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Activins in reproductive biology and beyond

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BACKGROUND: Activins are members of the pleiotrophic family of the transforming growth factor-beta (TGF-β) superfamily of cytokines, initially isolated for their capacity to induce the release of FSH from pituitary extracts. Subsequent research has demonstrated that activins are involved in multiple biological functions including the control of inflammation, fibrosis, developmental biology and tumourigenesis. This review summarizes the current knowledge on the roles of activin in reproductive and developmental biology. It also discusses interesting advances in the field of modulating the bioactivity of activins as a therapeutic target, which would undoubtedly be beneficial for patients with reproductive pathology.

METHODS: A comprehensive literature search was carried out using PUBMED and Google Scholar databases to identify studies in the English language which have contributed to the advancement of the field of activin biology, since its initial isolation in 1987 until July 2015. 'Activin', 'testis', 'ovary', 'embryonic development' and 'therapeutic targets' were used as the keywords in combination with other search phrases relevant to the topic of activin biology.

RESULTS: Activins, which are dimers of inhibin β subunits, act via a classical TGF-β signalling pathway. The bioactivity of activin is regulated by two endogenous inhibitors, inhibin and follistatin. Activin is a major regulator of testicular and ovarian development. In the ovary, activin A promotes oocyte maturation and regulates granulosa cell steroidogenesis. It is also essential in endometrial repair following menstruation, decidualization and maintaining pregnancy. Dysregulation of the activin-follistatin-inhibin system leads to disorders of female reproduction and

pregnancy, including polycystic ovary syndrome, ectopic pregnancy, miscarriage, fetal growth restriction, gestational diabetes, pre-eclampsia and pre-term birth. Moreover, a rise in serum activin A, accompanied by elevated FSH, is characteristic of female reproductive aging. In the male, activin A is an autocrine and paracrine modulator of germ cell development and Sertoli cell proliferation. Disruption of normal activin signalling is characteristic of many tumours affecting reproductive organs, including endometrial carcinoma, cervical cancer, testicular and ovarian cancer as well as prostate cancer. While activin A and B aid the progression of many tumours of the reproductive organs, activin C acts as a tumour suppressor. Activins are important in embryonic induction, morphogenesis of branched glandular organs, development of limbs and nervous system, craniofacial and dental development and morphogenesis of the Wolffian duct.

CONCLUSIONS: The field of activin biology has advanced considerably since its initial discovery as an FSH stimulating agent. Now, activin is well known as a growth factor and cytokine that regulates many aspects of reproductive biology, developmental biology and also inflammation and immunological mechanisms. Current research provides evidence for novel roles of activins in maintaining the structure and function of reproductive and other organ systems. The fact that activin A is elevated both locally as well as systemically in major disorders of the reproductive system makes it an important biomarker. Given the established role of activin A as a pro-inflammatory and pro-fibrotic agent, studies of its involvement in disorders of reproduction resulting from these processes should be examined. Follistatin, as a key regulator of the biological actions of activin, should be evaluated as a therapeutic agent in conditions where activin A overexpression is established as a contributing factor.

Key words: activin / follistatin / inhibin / ovary / testis / placenta / pregnancy / pre-eclampsia / cancer / inflammation

Introduction

Activins belong to the pleiotrophic family of the transforming growth factor-beta (TGF-β) superfamily of cytokines, and were initially isolated for their capacity to induce the release of FSH (Vale *et al.*, 1986; Ling *et al.*, 1987). Activins are disulphide-linked dimers of the inhibin β subunits, β_A, β_B and β_C (Vale *et al.*, 1986; Nakamura *et al.*, 1992). Based on the type of β subunits, a number of activins have been identified. Of these, the best studied are activin A ($\beta_A\beta_A$) and activin B ($\beta_B\beta_B$). Studies have also shown the presence of activin C ($\beta_C\beta_C$) (Hotten *et al.*, 1995; Loveland *et al.*, 1996), activin D ($\beta_D\beta_D$) (Oda *et al.*, 1995) and activin E ($\beta_E\beta_E$) (Fang *et al.*, 1996).

Some of the subunits that dimerize to form the activins are also involved in the formation of inhibins, which are inhibitors of pituitary FSH secretion (Ling *et al.*, 1985; Robertson *et al.*, 1985). Inhibins are heterodimers composed of an inhibin β_A or β_B subunit and an inhibin α subunit bridged by disulphide links forming inhibin A ($\alpha\beta_A$) and inhibin B ($\alpha\beta_B$) (Ling *et al.*, 1985; Robertson *et al.*, 1985; de Kretser and Robertson, 1989).

Both activin and inhibin are well known for their role in regulation of reproduction, and were initially discovered for their actions in modulating FSH activity (Fig. 1) . Activin A stimulates the release of FSH from the anterior pituitary (Vale *et al.*, 1986; Ling *et al.*, 1987), whereas inhibin suppresses FSH secretion (Keogh *et al.*, 1976; Robertson *et al.*, 1985; de Kretser *et al.*, 2002). However subsequent studies have shown that activin regulates a plethora of biological activities, ranging from embryonic development to inflammation. Similarly, its importance in reproductive biology has expanded well beyond the role of an FSH regulator. Several inhibin subunit and follistatin knockouts as well as transgenic over-expressing mouse models have aided the discovery of the multi-dimensional roles of activin and its regulators (Table I). Similarly, the development of specific immunoassays to measure these proteins in body fluids and tissues was instrumental in the advancement of the field.

This review summarizes the current body of knowledge on the roles of activins in reproductive and developmental biology. The association of activin dysregulation with male and female reproductive pathologies is also described. The paper also discusses interesting advances in the availability of agents that have the capacity to modulate the bioactivity of activins as a therapeutic target. Some of these approaches may provide significant benefits to patients with reproductive pathology.

Methods

A comprehensive literature search was carried out using PUBMED and Google Scholar databases to identify studies which have contributed to the advancement of the field of activin biology, since its initial isolation in 1987 until July 2015. Studies published in a language other than English were excluded. 'Activin', 'testis', 'ovary', 'embryonic development' and 'therapeutic targets' were used as the keywords in combination with other search phrases relevant to the topic of activin biology.

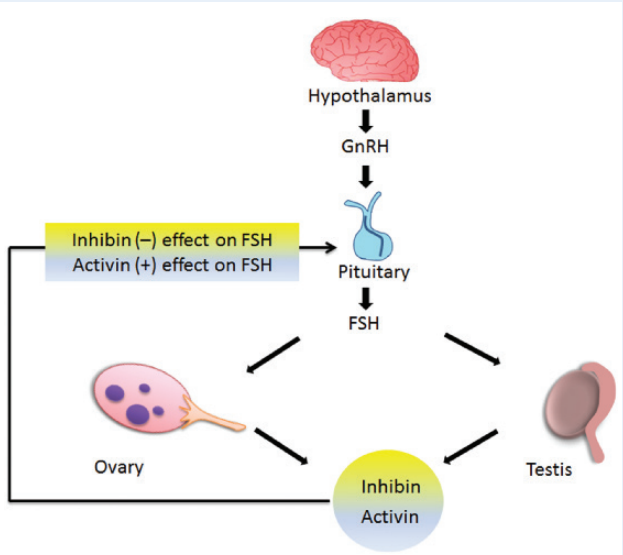

Figure 1 Activin and inhibin regulation of the hypothalamo-pituitary-gonadal axis. The hypothalamus produces GnRH which stimulates FSH release from the anterior pituitary. Activin stimulates the release of FSH whereas inhibin inhibits FSH secretion.

Table I Phenotypes of transgenic and knockout mouse models with altered activin, follistatin and inhibin levels.

