REPRODUCTION

Nodal signalling and apoptosis

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Abstract

Nodal, a member of the transforming growth factor β family, was first cloned from a 7.5 day post-coitum mouse embryo cDNA library. Nodal exerts its biological effects by signalling through its types I and II serine/threonine kinase receptor complex and intracellular Smad proteins. The type II receptors for Nodal are Activin type II receptors ActRIIA and ActRIIB, whereas the putative type I receptors are Activin receptor like kinase (ALK) 4 and ALK7. The main Smad proteins involved in Nodal signalling are Smad2 and Smad3. Studies of Nodal in adult tissues indicate that it is pro-apoptotic in rat ovarian granulosa cells, human trophoblast cells and human ovarian epithelial cancer cells and is growth inhibitory in the latter two cell types. This review summarises the progress made on the functions of Nodal in the apoptosis of adult tissues, especially in the ovary and placenta.

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Introduction

Apoptosis is an evolutionarily conserved mode of programmed cell death across the animal kingdom. It plays important roles in numerous physiological events in mammals, including ovarian follicular atresia, decidualisation and placentation during embryo implantation, as well as many pathological conditions. Nodal, a member of the transforming growth factor (TGF) β family, exerts its biological effects by signalling through a cell surface serine/threonine kinase receptor complex of types II and I receptors and intracellular Smad proteins. Nodal plays an important role in the induction of dorsal mesoderm, anterior patterning and formation of left–right asymmetry during early embryo development. In extra-embryonic tissues, studies have been limited. Nodal is capable of inducing apoptosis and inhibiting cell growth. This review will focus on the role of Nodal in apoptosis in the ovary and placenta, as well as its possible physiological and pathophysiological roles in folliculogenesis, follicular atresia, ovarian cancers and placentation.

TGFβ family members and their signalling mechanisms

The transforming growth factor (TGF) β family consists of a large group of growth and differentiation factors, including several TGFβ proteins, bone morphogenetic proteins (BMPs), Activins/Inhibins, growth and differentiation factors (GDFs), as well as Nodal and its related proteins (Massague & Chen 2000, Chang *et al.* 2002). Members of TGFβ family are multifunctional proteins that regulate cell proliferation and differentiation, extracellular matrix modification, angiogenesis, apoptosis and immunosuppression (Schier & Shen 2000, Feng & Derynck 2005, Jones *et al.* 2006).

A TGFβ protein exerts its function by binding to and bringing together on the cell surface types I and II receptors to form a ternary ligand–receptor complex (Massague 1998). Five members of type II and seven members of type I receptors (Activin receptor like kinase (ALK) 1–7) have been characterised in mammals (Peng 2003). Upon phosphorylation by the type II receptor, the type I receptor phosphorylates and activates Smads, which are intracellular signalling molecules for members of the TGFβ superfamily. Smad2 and Smad3 respond to TGFβs, Activins, Nodal and Lefty, whereas Smad1, Smad5 and Smad8 mediate BMP signalling (Miyazawa *et al.* 2002). Following receptor activation, phosphorylated Smads are released from the receptors and form oligomeric complexes with common-partner Smad, Smad4, and translocate into the nucleus to regulate the transcription of target genes.

Nodal

Nodal was first cloned from a 7.5 day post-coitum mouse embryo cDNA library (Zhou *et al.* 1993). Like other members of the TGFβ family, Nodal exerts its biological effects by signalling through its type I and type II serine/threonine kinase receptor complex and Smad2, Smad3 and Smad4. The receptor-activated Smad2 and Smad3 form a complex with Smad4, which is imported into the nucleus where they, with the aid of transcriptional activators or inhibitors, bind to the *cis*-acting elements in the promoter region of the target gene to modulate gene transcription (Findlay *et al.* 2001).

