidity and mortality. Importantly, activin levels have been shown to be elevated prior to the onset of maternal symptoms (Muttukrishna *et al.* 2000). The combined measurement of placental factors (e.g. hCG, pregnancy associated placental protein-A, AFP, oestriol) together with Doppler ultrasound analysis of uterine artery blood flow, has been trialled as a potential screening test in the second trimester. Addition of serum inhibin A and/or activin A levels can improve predictive efficacy (Ay *et al.* 2005, Madazli *et al.* 2005, Spencer *et al.* 2006), particularly of early onset PET (Muttukrishna *et al.* 2000), but so far does not appear to be of great clinical significance. The roles that inhibins and activins play in the pathogenesis of PET are not understood, although the elevation of placental of inhibin/activin α and βA subunits, in the absence of elevated follistatin, indicates increased levels of bioactive activin within the feto-placental unit (Casagrandi *et al.* 2003). Activin A levels are also elevated in maternal serum in pregnancies complicated by intrauterine growth restriction (Wallace *et al.* 2003), suggesting that abnormal inhibin/activin levels may be useful as a screening tool for high risk pregnancies requiring greater obstetric attention.

Regulation of uterine immune responses

Both activin A and TGFβ also have immunomodulatory/inflammatory actions. This is of potential importance in regard to their functions in the endometrium – a site of highly specialised immune responses. TGFβ fulfils a pivotal role in the peripheral immune system through mediating the acquisition of immune tolerance (Schmidt-Weber & Blaser 2004). Tolerance is essential for preventing inappropriate immune responses, for example, to self-antigens, and is undoubtedly a significant component of the uterine immune environment, enabling embryo implantation and gestation. Elegant experiments in the mouse have demonstrated an interaction between seminal plasma and the endometrial mucosa. Immediately after mating, seminal plasma triggers an acute inflammatory response – involving recruitment of antigen presenting cells and controlled cytokine (e.g. GM-CSF, chemokines) production – proposed to instigate the tolerance response to paternal antigens (Robertson *et al.* 1997, Johansson *et al.* 2004). TGFβ is a major constituent of seminal plasma in rodents and humans, and appears to be a major contributor to these actions (Robertson *et al.* 2002). Recent studies of effects of seminal plasma on epithelial cells in culture suggest similar mechanisms may take place in the human reproductive tract (Gutsche *et al.* 2003). In addition, TGFβ can inhibit T helper type 1 (Th1) responses, which may be detrimental to pregnancy (Raghupathy 2001), and is an important regulator of NK cell behaviour, down-regulating IFN-γ induced activation and inflammatory cytokine production (Rook *et al.* 1986). TGFβ2 is abundantly produced by uterine-specific NK cells (Clark *et al.* 1994, Nagaeva *et al.* 2002), where it may be involved in the generation of their low cytotoxic and immunosuppressive phenotype (Saito *et al.* 1993, Eriksson *et al.* 2004, Fig. 2). Indeed in mice prone to a high pregnancy failure rate, TGFβ mRNA is significantly decreased in both uterine epithelial and metrial gland (NK) cells (Gorivodsky *et al.* 1999). Thus, TGFβ actions in the peri-implantation endometrium – both endogenously from endometrial and leukocyte expression, and exogenously from seminal plasma – potentially are instrumental in the establishment of anti-rejection strategies to allow implantation of the semi-allogeneic embryo. An immunomodulatory role for MIC-1 has also been proposed, as an overall immunosuppressive factor, due to its high maternal serum concentrations during pregnancy (Moore *et al.* 2000). Although its expression by uterine immune cells has not been described, MIC-1 is abundantly produced by the placenta, and is present in very high concentrations in amniotic fluid, suggesting systemic and intrauterine anti-inflammatory/immunosuppressive actions.

Activin A has also roles within the systemic immune system; it was first described as erythroid differentiation factor (Murata *et al.* 1988), responsible for promoting erythropoiesis, regulating B lymphocyte generation (Zipori & Barda-Saad 2001) and promoting mast cell differentiation and migration *in vitro* (Funaba *et al.* 2003). Activin A is also involved in inflammatory reactions: elevated activin A levels are associated with inflammatory pathologies in humans (Yu & Dolter 1997), and activin A is acutely and transiently released into the peripheral circulation after an inflammatory insult in sheep models (Jones *et al.* 2004). This precedes the elevation in serum tumour necrosis factor-α, indicating pro-inflammatory actions. Previous studies examining the effect of activin on systemic and local cytokine production have produced conflicting evidence, suggesting that activin possesses both pro- and anti-inflammatory qualities (de Kretser *et al.* 1999, Keelan *et al.* 2000).