Mouse model	Phenotype summary	References
Inhibin βA subunit gene knockout (*Inhba*$^{-/-}$)	Neonatal death due to failure in suckling as a result of mandibular and palate defects; Absence of coiling in the epididymis Low Sertoli cell numbers contributing to smaller testes at birth	Matzuk *et al.* (1995a), Tomaszewski *et al.* (2007) and Mithraprabhu *et al.* (2010)
Inhibin βB subunit gene knockout (*Inhbb*$^{-/-}$)	Born with open eyes, resulting in permanent ocular damage; Females show prolonged gestation and poor nursing behaviour	Schrewe *et al.* (1994) and Vassalli *et al.* (1994)
Inhibin α subunit gene knockout (*Inha*$^{-/-}$)	Testicular and ovarian tumours; Infertility; kyphoscoliosis; cancer cachexia	Matzuk *et al.* (1992)
Inhba and Inhbb double mutants	Exhibit the defects seen in *Inhba*$^{-/-}$ and *Inhbb*$^{-/-}$ mice, but no additional abnormalities	Matzuk *et al.* (1995a)
Testis specific over-expressers of *Inhba* under the control of mouse metallothionein promoter	Small degenerated testes; Infertile at 10 weeks of age	Tanimoto *et al.* (1999)
Activin receptor type II deficient mice (Acvr2$^{-/-}$)	Few develop craniofacial abnormalities and die at birth; small testes; reduction in Sertoli cell number; males have a 3 week delay in the onset of fertility ; females are infertile	Matzuk *et al.* (1995b)
Mice deficient in *Inhba*, but over-expressing *Inhbb* (*Inhba*$^{BK/BK}$)	Reduced body weight; slow hair growth; rough hair coat and sunken eyes; large external genitalia and smaller testes; delayed onset of fertility in males by 9 days and death by 26 weeks	Brown *et al.* (2000)
Follistatin gene knockout mice (*Fst*$^{-/-}$)	Die a few hours after birth because of difficulties in respiration; poorly developed muscles; runty; shiny, taut skin due to hyperkeratosis of the skin; cleft palate, whisker defects and absence of incisors; absence of the 13th pair of ribs	Matzuk *et al.* (1995c)
Follistatin isoform-specific transgenic mouse models	*Mouse Follistatin knockout mice with a human FST288 transgene (TghFST288)*: Die at birth due to respiratory difficulty; runty; shiny taut skin similar to *Fst*$^{-/-}$; poorly developed whiskers; flat urogenital tubercle	Lin *et al.* (2008) and Holdsworth-Carson *et al.* (2014)
	Mouse Follistatin knockout mice with a human FST315 transgene (TghFST315): Survive to adulthood; slow growth; slightly flat urogenital tubercle; disorientation of whiskers; tail tip necrosis; micro-ophthalmic; females are infertile due to malformations and chronic inflammation within the female reproductive tract	

Activin biology

The isolation and characterization of activin

Nearly three decades ago, during the purification of inhibin from porcine ovarian follicular fluid, it was identified that certain fractions of follicular fluid possessed the ability to release FSH from rat anterior pituitary cell cultures. This discovery led to the isolation and characterization of the activins. In 1986, Vale and colleagues isolated a homodimer of two inhibin β_A subunits with a molecular weight of 28 kDa, and named it the 'FSH releasing protein' (Vale *et al.*, 1986). In the same year, Ling *et al.* isolated a heterodimer composed of an inhibin β_A and an inhibin β_B subunit having a molecular weight of 24 kDa (Ling *et al.*, 1987) and called the substance 'activin' since it stimulated FSH, in contrast to the FSH suppressive effects of the inhibins.

Because the inhibins and activins showed considerable structural homology to TGF-β, they were classified as members of this protein superfamily (Ling *et al.*, 1987). Although both the above research groups proposed the existence of a β_B homodimer, the isolation of activin B as a 25 kDa protein from porcine ovarian follicular fluid was finally achieved by Nakamura and colleagues (Nakamura *et al.*, 1992). Several groups identified the presence of β_C and β_E subunits by molecular cloning (Hotten *et al.*, 1995; Fang *et al.*, 1996). A β_D subunit has been isolated in *Xenopus laevis*, but not in mammalian tissues (Oda *et al.*, 1995; Hedger *et al.*, 2011).

Activin is synthesized as a large precursor molecule with an N-terminal prodomain, which mediates the folding of the mature C-terminal domain (Vale *et al.*, 1986). Mutagenesis studies and crystallography of receptor-bound activin A indicate that the bonds between the prodomain and the mature domain are non-covalent (Walton *et al.*, 2009), unlike other TGF-β superfamily members which attach to their prodomains with high affinity (Walton *et al.*, 2010, 2012). Activin A, when secreted by cells, still contains the prodomain. As the propeptide is easily released from the mature form when activin A binds to cell surface receptors, it is considered to be secreted in an active form, unlike TGF-β1, which is secreted in a latent form due to its tightly attached prodomain (Walton *et al.*, 2009, 2010).

Activin receptors, regulation of secretion and modulation of bioactivity

Activins A and B act through the serine/threonine kinase pathway common to other TGF-β superfamily members (Mathews and Vale, 1991; Tsuchida *et al.*, 2004) (Fig. 2). Activin binds to one of two type II

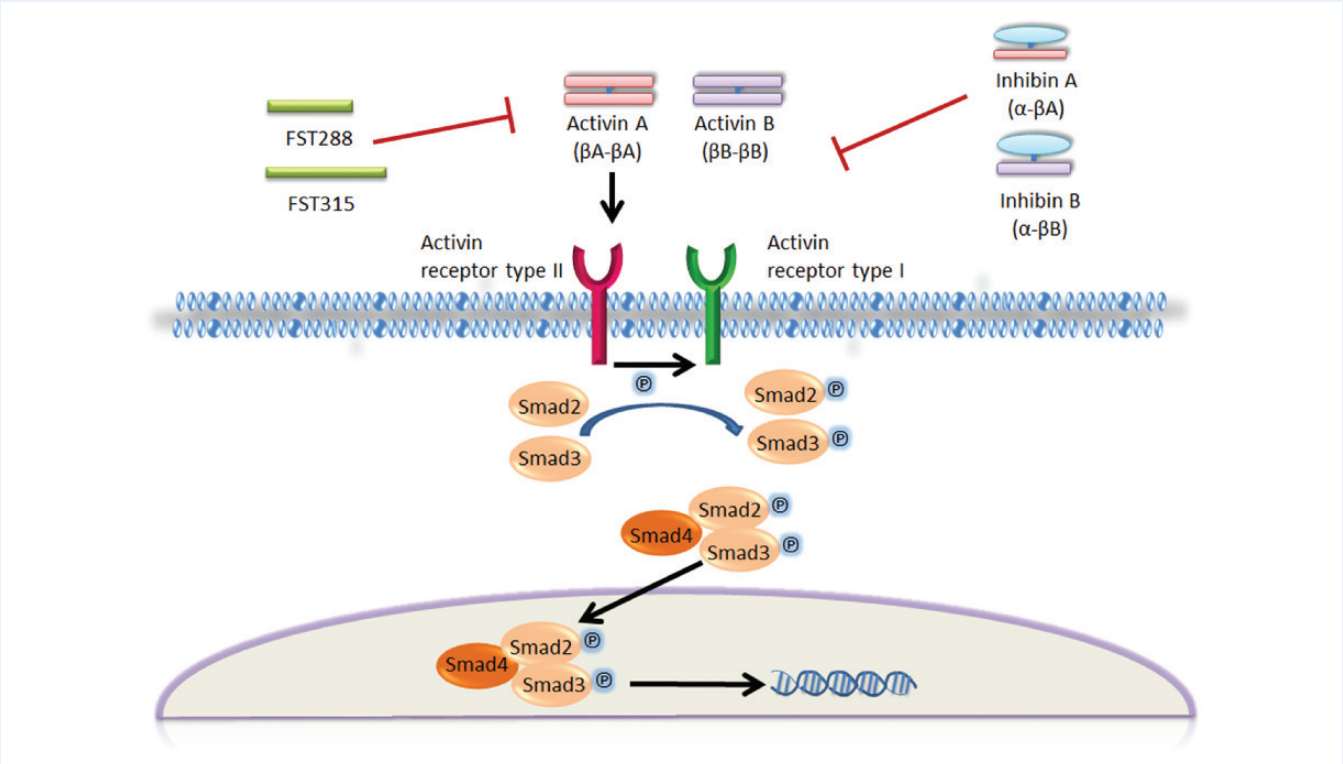

Figure 2 The activin signalling pathway. Activin binds to a type II receptor on the target cell surface. The type II receptor then binds to a type I receptor and causes phosphorylation of serine-threonine kinase residues. This leads to phosphorylation of SMAD2 and SMAD3, which form heteromeric transcription factor complexes with the common mediator, SMAD4. This complex is translocated to the nucleus, where they modulate target gene transcription. FST: follistatin.

activin receptors (ACVR2A or ACVR2B) present on target cell surfaces (Tsuchida *et al.*, 2004; Hedger and de Kretser, 2013). The type II receptor then binds to a type I receptor (Wrana *et al.*, 1994; Heldin *et al.*, 1997). Activin type I receptor is also known as activin receptor-like kinase (Alk) (Tsuchida *et al.*, 2004). While activin A acts via Alk 4 (activin receptor IB), activin B and activin AB can act via both Alk 4 and Alk 7 (Tsuchida *et al.*, 2004). Activin type I receptors contain serine/threonine kinase residues in their intracellular domains (Mathews and Vale, 1991). Binding of the type II receptors to type I receptors leads to phosphorylation of these residues. The type I receptors then induce phosphorylation of SMAD2 and SMAD3, which are intracellular proteins. These proteins form heteromeric transcription factor complexes with the common mediator, SMAD4. These complexes are translocated into the nucleus, where they modulate target gene transcription, to produce effects such as cellular proliferation, differentiation and apoptosis (Lin *et al.*, 2006; Hedger *et al.*, 2011).