ALK4 and ALK7 are the two putative type I receptors for Nodal (Tsuchida *et al.* 1996, Reissmann *et al.* 2001). The type II receptors for Nodal are thought to be Activin type II receptors ActRIIA and ActRIIB. ALK7 was first cloned from the rat as an orphan receptor (Tsuchida *et al.* 1996). The serine/threonine kinase domain of ALK7 is similar to that of ALK4 but its extracellular domain is unique and bears no similarity to that of any other ALKs. These features suggest that ALK7 may possess signalling properties similar to those of other TGFβ and Activin receptors but have different ligand specificity. Indeed, ALK7 is not capable of binding TGFβ, Activin or BMP7 even in the presence of the type II receptors as reported by Reissmann *et al.* (2001). However, Activin AB and Activin B are thought to be ligands for ALK7 in the pancreatic β cell line MIN6 (Tsuchida *et al.* 2004). Recent study with gonadotrope cells also revealed that Activin B signals through both ALK4 and ALK7 (Bernard *et al.* 2006).

Based on studies in the mouse, *Xenopus* and zebrafish (Zhang *et al.* 2004), Nodal is identified as a critical regulator of early vertebrate development which is involved in the induction of dorsal mesoderm, anterior patterning and formation of left–right asymmetry (Iannaccone *et al.* 1992, Brennan *et al.* 2002, Eimon & Harland 2002). Nodal knockout is lethal, resulting from defects in primitive streak formation (Gu *et al.* 1998). On the other hand, Nodal inhibits the differentiation of rat trophoblast stem cells into giant cells (Iannaccone *et al.* 1992, Ma *et al.* 2001). Similarly, it has been suggested that Nodal regulates human placental development (Roberts *et al.* 2003). Pro-apoptotic and growth inhibitory effects of Nodal have been reported in ovarian granulosa cells (Wang *et al.* 2006), human trophoblast cells (Munir *et al.* 2004) and ovarian epithelial cancer cells (Xu *et al.* 2004, 2006). Moreover, activation of ALK7 is capable of inducing apoptosis in hepatoma cells (Kim *et al.* 2004) and pancreatic β cells (Zhang *et al.* 2006).

Nodal and ovarian follicular atresia

In the ovary, more than 99% of the developing follicles fail to mature fully but instead undergo atresia (Amsterdam *et al.* 1997). Follicular atresia is a negative and selective degenerative process during follicular growth and development, and occurs as a consequence of apoptotic cell death of the follicular somatic cells and of the oocyte (Chun & Hsueh 1998, Asselin *et al.* 2001). The destiny of the developing follicles (continual growth and eventual ovulation or atresia) is dependent on the fate of the granulosa cells within them (i.e. proliferation, differentiation or apoptosis), which is determined by the coordinated actions and interactions of cell survival and death factors, including follicle-stimulating hormone (FSH), GDF-9, Nodal and other growth factors and cytokines (Jiang *et al.* 2003, Craig *et al.* 2007). In mammals, two basic apoptotic pathways have been defined: the mitochondria-mediated pathway and the death receptor pathway (Hussein *et al.* 2003). Interaction between pro- and anti-apoptotic members of the Bcl-2 family of proteins in the mitochondrion determines whether pathways for apoptosis are activated or suppressed (Zinkel *et al.* 2006). Release of mitochondrial cytochrome *c*, second mitochondria-derived activator of caspase (Smac/DIABLO) and Omi/HtrA2 activates apoptosis (Labedzka *et al.* 2006). Cytochrome *c* forms a complex (apoptosome) with apoptosis-activating factor-1 (Apaf1) and procaspase-9, thus activating caspase-9. Activated caspase-9 cleaves and activates downstream effector caspases, including caspase-3, resulting in induction of apoptosis (Hussein 2005).

Nodal and its type I receptor ALK7 are expressed in a cell type-specific and follicular stage-dependent manner during folliculogenesis (Wang *et al.* 2006). Nodal immunoreactivity in granulosa cells is the strongest in pre-antral follicles when compared with the signals from later stages of development. In addition, immunosignals of Nodal are also detected in the theca cell layer of antral follicles. In contrast, ALK7 was mainly detected in the interstitial cells at the pre-antral stage of development, while it was present in granulosa cells, but not theca cells in antral follicles. Throughout antral growth, ALK7 is highly expressed in granulosa cells, whereas Nodal content in the granulosa cells decreases as the follicle transits from the pre-antral to antral stage of development, implying that during development through the penultimate stage, the granulosa cell acquires their ability to undergo apoptosis and initiate atresia (due to the presence of ALK7) but fail to do so due to low level of its ligand (Wang *et al.* 2006). During declining gonadotrophin support (e.g. artificial gonadotrophin withdrawal; Boone *et al.* 1997), the antral follicle destined for atresia not only expresses an increased level of Nodal but also shows co-localisation of both the ligand and its receptor in the granulosa cells. In addition, the relative abundance of Nodal mRNA is significantly higher in granulosa cells from atretic antral follicles than healthy ones, suggesting that increased granulosa cell Nodal expression may be a physiological signal for the induction of atresia.