Regulation of menstruation and repair

It is, therefore, not surprising that activin A and TGFβ are also abundant in the pre-menstrual endometrium, corresponding to immune cell infiltration and other inflammatory events. Activin βA subunits are intensely expressed by neutrophils and macrophages in the premenstrual and menstrual endometrium (Leung *et al.* 1998), whilst TGFβ is expressed by endometrial immune cells (Lea & Clark 1991). Leukocytes are probably effectors of endometrial breakdown (Salamonsen & Lathbury 2000), and a number of actions for activin and TGFβ could be envisaged in this process, through autocrine or paracrine upregulation of MMPs and cytokines. Whilst activin A could promote endometrial breakdown via upregulation of MMPs in endometrial cells and leukocytes (Ogawa *et al.* 2000, Jones *et al.* 2006), TGFβ is an established suppressor of MMP production by endometrial cells (Fig. 2). Progesterone acts as a 'blanket-repressor' of the majority of MMPs to prevent precocious endometrial breakdown, during the preparation for implantation, and TGFβ has been demonstrated to fulfil a critical role as a paracrine transducer of progesterone action (Osteen *et al.* 2003). This suppressive effect is overcome by the production of TGFβ antagonist lefty A, first described as endometrial bleeding-associated factor (Kothapalli *et al.* 1997). Lefty A antagonises TGFβ signalling at the level of the type II receptor and by interference with Smad 2 phosphorylation, and its dramatic upregulation in the perimenstrual phase is coincident with the release of MMP suppression (Tabibzadeh 2002). Furthermore, *in vitro*, lefty A directly stimulates the expression of proMMP-3 and -7 (Cornet *et al.* 2002).

In addition to inflammatory actions, both activin A and TGFβ have apparently contrasting roles in the resolution of inflammation and wound healing. TGFβ isoforms are selectively expressed during wound healing (reviewed by O'Kane & Ferguson (1997)). TGFβ1 and TGFβ2 are acutely upregulated after wounding, followed by an upregulation and dominant expression of TGFβ3. The healing process is dependent on isoform-specific functions, TGFβ3 instrumental for wound closure and collagen deposition. Neutralisation of TGFβ1 and TGFβ2 activity results in decreased scarring (Shah *et al.* 1994), and indeed the temporal shift in isoform expression during wound healing, that is an elevated ratio of TGFβ3:TGFβ1, appears to be critical for minimal scarring. Differential activin expression is also integral to the wound healing process. In skin wounds, activin βA is acutely upregulated in the wound site particularly in infiltrating immune cells, followed by an upregulation in βB subunits in migrating keratinocytes that is maintained until wound closure (Munz *et al.* 1999*a*). Overexpression of activin βA in the mouse epidermis accelerates wounding healing and scarring (Munz *et al.* 1999*b*), whilst overexpression of follistatin in keratinocytes results in delayed healing, and also in a reduction in collagen scar tissue deposition (Wankell *et al.* 2001). The importance of TGFβ and activin in modulating tissue repair are further confirmed by the phenotype of the Smad 3 knockout mouse, which conversely experiences accelerated healing, characterised by reduced inflammation and scar deposition (Ashcroft *et al.* 1999). Overall, these mediators are clearly important, but act in a tightly coordinated manner, both spatially and temporally, to mediate wound healing.

Interestingly, the pattern of βA and βB expression in peri-menstrual endometrium is markedly similar to that in wound healing, with βA-expressing inflammatory cells infiltrating in the acute inflammatory response, whilst βB-positive macrophages are resident in the endometrium during endometrial regeneration and proliferation (Jones *et al.* 2000). These contrasting expression patterns suggest differential actions for activin A and B in the endometrium during menstruation and endometrial repair. TGFβ expression during endometrial repair has not been closely examined, although recent *in vitro* studies of wound repair using endometrial stroma provide some evidence for modulatory effects of TGFβ on stromal cell motility and collagen gel contractility (Nasu *et al.* 2005).

Summary

This review describes the expression and potential roles for a number of TGFβ superfamily members in the uterus and placenta. The actions of activins and TGFβs have been studied in the greatest depth, leading to proposed roles in modulating cell turnover and tissue remodelling across the menstrual cycle and during the establishment of pregnancy. Interestingly, activins and TGFβs often appear to have opposing actions, indicating that a greater degree of specificity exists in the downstream signalling pathways of these two closely related ligands that are claimed to utilise the same Smads. More recently, the expression of a number of less well-known TGFβ superfamily ligands by the uterus, embryo and placenta has been described. Their involvement in endometrial biology and the events involved in the establishment of pregnancy is not surprising, given their association with dynamic cellular/tissue development. The identification of receptors, binding proteins and accessory/soluble receptors by endometrial and placental cells reinforces the tight regulation of action that is so characteristic of the TGFβ superfamily. Further complexity exists within this family, as ligands are synthesised as inactive proforms, requiring activating by photolytic enzymes (e.g. furin), meaning that proof of synthesis does not necessarily demonstrate biological activity. Future descriptive, biochemical and functional studies (*in vitro* and *in vivo*) will undoubtedly produce a greater understanding of the ever-expanding roles for, and complex interactions of, these factors in relation to reproductive biology.

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