Activin A can also act via the mitogen-activated protein (MAP) kinase signalling pathway, which is induced in inflammatory states (Hedger and de Kretser, 2013). MAP Kinases, such as ERK 1/2, JNK and p38, are important regulators of genes involved in inflammation and immunity (Kaminska, 2005; Zarubin and Han, 2005). Activin A production is stimulated by inflammatory mediators such as interleukin-1 and Toll-like receptor ligands which activate MAP Kinase signalling, via the MyD88/TRAP pathway (Jones *et al.*, 2007; Hedger *et al.*, 2011).

The bioactivity of activin is regulated by several endogenous inhibitors. While inhibin is capable of inhibiting the ability of activin to stimulate FSH secretion, it also regulates the amount of activin produced, because the formation of inhibin (α-β heterodimer) limits the availability of β subunits for activin synthesis (de Kretser *et al.*, 2002). Furthermore, activin C is also known to act as an activin A antagonist, mainly by competitively binding to β_A and β_B subunits, thus decreasing activin A levels with a resultant down-regulation of the Smad signalling pathway (Mellor *et al.*, 2000, 2003; Gold *et al.*, 2013). In addition, activin C can bind to the receptors ActRIIA and ActRIIB (Marino *et al.*, 2014).

Another important regulator of activin bioactivity is follistatin, originally called the FSH suppressing protein (Robertson *et al.*, 1987; Ueno *et al.*, 1987). The capacity of follistatin to bind to activin A and B with high affinity virtually irreversibly regulates activin bioactivity (Nakamura *et al.*, 1990, 1991). Unlike inhibin, follistatin does not share structural similarity with activin (Robertson *et al.*, 1987; Ueno *et al.*, 1987). It is a monomeric polypeptide, initially produced as a preprotein composed of 344 amino acids (Inouye *et al.*, 1991). Alternative splicing produces two mature polypeptide forms (Shimasaki *et al.*, 1988). These are termed FST 315 and FST 288, based on the length of the polypeptide chains (Inouye *et al.*, 1991). Both proteins have a heparin-binding site composed of a series of arginine residues (Esch *et al.*, 1987; Lerch *et al.*, 2007). However, the tertiary structure of FST 315 is such that the amino acids comprising the carboxy terminus fold back to obscure the binding site. Thus, FST 315 is more able to circulate freely within the body. FST 288, on the other hand, is rapidly bound by its positively charged sites to negatively charged heparin sulphate proteoglycans on cell surfaces, and exists as a tissue-bound form (Sugino *et al.*, 1993; Lerch *et al.*, 2007).

Each activin subunit binds to one follistatin molecule, i.e. the activin-follistatin complex is composed of one activin dimer and two follistatin molecules (Thompson *et al.*, 2005). Once activin binds to FST 288, this complex undergoes rapid endocytosis and lysosomal degradation (Hashimoto *et al.*, 1997). Binding of activin to FST 315 produces a conformational change in the protein, thus exposing the heparin binding site on the FST 315 protein, targeting the complex to a lysosomal degradation pathway (Nakamura *et al.*, 1990; Hashimoto *et al.*, 1997). Thus, follistatin causes an irreversible inhibition of activin (Hashimoto *et al.*, 1997; Phillips and de Kretser, 1998).

Activins in female reproductive biology

Apart from its originally identified role as a protein capable of stimulating FSH release from the anterior pituitary, the activins also regulate many other aspects of female reproductive biology. The inhibin β subunits and activin receptors are widespread throughout the female reproductive system.

In the ovary

Activin receptors and subunits are expressed in the human fetal ovary from 14–21 weeks of gestation, with β_A subunits in oogonia while β_B subunits were found in both oogonia and somatic cells. This specific pattern of expression indicates that activin regulates germ cell proliferation during development. Furthermore, stimulating ovarian fragment cultures with activin A increased the proliferation of oogonia (Martins da Silva *et al.*, 2004). Adult mice with an activin receptor II gene deletion showed an increase in the number of atretic ovarian follicles and very few corpora lutea, indicating a role for activin in post-pubertal follicular development (Matzuk *et al.*, 1995b). Expression patterns of activin receptors and signalling pathway components during post-natal follicular development in rodents and humans indicate that activin A stimulates the differentiation of primordial follicles into antral follicles but can also cause follicular atresia (Woodruff *et al.*, 1990; Roberts *et al.*, 1993; Drummond *et al.*, 2002). Furthermore, activin A promotes granulosa cell proliferation in both rats and mice (Matzuk *et al.*, 1992; Li *et al.*, 1995) and also acts as a promoter of oocyte maturation, and a suppressor of granulosa cell steroidogenesis (Alak *et al.*, 1998). This observation is further supported by the fact that mutant mice in which the activin A encoding *Inhba* gene has been replaced by the activin B encoding *Inhbb* gene ($Inhba^{BK/BK}$ mice) are infertile with lower numbers of pre-ovulatory follicles and reduced estrogen and progesterone levels (Brown *et al.*, 2000). Thus, activin B cannot completely replace activin A in the regulation of the female reproductive cycle.

In the uterus

Activin A and activin receptor proteins have been detected in the myometrium of both pregnant and non-pregnant women (Schneider-Kolsky *et al.*, 2001). A study which used primary cultures of the endometrium from normal young women in the mid to late proliferative or mid-secretory phase of the uterine cycle assessed the mRNA expression levels for inhibin subunit and activin receptor genes. This study showed that both endometrial stromal cells and epithelial cells expressed mRNA for the inhibin β_A, β_B and α genes, as well as ActRII and ActRIIB receptor genes (Petraglia *et al.*, 1998). Using immunohistochemistry, the β_A and β_B subunits were detected in the human endometrial luminal and glandular epithelium, and in migrating cells, which were identified as macrophages and neutrophils (Leung *et al.*, 1998; Jones *et al.*, 2000b). A similar pattern of expression was observed in the mouse endometrium (Kaitu'u-Lino *et al.*, 2009).

The endometrial expression of the inhibin β_A subunit showed a dynamic pattern across the menstrual cycle, while the inhibin α subunit expression remains relatively stable, suggesting an important role for activin A in the control of menstrual events including inflammation and endometrial repair (Leung *et al.*, 1998; Otani *et al.*, 1998; Jones *et al.*, 2002a, b). During the proliferative phase, inhibin β_A immunostaining was reduced in both the luminal and glandular epithelium whereas in the secretory phase immunostaining in these cells was increased. Further, while the immunostaining remained constant in the luminal epithelium for the rest of the menstrual cycle, it was decreased in the glandular epithelium at menstruation (Leung *et al.*, 1998).

Activins are important autocrine/paracrine regulators of endometrial decidualization and the priming of the endometrium for implantation. At the onset of decidualization, inhibin β_A, β_B and α subunit expression was increased in decidualized stromal cells (Petraglia *et al.*, 1990; Jones *et al.*, 2002a, b; Kaitu'u-Lino *et al.*, 2009). Using similar techniques, the activin subunits were localized to macrophages, neutrophils and mast cells indicating the involvement of activins and immune cells in inflammation and endometrial remodelling (Jones *et al.*, 2000b; Kaitu'u-Lino *et al.*, 2009). Importantly, activin A protein was secreted by decidual cells *in vitro*. Further, activin A stimulated markers of decidualization when added to decidual cells in culture. Moreover, this response was inhibited by the neutralization of activin using follistatin (Jones *et al.*, 2002a).