Addition of recombinant Nodal to cultures of granulosa cells from large antral and pre-ovulatory follicles significantly increases granulosa cell apoptosis (Wang *et al.* 2006). Increased apoptosis can also be observed in granulosa cells infected with adenoviral Nodal when compared with LacZ control (Wang *et al.* 2006), suggesting that Nodal is a death signal for granulosa cells. Granulosa cells co-infected with adenoviral-dominant negative ALK7 and Nodal exhibit lower extent of apoptosis when compared with the cells infected with Nodal alone, providing support to the concept that Nodal is able to activate endogenous ALK7 (Wang *et al.* 2006). In addition, forced expression of an activated ALK7 mimics the pro-apoptotic effect of Nodal and significantly increases granulosa cell apoptosis, thus providing support that the Nodal/ALK7-signalling pathway is pro-apoptotic in granulosa cells (Wang *et al.* 2006).

It has been demonstrated that Nodal binds to ALK7 or ALK4 and activates the Smad2/3 signalling pathway (Findlay *et al.* 2001). Granulosa cells infected with adenoviral Nodal or an activated ALK7 exhibit increased phospho-Smad2 (ser465/467) level and phospho-Smad2/Smad2 ratio, and increased nuclear import of phopsho-Smad2 and apoptosis in the granulosa cells, suggesting a role of Smad2 in Nodal/ALK7-mediated granulosa cell apoptosis (Wang *et al.* 2006).

It has been demonstrated that Nodal or ALK7 activation down-regulates granulosa cell X-linked inhibitor of apoptosis protein (XIAP) mRNA and protein content (Wang *et al.*). XIAP, a well studied member of the inhibitor of apoptosis protein family, is a determinant of granulosa cell survival and follicular growth (Asselin *et al.* 2001). XIAP is an intracellular anti-apoptotic protein, which functions as a direct inhibitor of caspases-3, -7 (Deveraux *et al.* 1997) and -9 (Deveraux *et al.* 1998). XIAP can also function as a E3 ubiquitin ligase for caspase-3 (Yang *et al.* 2000, Suzuki *et al.* 2001) and Smac (Hu & Yang 2003). In addition, XIAP can bind to Smac/DIABLO and HtrA2/Omi, leading to the neutralisation of death signals in the cytosol and preventing apoptosis (Datta *et al.* 1999, Chung *et al.* 2002). Furthermore, ALK7-mediated apoptosis can be attenuated by forced expression of XIAP or facilitated by XIAP down-regulation (Wang *et al.* 2006). Dominant negative Smad2 expression attenuates ALK7-mediated XIAP down-regulation, suggesting that the decrease in XIAP content by Nodal involves Smad signalling.

The phosphotidyl inositide-3 kinase (PI3K)/Akt pathway plays an important role in regulating granulosa cell apoptosis (Asselin *et al.* 2001). Akt, also known as protein kinase (PK) B, is a serine/threonine PK downstream of PI3K (Bellacosa *et al.* 1991, Cheng *et al.* 1992, Nakatani *et al.* 1999) and an important anti-apoptotic factor. A number of Akt substrates are pro-apoptotic, including Bcl-2-associated death promoter (BAD), caspase-9, forkhead transcription factors and apoptosis

signal-regulating kinase 1 (ASK1) (Datta *et al.* 1999, Kim *et al.* 2001). Their function is attenuated by Akt phosphorylation. Overexpression of Nodal or ALK7 activation can significantly decrease phospho-Akt and phospho-Akt/Akt ratio. Furthermore, activation of Akt decreases ALK7-mediated mitochondrial Smac, Omi and cytochrome *c* release and attenuates granulosa cell apoptosis (Wang *et al.* 2006).