The functional role of activin A in endometrial repair following menstruation has been demonstrated using an *in vitro* wound repair model and a mouse model of endometrial breakdown (Kaitu'u-Lino *et al.*, 2009). They showed that the addition of 50 ng/ml of activin A to a wounded human endometrial cell line promoted healing. Simultaneous addition of follistatin to the cultures significantly delayed repair, consistent with the fact that mice over-expressing follistatin showed a considerable delay in endometrial repair (Kaitu'u-Lino *et al.*, 2009).

In pregnancy

During early pregnancy in women, the pattern of inhibin β_A, β_B and α subunit expression in the endometrium was similar to that described for decidualization at the end of the menstrual cycle (Jones *et al.* 2000b). Serum activin A levels gradually rise from the third trimester onwards, peaking at 38–39 weeks of gestation during normal human pregnancy (O'Connor *et al.*, 1999) and then decline abruptly following parturition (Harada *et al.*, 1996; Reddy *et al.*, 2009). These data indicate the importance of activin A in maintaining pregnancy. The feto-maternal unit is thought to contribute to this increase in serum activin A level (O'Connor *et al.*, 1999). Indeed, the human placenta was shown to be a novel source of inhibin by radioimmunoassay (McLachlan *et al.*, 1986), a finding later confirmed by the demonstration of inhibin β_A and β_B expression in the extravillous trophoblast, syncytiotrophoblast cells (Mylonas *et al.*, 2006), and in chorionic villi (Muttukrishna *et al.*, 2004a, b). Inhibin β_C was immunolocalized to the syncytiotrophoblast cells mainly, with weak staining in extra-villous trophoblast cells (Mylonas *et al.*, 2011). In a study using primary cultures of human placenta, activin A was shown to modulate the release of progesterone,

an important hormone in maintaining pregnancy (Petraglia *et al.*, 1989). They also showed that activin A promoted the production of GnRH, and thereby potentiated the release of hCG, another vital gestational hormone.

Role of activins in female reproductive pathology

Dysregulation of the activin signalling pathway leads to several disorders in females, with many of these having serious implications on fertility and child birth (Fig. 3). Several authors have reported that the activin:follistatin ratio is altered in patients with polycystic ovary syndrome (PCOS). In two clinic based cohorts, serum activin was significantly lower in patients with PCOS compared with controls, while follistatin was increased (Eldar-Geva *et al.*, 2001; Norman *et al.*, 2001). A further study showed that both follistatin and inhibin B were increased, while circulating activin A was decreased in patients with PCOS (Shen *et al.*, 2005). However, a study which assessed a community based population reported that activin A and B were not significantly different between women with PCOS and controls, although follistatin was elevated (Teede *et al.*, 2013). Nevertheless, all authors show that PCOS is associated with an imbalance in the ratio of activin and its inhibitors, follistatin and inhibin. A delicate balance between activin and inhibin levels regulates pre-ovulatory follicle development. Thus, dysregulation of this system might be a causative factor of PCOS.

Interestingly, serum activin A shows a massive increase in several pregnancy-related disorders. Activin A was significantly elevated in women with pre-eclampsia at each gestational period, compared with matched healthy controls (Muttukrishna *et al.*, 1997; Silver *et al.*, 1999; Ong *et al.*, 2004), making it a potential marker of pre-eclampsia. This was also consistent with the excessive release of anti-angiogenic substances from the placenta (Williamson *et al.*, 2015). Time course studies showed that serum activin A increased significantly prior to the onset of pre-eclampsia (27–30 weeks) (Muttukrishna *et al.*, 2000; Diesch *et al.*, 2006), with activin A being elevated from 20 weeks onwards in early onset pre-eclampsia (Muttukrishna *et al.*, 2000). Moreover, serum activin A increased significantly during labour in pre-eclamptic patients compared with their levels before labour. This was in contrast to women undergoing normal labour, where the activin A level did not change significantly from the pre-labour level. Following delivery, serum activin A levels declined rapidly in both the normal and pre-eclamptic group (Reddy *et al.*, 2009). In comparison to normal pregnant women who had very low activin A in urine, women with pre-eclampsia showed significantly elevated levels of activin A in urine (Muttukrishna *et al.*, 2006). Thus, urine and/or serum levels of activin A would be useful in a panel of biomarkers for pre-eclampsia.

Subcutaneous administration of 50 μg activin A to mice from Day 10 of gestation using mini-osmotic pumps resulted in 9-fold increased serum levels and a syndrome similar to pre-eclampsia, as they exhibited hypertension and proteinuria, which are hallmarks of pre-eclampsia, together with preterm littering and endothelial oxidative stress (Lim *et al.*, 2015). Moreover, human umbilical vein endothelial cells (HUVECs), when treated with activin, showed up-regulation of NADPH oxidase, a marker of oxidative stress and endothelial dysfunction. These effects were alleviated by treating the cells with follistatin. Similar effects were seen when HUVECs were cultured with serum obtained from pre-eclamptic patients (Lim *et al.*, 2015). These studies suggest that activin A is directly involved in the pathogenesis of pre-eclampsia. In fact, variation in activin and its receptors has been implicated as a genetic risk factor for pre-eclampsia. Five single nucleotide polymorphisms (SNP) of the activin receptor *ACVR2A* gene were shown to be associated with pre-eclampsia, with one SNP (rs1424954) being strongly associated, in a study based on 34 Australian/ New Zealand families with a history of pre-eclampsia (Moses *et al.*, 2006). Although further analysis of 74 Australian/ New Zealand pre-eclamptic families including this cohort

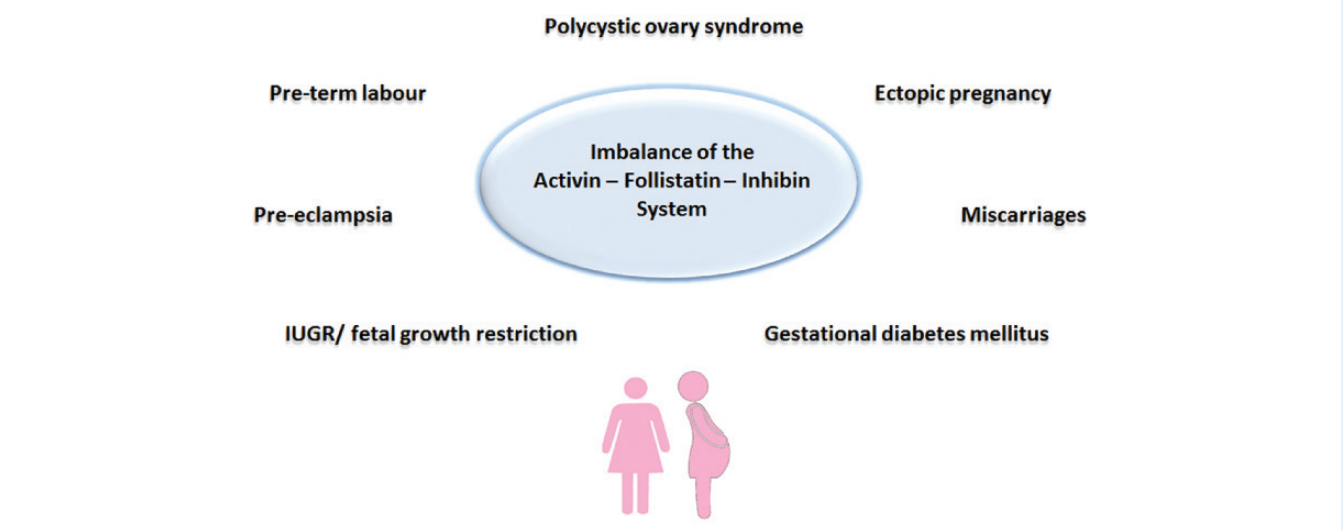

Figure 3 Several reproductive disorders of females are characterized by altered activin:follistatin or activin:inhibin ratios in serum. Activin A is elevated in pre-eclampsia, intrauterine growth restriction (IUGR)/fetal growth restriction and pre-term labour. Reduced activin:follistatin ratio is seen in gestational diabetes mellitus, while increased activin:follistatin ratio is a hallmark of polycystic ovary syndrome. Serum activin A and B are both low in women with ectopic pregnancies. Low or normal serum activin levels accompanied by reduced inhibin levels are observed in women with miscarriages.

did not reveal a strong association between *ACVR2A* and pre-eclampsia (Fitzpatrick *et al.*, 2009), a large Norwegian population-based study (the HUNT study) showed a significant association between *ACVR2A* SNPs and pre-eclampsia (Roten *et al.*, 2008). Thus, defects in the activin receptor gene may lead to abnormal elevation of activin A during implantation and placentation, contributing to the pathogenesis of pre-eclampsia (Fitzpatrick *et al.*, 2009).

In patients with gestational diabetes mellitus, a metabolic disorder which arises in late pregnancy, the serum activin:follistatin ratio has been shown to be increased (Näf *et al.*, 2014). Activin A was significantly elevated in patients with gestational diabetes mellitus compared with controls, and activin levels decreased following improved diabetic control using insulin (Petraglia *et al.*, 1995; Gallinelli *et al.*, 1996).

Activin A is also increased in patients showing fetal growth restriction and those who enter pre-term labour (Gallinelli *et al.*, 1996; Muttukrishna *et al.*, 2004a). Interestingly, maternal serum activin A was 3-fold higher in cases of intra-uterine growth restriction (IUGR), compared with normal pregnancies with constitutionally small fetuses (Wallace *et al.*, 2003). Furthermore, activin A was increased in the circulation of IUGR fetuses (Morpurgo *et al.*, 2004). Moreover, administration of activin A to pregnant mice at the 10th day of gestation caused fetal growth restriction (Lim *et al.*, 2015). Inhibin β_B immunostaining was reduced in the syncytiotrophoblast cells in human placental tissue obtained from pregnancies complicated with IUGR, although the significance of this is not clear (Mylonas *et al.*, 2006). Nevertheless, the reports on gestational diabetes mellitus and IUGR suggest that the activin-follistatin system is involved in feto-maternal metabolism, and that an imbalance may lead to fetal growth inhibition and altered maternal metabolism.

On the other hand, circulating activin A has been reported to be significantly lower in patients with ectopic pregnancies, compared with normal intrauterine pregnancies (Florio *et al.*, 2007; Daponte *et al.*, 2013; Elito *et al.*, 2014), possibly because of the failure of the embryo to implant in the endometrium. Similarly, serum activin B was also significantly decreased in women with ectopic pregnancies, and the inhibin β_B subunit expression was reduced in the decidualized endometrium of these patients (Horne *et al.*, 2008).

Serum activin A levels in patients with miscarriages, however, have shown variable results with levels significantly lower in women just after complete miscarriage compared with gestation-matched controls and those with incomplete miscarriages (Luisi *et al.*, 2003). However other studies did not confirm these outcomes (Muttukrishna *et al.*, 2002, 2004b; Wallace *et al.*, 2004; Warrick *et al.*, 2012). A lower inhibin β_A subunit expression in luteal phase endometrial stromal cells in women with recurrent miscarriage compared with those with no history of miscarriage was shown in one study by immunohistochemistry (Prakash *et al.*, 2006). Nevertheless, all studies agree that a reduction in serum inhibin A levels is a more reliable predictor of miscarriages.

Activin A and reproductive aging in the female

Reproductive aging, the gradual decline and eventual cessation of ovarian activity, is characterized by an increase in FSH levels resulting from an elevation of activin and decreasing inhibin levels, as shown by the following studies. Serum activin A was increased in females of mid-reproductive age and peri-menopausal women, compared with younger, cycling women (Harada *et al.*, 1996; Santoro *et al.*, 1999; Reame *et al.*, 2007). In females aged 43–47 years, follicular phase serum inhibin B levels were significantly reduced and serum inhibin A was low in the luteal phase compared with younger women (19–38 years old) whereas serum activin A levels increased two fold in these women compared with the younger females, throughout the menstrual cycle (Santoro *et al.*, 1999).

Activin A and follistatin, but not the inhibins, were significantly increased in fluid obtained from dominant follicles of 40–45 year old women having normal menstrual cycles, compared with that of women aged 20–25 years (Klein *et al.*, 2000). The authors suggested that elevated activin levels might be causing premature ovulation in older women, as activin A was shown to have such effects in rats (Erickson *et al.*, 1995). As FSH levels were not affected, it is very likely that this is a result of actions at the ovarian level. Interestingly, a transcriptome profile study on cumulus cells showed that *ACVR2B* and *Inha* gene expression were down-regulated in women above the age of 37 years (Al-Edani *et al.*, 2014). These findings suggest that activin A levels are an important marker of female reproductive age.

Activins in male reproductive biology

The role of activin in the testis

Measurements of activin A and B protein using specific enzyme-linked immunosorbent assays (ELISAs) have shown that the testis is one of the major sites of activin production (Buzzard *et al.*, 2004; Barakat *et al.*, 2008; Winnall *et al.*, 2013). Many cell types in both developing and adult testes show inhibin β_A and β_B subunit gene expression, while activin receptors are also highly expressed in testicular tissue. Whole mount *in situ* hybridization showed *Inhba* to be expressed in the fetal testis at embryonic day 16.5 in the mouse. Activin receptors ACVR2A and ACVR2B were shown to be localized mainly in the gonocytes, interstitial cells and Sertoli cells in the developing murine testis (Mendis *et al.*, 2011). Furthermore, fetal Leydig cells act as a source of activin A in the mouse (Archambeault and Yao, 2010). In the post-natal human, mouse and rat testes, β_A subunits have been demonstrated in gonocytes and Sertoli cells, using *in situ* hybridization and immunohistochemistry (Anderson *et al.*, 1998; Meehan *et al.*, 2000; Buzzard *et al.*, 2003; Okuma *et al.*, 2006; Barakat *et al.*, 2008).

Both inhibin β_A and β_B subunits have been detected by *in situ* hybridization in spermatogonia and primary spermatocytes in the post-natal rat (Buzzard *et al.*, 2004). In the adult human testis, inhibin β_B mRNA has been detected in spermatogonia, round spermatids and Sertoli cells, whereas immunolabelling techniques have shown β_B subunits to be expressed in pachytene and round spermatids (Marchetti *et al.*, 2003). The rat peritubular myoid cells were shown to produce activin A protein, while mRNA for both inhibin β_A and β_B were detected (de Winter *et al.*, 1994). Furthermore, mouse, rat and human Leydig cells also produce activin A and B (Lee *et al.*, 1989; Anderson *et al.*, 1998; Marchetti *et al.*, 2003). The limited existing data show that activin C is produced in the human and rat testis. While β_C subunit localization in the human is confined mainly to the round spermatids and spermatogonia (Marino *et al.*, 2014), localization in the rat is seen in

the spermatogonia, pachytene spermatocytes as well as the Leydig cells (Gold *et al.*, 2004).

Dynamic changes occur in the expression of inhibin β subunits in the testis during embryonic development and subsequent post-natal maturation. At about embryonic day 12.5 in mice, just after sex determination, activin A levels increase, reaching a peak at the day of birth (Mendis *et al.*, 2011). Post-natally, activin A levels are highest in the first week of life in mice (Barakat *et al.*, 2008; Mithraprabhu *et al.*, 2010). A similar pattern occurred in rats, with both serum and testicular activin A protein levels peaking at Day 6 post-partum, but drastically decreasing by Day 20, and even further at Day 90 post-partum (Buzzard *et al.*, 2004).