Although the above studies have significantly advanced our current understanding on the molecular and cellular mechanisms regulating follicular development and atresia, much remains to be learned on the regulation on follicular Nodal and ALK7 expression during folliculogenesis and their downstream signalling pathways involved in the regulation of follicular cell fate and follicle destiny. Wang *et al.* (2006) proposed a hypothetical model to guide possible future investigations. In the healthy antral follicles, Nodal is localised in the theca cells, whereas its type I receptor ALK7 is in the granulosa cells. Uncoupling of the ligand from its receptor renders the Nodal-signalling pathway silent. FSH increases XIAP expression and activates the PI3K–Akt pathway. The up-regulation of Akt and XIAP suppresses the release of mitochondrial death proteins and therefore inhibits caspase activation and activities, and promotes cell survival (Wang *et al.* 2003). However, in mid- to late-follicular stages, FSH levels decline and induce the co-localisation of Nodal and ALK7 in granulosa cells, triggering ALK7 signalling and downstream events, including Smad2 activation, Akt inhibition and XIAP down-regulation. Inactivation of Akt increases mitochondrial release of Smac, Omi and cytochrome *c*, thus activating granulosa cell caspases and apoptosis, and eventually follicular atresia.

Nodal and apoptosis in ovarian cancer cells

Ovarian cancer is the most fatal gynaecological malignancy in the Western world and ranks fifth among the most common female cancers (Ries *et al.* 2000). Ovarian cancer that originates from the ovarian surface epithelium constitutes ∼90% of all cancers in the ovaries (Auersperg *et al.* 2001). The homeostasis of human ovarian surface epithelial cells is maintained by a delicate balance in the expression and actions of tumour suppressors and cell survival factors (Cheng *et al.* 2002). Dysregulation of the homeostatic mechanism(s) may lead to ovarian cancers and to chemoresistance. mRNAs for Nodal, ActRIIB, the type II receptor partner of ALK7 and Smad2, 3 and 4, as well as four transcripts of ALK7 (ALK7-1, -2, -3 and -4) are expressed in both chemosensitive and chemoresistant ovarian cancer cell lines (Xu *et al.* 2004). Overexpression of Nodal significantly increases apoptosis, a response that could be mimicked by introduction of the constitutively active form of ALK7 (ALK7-ca; Xu *et al.* 2004). Interestingly, ALK7-ca and Nodal induce a greater extent of apoptosis in the

chemosensitive cells (A2780-s) when compared with its chemoresistant counterpart (A2780-cp), which is associated with decreased XIAP expression at both mRNA and protein levels (Xu *et al.* 2004). XIAP levels in Nodal-transfected or ALK7-activated chemosensitive cells are also significantly lower than those in the chemoresistant variant, implying that the responsiveness of the ovarian cancer cells to Nodal and ALK7 activation may be related to the cellular level of XIAP (Xu *et al.* 2004). This is also consistent with the observation that activation of the Nodal/ALK7 pathway also significantly increases the cleavage/activation of caspases-9 and -3 (Xu *et al.* 2004). Since XIAP is an inhibitor of caspases-9 and -3, down-regulation of XIAP by Nodal may be the mechanism by which Nodal activates caspases-3 and -9 and induces apoptosis in ovarian cancer cells (Xu *et al.* 2004).

Using the immortalised ovarian surface epithelial cell line IOSE397 and the ovarian cancer cell line OV2008 Xu *et al.* (2006) found that both normal and malignant ovarian epithelial cell lines expressed the signalling molecules of the Nodal–ALK7 pathway, including the ligand, receptors and Smads. Activation of ALK7 effectively induces apoptosis, which is accompanied by up-regulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2 and Bcl-XL, disruption of the mitochondrial membrane potential as well as increased mitochondrial release of cytochrome *c* (Xu *et al.* 2006). It has been proposed that Nodal activates ActRIIB and ALK7 and induces Smad2/3 activation, which in turn regulates the expression of Bax and Bcl-2. The decrease in Bcl-2/Bax ratio results in the release of cytochrome *c*, activation of caspase-3 and subsequent apoptosis (Xu *et al.* 2006).