These data implicate activin A as an autocrine and paracrine modulator of germ cell development and Sertoli cell proliferation. *Inhba*$^{-/-}$ mice, which lack activin A, showed a significant reduction in testis weight compared with wildtype littermates at the day of birth (Mendis *et al.*, 2011). Their testes, interestingly, had a significantly lower number of Sertoli cells, but twice the number of gonocytes, indicating that at this age, activin A is essential in maintaining a normal germ cell: Sertoli cell ratio. Abrogation of activin signalling in the fetal mouse testis resulted in testicular dysgenesis characterized by reduced Sertoli cell numbers and failure in testicular cord elongation (Archambeault and Yao, 2010). A study using 3-day-old rats also showed that activin increased gonocyte numbers and reduced Sertoli cell mitosis in culture (Meehan *et al.*, 2000). Further *in vitro* studies demonstrated that activin promotes rat Sertoli cell proliferation at the ages of 6 and 9 days post-partum, but not at Day 3 (Boitani *et al.*, 1995; Buzzard *et al.*, 2003). The study by Buzzard *et al.* (2003), which used a specific ELISA to measure protein levels, showed that high levels of activin A were produced by peritubular cells of 6 day old rats, and that activin A also stimulated the release of inhibin in culture.

Similarly, 21 day old rat peritubular and Sertoli cells in culture secrete activin A that stimulate inhibin secretion and regulate Sertoli cell aromatase activity in an autocrine manner (de Winter *et al.*, 1993, 1994). Both activin A and B were shown to stimulate the *in vitro* proliferation of spermatogonia in 25 day old rats (Mather *et al.*, 1990). Furthermore, this study also showed that activin was bound directly to isolated spermatogonia, supporting the fact that activin acts locally to regulate testicular growth. Suppressing activin by over-expressing follistatin in transgenic mice resulted in spermatogenic arrest, seminiferous tubule degeneration and infertility (Guo *et al.*, 1998), highlighting the importance of normal activin levels in maintaining fertility.

In adult rats, activin A production by the seminiferous tubule is regulated in a stage dependent manner related to the seminiferous epithelial cycle with lowest levels at stage VI and maximum levels during stage VIII, which coincided with release of sperm into the tubular lumen (Okuma *et al.*, 2006). These data suggest activin A is involved in the control of releasing sperm into the lumen, and possibly in the process of opening up of the blood–testis barrier to enable transit of preleptotene spermatocytes to the intraluminal side of the tubule. This action is supported by the capacity of activin A to disrupt Sertoli cell tight junctions that constitute the blood–testis barrier in an *in vitro* model (Nicholls *et al.*, 2012) and the fact that spermatids are 'anchored' by cell junctions that are, in effect, hemi-inter-Sertoli cell junctions.

The *Inhba*$^{BK/BK}$ mouse in which the *Inhba* locus has been replaced by the *Inhbb* gene, has been useful in dissecting out the specific roles of activin A and B. The testis size is reduced in these mice (Brown *et al.*, 2000), and maturation of type A spermatogonia into spermatocytes was delayed (Mithraprabhu *et al.*, 2010). Furthermore, *Inhba*$^{BK/BK}$ mice exhibit a 9 day delay in the onset of fertility (Brown *et al.*, 2000). This indicates that activin B cannot entirely replace activin A in post-natal germ cell maturation and testicular development. While activin A is essential for male fertility, the activin B deficient *Inhbb*$^{-/-}$ mice remain fertile, confirming that activin B does not play a major role in testicular development (Vassalli *et al.*, 1994).

Activins in the rest of the male reproductive tract

Activin receptor mRNA was expressed in the testis, epididymis, vas deferens and prostate in both the rat and mouse (Feng *et al.*, 1993; Winnall *et al.*, 2013). In the adult murine male reproductive tract, activin A and its binding protein follistatin, showed a specific pattern of expression with activin A being highest in the caput epididymis and low in the vas deferens (Winnall *et al.*, 2013). Follistatin showed the exact opposite pattern of expression, with low levels in the epididymis, but very high levels in the vas deferens. This expression pattern suggests that activin A and follistatin are important in maintaining the structure of the epididymis and vas deferens.

In studies using rodents, *Inhba* and *Inhbb* mRNA were shown to be expressed in the seminal vesicles and prostate (Ying *et al.*, 1997; Winnall *et al.*, 2013). However, the absence of activin A in the seminal plasma of vasectomized men (Anderson *et al.*, 1998) suggests that the testis and epididymis are the major sources of activin A in the male reproductive tract. While it is known that normal men have high activin A levels in seminal plasma (Anderson *et al.*, 1998), it is yet to be discovered if activin has a specific role in maintaining the fertilizing capacity of sperm or facilitating the passage of sperm within the female tract.

Role of activins in the pathology of the male reproductive tract

As activin A has an involvement in many major biological pathways, not just those governing reproductive biology, dysregulation of activin signalling can cause severe adverse effects. Activin A is systemically elevated in many acute and chronic inflammatory conditions (Jones *et al.*, 2000a; Michel *et al.*, 2003; Leto *et al.*, 2006; Zhang *et al.*, 2009; Wu *et al.*, 2013). Thus, it is important to consider the effects of elevated activin levels on male fertility.

In mice with the inhibin α gene deletion, activin is over-produced and results in the development of testicular tumours (Matzuk *et al.*, 1992). These invasive tumours which develop as early as 4 weeks of age completely destroy the testicular architecture, and render the mice infertile (Matzuk *et al.*, 1992). Transgenic mice over-expressing the *Inhba* gene in the testes showed disrupted spermatogenesis and infertility (Tanimoto *et al.*, 1999). Chronic activin signalling in mice reduced testicular mass by 23.5% and caused hypospermatogenesis (Nicholls *et al.*, 2012). The authors also showed that elevated activin levels caused terminally differentiated adult Sertoli cells to revert back to an immature, proliferating phenotype, highlighting the importance of normal activin balance in maintaining male testicular function and fertility.

Sertoli cell only syndrome is a common cause of non-obstructive azoospermia in men. Several studies have shown that the activin signalling pathway is activated in patients with Sertoli cell only syndrome. SMAD2, a component of the activin signalling pathway, was shown to be elevated

in testicular biopsies from men with Sertoli cell only syndrome (Sun *et al.*, 2008). Another group of men with Sertoli cell only syndrome and Leydig cell hyperplasia had an increase in phosphorylated SMADs 2/3 in Leydig cells (Gonzalez *et al.*, 2010). Leydig cell hyperplasia together with germ cell apoptosis was observed in transgenic mice over-expressing SMAD4 (Narula *et al.*, 2002).

Role of activin A in tumours of the reproductive tract

Activin A is a well-known modulator of many cancers involving various tissues and organs in the body (Harada *et al.*, 1996; Loomans and Andl, 2014; Loumaye *et al.*, 2015). While activin A aids the progression of certain tumours (Togashi *et al.*, 2015), in some instances it is known to inhibit tumourigenesis (Loomans and Andl, 2014), depending on the cell types involved and other interacting pathways. Activin B acts in a similar fashion to activin A in tumour progression (Wacker *et al.*, 2009; Tamminen *et al.*, 2015), while activin C is a tumour suppressor (Gold *et al.*, 2013). This section of the review will focus on the involvement of activin in tumours of both the male and female reproductive system.

Endometrial carcinoma is one of the most common reproductive tract tumours in the female. Protein levels of ACVR2 receptors, through which activin signalling occurs, were found to be increased in endometrial tissue obtained from patients with endometrial carcinoma (Piestrzeniewicz-Ulanska *et al.*, 2002). Activin A was significantly elevated in serum as well as in the fluid obtained from uterine washes of patients with endometrial carcinoma compared with healthy controls. Following surgery, activin A levels decreased significantly (Petraglia *et al.*, 1998). In patients with uterine endometrioid adenocarcinoma, inhibin β_B subunit expression was significantly associated with cervical metastasis of the tumour (Mylonas, 2011). Similar to patients with endometrial carcinoma, women with cervical cancer were found to have elevated serum activin A levels, which declined significantly a month following surgery (Petraglia *et al.*, 1998).

Activin signalling is increased in human testicular and ovarian cancers (Marino *et al.*, 2014). Human granulosa cell tumour cell lines respond to both activin A and B by increased proliferation (Cheng *et al.*, 2014; Marino *et al.*, 2014). Follistatin, as well as activin receptor type I inhibitors, were able to block the activin-induced cellular proliferation of granulosa cell tumours *in vitro* (Cheng *et al.*, 2014). This proliferative effect of activin on gonadal tumours is further confirmed by the fact that mice over-expressing activin A as a result of inhibin α subunit gene deletion develop ovarian and testicular tumours at a very early age (Matzuk *et al.*, 1992). Furthermore, activin B was shown to be elevated in the serum of patients with granulosa cell tumours (Vihko *et al.*, 2003). In addition, inhibin β_A and β_B subunits were expressed in dysgerminoma and yolk sac tumours of the ovary (Cobellis *et al.*, 2001), indicating the involvement of activins in various ovarian tumours.

Phosphorylated SMAD2/3 was increased in nuclei of all human seminoma samples examined, suggesting that the activin signalling pathway is active in these tumours (Dias *et al.*, 2009). Furthermore, activin type I and type II receptor mRNA expression was observed in seminomas and non-seminomas of adolescent boys as well as adults. Moreover, seminomas also expressed high levels of the β_A subunit and follistatin (van Schaik *et al.*, 1997). In addition to seminomas, inhibin β_A and β_B subunit immunolocalization was also reported in embryonal carcinoma, choriocarcinoma and yolk sac tumours of adult men (Cobellis *et al.*, 2001). These studies strongly suggest an autocrine involvement of activin in testicular tumours. Interestingly, a mutation in the *SMAD4* gene was observed in 2 patients with seminomas, out of 20 biopsies examined (Bouras *et al.*, 2000). This mutation in the carboxy terminal domain of SMAD4 causes premature termination of the protein, thereby disrupting the activin signalling pathway. Thus, dysregulation of the activin signalling pathway appears to be a factor in the development of human germ cell tumours.

Prostate cancer is one of the most prevalent and commonly diagnosed cancers. Dysregulation of activin signalling is thought to contribute to prostate cancer (Gold and Risbridger, 2012). Activin A and B inhibited the proliferation of the human prostate epithelial tumour line LNCaP in a dose dependent manner (McPherson *et al.*, 1997; Mellor *et al.*, 2003). However, another study showed that activin A aided the proliferation of LNCaP cells, and caused up-regulation of specific differentiation markers such as prostate specific antigen and prostatic acid phosphatase (Zhang *et al.*, 1997). Furthermore, LNCaP cells did not produce activin A in culture whereas activin A and follistatin were produced by a more aggressive prostate tumour cell line PC3 (McPherson *et al.*, 1999; Hofland *et al.*, 2012). Moreover, PC3 cells did not respond to the anti-proliferative effects of exogenous activin (McPherson *et al.*, 1999). Thus, activin A in particular, appears to have variable effects on prostate cancer depending on the stage of disease. Nevertheless, activin C did not alter DNA synthesis of LNCaP cells, indicating that it does not act in a similar fashion to activins A and B in prostate cancer (Mellor *et al.*, 2003). For a more comprehensive review on the role of activins in prostate cancer, the reader is referred to Gold and Risbridger (2012).

The expression of the activin βc subunit at sites wherein β_A is also produced, results in decreased levels of activin A due to competition by the formation of β_A-β_C dimers. Based on this capacity, β_C has been shown to be a tumour suppressor (Marino *et al.*, 2014). In inhibin α gene knock-out mice over-expressing activin C, the incidence of gonadal tumours was reduced. Activin C inhibited the activin signalling pathway and also reduced serum and tissue activin A levels in these mice (Gold *et al.*, 2013; Marino *et al.*, 2015). Furthermore, overexpression of the inhibin β_C subunit reduced activin A levels in the PC3 human prostate cell line, by the formation of activin AC (Mellor *et al.*, 2003), and this could be a regulatory mechanism by which the tumorigenic activity of activin A is regulated in the prostate.

Activins in developmental biology

Activins act within the developing embryo to determine the fate of cell populations (Stern *et al.*, 1995; Ball and Risbridger, 2001). Embryonic induction, the process by which cellular signals of one cell population affects the developmental outcome of another group of cells, is an important part of early embryonic development. Many TGF-β superfamily proteins, including activin, play a vital role in this process (Nakamura *et al.*, 1992; Hogan *et al.*, 1994; Stern *et al.*, 1995).

Activins A, AB and B have been shown to possess the capability of mesoderm induction in embryos, and lead to the formation of a secondary body axis (Sokol *et al.*, 1991; Nakamura *et al.*, 1992). Of these growth factors, activin B was the most potent protein in this aspect (Nakamura *et al.*, 1992). Although the injection of inhibin β_D mRNA into the four cell stage *Xenopus laevis* embryo induced

secondary body axis formation, this effect of β_D was weak compared with other activins (Oda *et al.*, 1995).

Activin A, working synergistically with other growth factors, directs embryonic stem cells into an endodermal fate (McLean *et al.*, 2007; Xu *et al.*, 2011; Guo *et al.*, 2014). Culturing human embryonic stem cells with basic fibroblast growth factor (bFGF) and bone morphogenetic factor 4 (BMP4), in the presence of activin A, for 3–4 days induced primitive streak and definitive-endoderm associated genes. Addition of the activin receptor inhibitor SB431542 to cells treated with bFGF and BMP4 inhibited the expression of the definitive-endoderm associated genes, confirming that activin A plays a key role in determining embryonic stem cell fate (Xu *et al.*, 2011). Furthermore, the Wnt- signalling pathway has also been shown to be acting in concert with activin A in the process of driving human embryonic stem cells into definitive endoderm (Naujok *et al.*, 2014).

Also, in development, activin is significantly involved in the morphogenesis of branched glandular organs. Several studies have shown that activin A and follistatin modulate the dichotomous division of the ureteric bud, so that the collecting duct system of the kidney is formed (Ritvos *et al.*, 1995; Maeshima *et al.*, 2003; Bush *et al.*, 2004). Similarly, activin inhibited branching of the developing prostate, whereas follistatin stimulated branching *in vitro* (Cancilla *et al.*, 2001). Furthermore, inhibition of the activin/TGF-β signalling components SMAD2, 3 and 4 stimulated branching of embryonic lung in culture (Zhao *et al.*, 1998). Exogenous activin A inhibited pancreatic and salivary gland lobulation in culture and this effect was reversible by the addition of follistatin (Ritvos *et al.*, 1995). Furthermore, activin A signalling was shown to be essential in the formation of many posterior fore-gut derived organs, such as the stomach, pancreas and the spleen (Kim *et al.*, 2000; Xu *et al.*, 2011).

Activins are also essential in the development of limbs (Stern *et al.*, 1995; Ball and Risbridger, 2001) with the expression patterns of the *Inhba* and *Inhbb* genes during bone and cartilage formation in embryos highlighting the importance of activins A and B in early bone morphogenesis (Merino *et al.*, 1999). The *Inhba* gene expression is initiated around the same time as the beginning of chondrogenesis of digits. On the other hand, *Inhbb* expression marks the formation of the last phalanx. Interestingly, exogenous administration of activin A, B or AB into the inter-digital areas of chicken embryos caused polydactyly. Furthermore, digit formation was inhibited by the administration of exogenous follistatin (Merino *et al.*, 1999). In addition to bone morphogenesis, activin has been shown to play a role in fetal muscle development, with activin inhibiting muscle growth whilst follistatin enhanced muscle formation (Link and Nishi, 1997).

The role of activin A in craniofacial development has been clearly demonstrated. Mice lacking *Inhba* died within 24 h of birth because they inhaled milk during suckling due to the presence of a cleft palate (Matzuk *et al.*, 1995a, 1996). These mice did not have whiskers or incisors and exhibited mandible defects. Interestingly, insertion of the *Inhbb* gene into the *Inhba* locus prevents these craniofacial malformations in mice (Brown *et al.*, 2000).

Accordingly, activin A has been identified as a key mesenchymal signal orchestrating the early events that lead to dental development in the mouse. Tooth development in *Inhba*$^{-/-}$ mice, that lacked incisors and mandibular molars, was rescued by implanting beads soaked in activin A protein in the mesenchyme of mandible explants at embryonic day 11.5 (Ferguson *et al.*, 1998). The balance between activin and follistatin was shown to be essential in normal dental enamel formation and molar teeth morphogenesis in the mouse (Wang *et al.*, 2004).

Activin B in particular, seems to play an important role in central nervous system development. *Inhbb* shows specific localization mainly to the fore and hindbrain as well as the spinal cord at embryonic day 10.5 and 12.5 in mice (Feijen *et al.*, 1994). A recent study has demonstrated that within the developing murine spinal cord, TGF-β and activin B act synergistically to promote proliferation and maturation of oligodendrocytes, the cells responsible for the formation of myelin (Dutta *et al.*, 2014). The authors reported that in *Inhbb*$^{-/-}$ embryos, which lack activin B, apoptosis of oligodendrocytes was increased. Furthermore, oligodendrocyte progenitor cells were also reduced during embryonic life.