Our knowledge on the mechanism(s) by which Nodal/ALK7 signalling induces apoptosis remains incomplete. Overexpression of an activated ALK7 can also induce apoptosis in hepatoma cells and pancreatic β cells (Kim *et al.* 2004, Zhang *et al.* 2006). However, since Activin AB and Activin B can also bind to ALK7 (Tsuchida *et al.* 2004, Bernard *et al.* 2006), the ability of ALK7 to induce apoptosis does not in itself implicate Nodal as a pro-apoptotic ligand. However, several mechanisms of ALK7-induced apoptosis have been proposed, although available evidence is limited (Kim *et al.* 2004, Zhang *et al.* 2006), including: (1) ALK7-mediated c-Jun N-terminal kinase (JNK) activation. It has been demonstrated that ALK7 activation increases levels of active and phosphorylated JNK, but not of p38 or extracellular signal-regulated kinase (Erk). Expression of dominant negative form of stress-activated PK/Erk kinase (SEK), an upstream kinase of JNK, abolishes ALK7-induced JNK activation and inhibits apoptosis (Kishimoto *et al.* 2003). (2) Activation of mitochondria- but not death receptor-mediated cell death pathway. This notion is supported by the observation that ALK7-induced apoptosis in FaO rat hepatoma cells is accompanied by activation of caspases-3 and -9, but not by caspases-7 and -8. In addition, the pan-caspase inhibitor Z-VAD-

fmk significantly attenuates ALK7-induced apoptosis which further support the conclusion that caspase activation is necessary for the induction of apoptosis. On the other hand, ALK7 induces SEK1-mediated mitochondrial cytochrome *c* release, which is necessary for the formation of the apoptosome with Apaf1 and procaspase-9 (Greenleaf *et al.* 1979). (3) Activation of Smad3 pathway. Overexpression of ALK7 in FaO rat hepatoma and Hep3B human hepatoma cells activates Smad2 and Smad3 (Kim *et al.* 2004). Smad3 RNA interference in the ALK7-infected Hep3B cells significantly inhibits caspase-3 cleavage (Kim *et al.* 2004), suggesting that Smad3 is indispensable in ALK7-induced apoptosis. (4) Suppression of Akt activation and the activation of the Smad2-caspase-3 cascade. This notion is supported by the observation that ALK7 activation in pancreatic β cells increases Smad2 phosphorylation, reduces Akt kinase activity and increases levels of active caspase-3 (Zhang *et al.* 2006).

Nodal and apoptosis during plancentation

During early placentation, the human trophoblast differentiates into the villous and extravillous trophoblasts (Huppertz *et al.* 2006). In both types of trophoblast, apoptosis plays a role in normal cell turnover, ensuring that non-functional cells are eliminated without a local inflammatory reaction from the maternal host (Huppertz *et al.* 2006). Nodal mRNA is expressed in the placenta at various gestational ages and in the choriocarcinoma cell line JEG-3 cells. mRNAs for ALK7 are also present in the human placentae, with all four transcripts of ALK7 present also in JEG-3 cells (Xu *et al.* 2004). Placental ALK7 from all three trimesters of pregnancy migrates on SDS–PAGE as two bands of 58 and 52 kDa, probably attributable to the glycosylated forms of the full-length and truncated ALK7 (tALK7) respectively. Full-length ALK7 content is constant throughout gestation, whereas tALK7 exhibits a significantly higher level in the third trimester than in the earlier stages. Overexpression of ALK7 and Nodal markedly increases apoptosis in JEG-3 cells (Munir *et al.* 2004). In addition, an increased level of active caspase-3 is detected in cells transfected with Nodal- and ALK7-ca, implying a role of Nodal in the induction of apoptosis during normal placentation (Munir *et al.* 2004). The physiological role of the Nodal/ALK7 pathway during pregnancy is not known, nor is its possible involvement in the aetiology of pregnancy complications such as preeclampsia, intrauterine growth restriction and habitual abortion.