Activin A plays an essential paracrine role in the morphogenesis of the straight Wolffian duct into the highly convoluted epididymis. *In situ* hybridization showed that *Inhba* was expressed mainly in the anterior and middle part of the mesonephric duct, the areas which transforms into the epididymis. Moreover, *Inhba* expression showed a gradient pattern, with the most amount of *Inhba* expressed in the anterior Wolffian duct, which corresponds to the caput, and a gradual reduction was observed along the rest of the duct. Thus, the degree of *Inhba* expression corresponded to the level of epididymal coiling. At embryonic day 15.5, *Inhba* mRNA was expressed only in the mesenchyme of the Wolffian duct, but not the epithelium. However, phosphorylated SMAD2/3 was observed in the epithelial nuclei, but not in the mesenchyme. Moreover, during the period of embryonic day 15.5 to 19.5, increased proliferation of epithelial cells occurred, leading to an expansion and coiling of the anterior Wolffian duct. In *Inhba*$^{-/-}$ mice lacking activin A, there was no such cellular proliferation during this period, and the epididymis remained uncoiled (Tomaszewski *et al.*, 2007).

Therapeutic potential of targeting activins in diseases with elevated activin levels

Since activin is a major regulator of inflammation, drives fibrosis, promotes proliferation of several cancers and causes cachexia (Jones *et al.*, 2007; de Kretser *et al.*, 2013; Chen *et al.*, 2014; Loumaye *et al.*, 2015), the therapeutic potential of antagonizing activin has been assessed over the past decade. Several studies have evaluated the potential of using endogenous activin inhibitors, such as follistatin, or synthetic activin antagonists, as therapeutic agents.

The ability of follistatin to counteract the pro-inflammatory and fibrotic actions of activin marks follistatin as a potential therapeutic in inflammatory diseases (Jones *et al.*, 2007; Maeshima *et al.*, 2014; Hardy *et al.*, 2015). Several studies have shown that the cachectic effects of activin A can be ameliorated by follistatin (Cipriano *et al.*, 2000; Chen *et al.*, 2014), raising the possibility of using follistatin in the treatment of patients suffering from cancer cachexia.

Several approaches to develop synthetic activin antagonists have been reported over the past years (Table II). A small molecule inhibitor, SB 431542, has been shown to antagonize activin signalling (Harrison *et al.*, 2005). SB 431542 inhibited SMAD2 phosphorylation, and inhibited the proliferation of human malignant glioma cell lines (Hjelmeland *et al.*, 2004). However, SB 431542 acts by inhibiting Alk4, Alk5 and Alk7, thus

Table II Exogenous activin antagonists.

Antagonist	Description	Mechanism of antagonism	References
SB 431542	Small molecule inhibitor	Inhibits ALK4 and ALK7 (Type I activin receptors)	Matsuyama *et al.* (2003), Inman *et al.* (2002) and Hjelmeland *et al.* (2004)
Soluble Activin Receptor II	Soluble fusion proteins composed of the extra-cellular ligand binding domain of the activin receptors (ActRIIA or Act RIIB) coupled to the Fc fragment of IgG	Inhibits activin signalling by trapping activin ligands	Cadena *et al.* (2010), Suragani *et al.* (2014), Chiu *et al.* (2013), Chantry *et al.* (2010) and Lotinun *et al.* (2010)
Modified prodomains of activin A and activin B	Fusion proteins composed of activin prodomains attached to the Fc fragment of IgG, together with fastner residues.	Bind with high affinity to activins A, or both activin A and B, to specifically inhibit their biological activity	Chen *et al.* (2015)

ALK, activin receptor-like kinase; IgG, immunoglobulin G.

TGF-β signalling in general (Inman *et al.*, 2002; Matsuyama *et al.*, 2003; Hjelmeland *et al.*, 2004).

Some of the most discussed activin-targeted therapeutics are the soluble activin receptors. These are fusion proteins composed of the extra-cellular ligand binding domain of the activin receptors coupled to the Fc fragment of immunoglobulin G (IgG) (Cadena *et al.*, 2010; Chiu *et al.*, 2013). Administration of a soluble ActRIIB to aged, castrated mice showed an improvement in muscular force and muscle mass, together with a rise in bone mineral density (Cadena *et al.*, 2010; Chiu *et al.*, 2013). Furthermore, soluble ActRIIA promoted osteogenesis and increased bone mass in healthy mice and non-human primates (Chantry *et al.*, 2010; Lotinun *et al.*, 2010), and also prevented osteolytic lesions in mice with myeloma and improved survival. Moreover, it also blocked bone metastasis in a mouse model of breast cancer (Chantry *et al.*, 2010). In addition to these effects on muscle and bone metabolism, soluble activin receptors have been shown to correct anaemia. ActRIIB promoted erythropoiesis in rodents and non-human primates, while reducing or preventing anaemia in mice (Suragani *et al.*, 2014). Furthermore, ActRIIA corrected anaemia by enhancing erythropoiesis, and minimized iron overload in a murine model of β-thalassemia. However, this effect was brought about by inhibiting another member of the TGF- β superfamily, the growth differentiation factor 11 (GDF 11). Thus, it is clear that soluble activin receptors can target other growth factors belonging to the TGF- β superfamily, which act via the same receptors.

While the effects of SB 431542, and in particular, the soluble activin receptors, are certainly promising, their ability to affect the widespread TGF-β superfamily members which are involved in a plethora of biological activities is undesired. Thus, it is imperative that more 'narrow spectrum' therapeutics, which target activins specifically, are developed. Such specific interventions will minimize adverse side effects and effectively provide the targeted outcome.

Recently, Chen *et al.* (2015) developed a more specific activin antagonist by modifying the prodomains of activin A and activin B. These molecules were formed by fusing the prodomains to the Fc fragment of IgG, and then adding fastner residues such as lysine, tyrosine histidine and alanine. The fastner residues, which are found in TGF-β1, contribute to latency of mature TGF- β1, a feature not found in activins. Incorporating these fastner residues and the fusion with IgG-Fc enables the modified prodomains to bind with high affinity to activins A and B, thus specifically inhibiting their biological activity. These activin antagonists have been shown to be capable of antagonizing activin-induced cachexia in mice. While the activin A prodomain specifically inhibited activin A, the activin B prodomain inhibited both activins A and B. Furthermore, these two molecules did not exhibit high affinity towards myostatin and GDF-11 (Chen *et al.*, 2015). While this is an exciting development, further studies would confirm the efficacy of these new activin antagonists. Although therapeutic targeting of activin in disorders of the reproductive system specifically has not yet been attempted, advancements in this area would definitely be beneficial to the field of reproductive pathology.

Conclusion

Since its initial discovery as an FSH stimulating hormone, activin A is now known for its multifaceted roles in reproductive biology, embryonic development and the control of inflammation and fibrosis in addition to other important physiological and pathological processes. While the essential roles of activins in gonadal development and regulation of gametogenesis is well established, current research identifies novel roles for these agents in maintaining the structure and function of the entire reproductive system and other organ systems. Further research is required to evaluate the extensive actions of activin A and to further understand the roles of activin B. These fields are rapidly evolving with data emerging as to the actions of these agents and their modulators in the physiology and pathology of humans and other mammals. The fact that activin A is elevated both locally and systemically in major reproductive system-related disorders makes it an important biomarker for diagnostic purposes. Moreover, given the established role of activin A as a pro-inflammatory and pro-fibrotic agent, studies of its role in disorders of reproduction resulting from these processes should be examined. Follistatin, as a key regulator of the biological actions of activin, should be evaluated as a therapeutic agent in conditions where overexpression of activin A is established as a contributing factor.

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Authors' roles

R.W. drafted the manuscript; D.M.d.K. provided critical comments and revised the manuscript.

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Conflict of interest

R.W.: no conflict of interest. D.M.d.K.: declares a conflict of interest as he is a shareholder in Paranta Biosciences, a company developing follistatin as a therapeutic.

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