Future studies

This review focuses on the function of Nodal in apoptosis in the ovaries and placenta. Apoptosis occurs in the mammalian ovaries under physiological conditions and
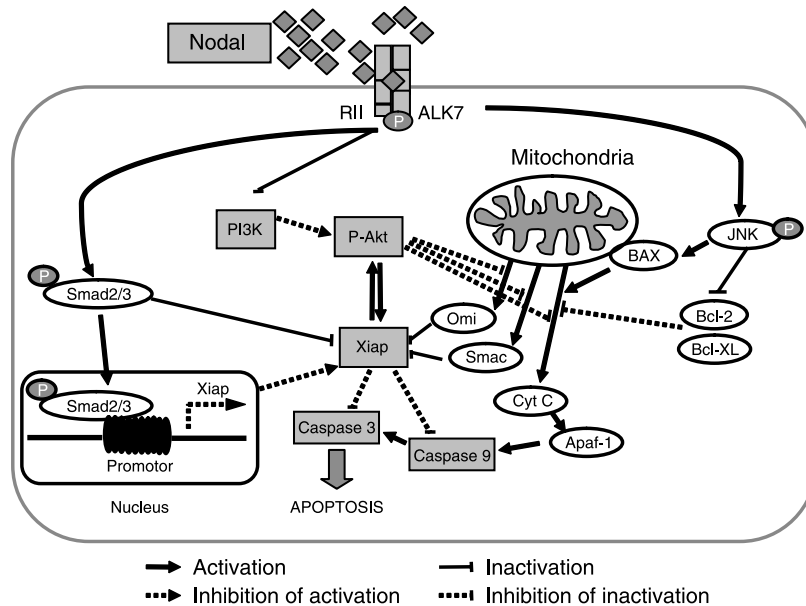
Figure 1 Summarised apoptotic pathways triggered by Nodal/ALK7 signalling. Nodal induces apoptosis via activation of its type I receptor, ALK7, which (1) stimulates the phosphorylation of Smad2 and Smad3, resulting in the down-regulation of XIAP, (2) activates JNK signalling which down-regulates Bcl-2 and Bcl-X and up-regulates Bax, (3) inhibits Akt and triggers the mitochondrial release of Smac, Omi and Cytochrome *c*. These responses in turn activate caspases and ultimately induce apoptosis.

plays important roles in ovarian folliculogenesis and follicular atresia. During embryo implantation, apoptosis is important for decidualisation and placentation (Huppertz *et al.* 2006). Dysregulation in apoptosis leads to pathological states, including cancers, pregnancy-related diseases or ovarian dysfunctions. Several recent studies have indicated that Nodal is able to induce apoptosis in ovarian granulosa cells, ovarian epithelial cancer cells and choriocarcinoma cells. Furthermore, these studies have established that Nodal and ALK7 activation down-regulates the cell survival factors XIAP and Akt, activates the mitochondrial death pathway as well as caspases-3 and -9, but not caspases-7 and -8. The signalling mechanism of Nodal-induced apoptosis is summarised in Fig. 1. However, much remains to be learned about the precise molecular mechanism of Nodal-induced apoptosis in these reproductive tissues and the physiological relevance of these findings in other reproductive events.

Nodal exerts its biological function via type I receptors ALK4 and ALK7 and intracellular Smad2/3. The above studies only shed light on the importance of ALK7, but not ALK4, in Nodal-induced apoptosis. Whether ALK4 is involved in Nodal-induced apoptosis has not been reported. In addition, Smad3 is only known to be necessary for ALK7-induced apoptosis in hepatoma cells. However, Smad2 is indispensable in Nodal/ALK7-induced apoptosis based on the results from ovarian granulosa cells and cancer cells, as well as from placental tissues. Whether indeed both Smad2 and Smad3 are equally important in Nodal-induced apoptosis and whether their involvement is cell type-dependent need

to be further investigated. Equal attention should also be drawn to study the molecular mechanisms involved in Nodal-induced apoptosis.

Combinatorial interactions of heteromeric TGFβ receptors and Smad complexes provide a high degree of signalling specificity and versatility. While Nodal, Activin AB and Activin B share the same type I receptor ALK7, TGFβs, Nodal, Activin, GDF-9 and Lefty activate the same R-Smads, suggesting that crosstalks between Nodal and other members of TGFβ family may be operational. Activation of Smads by different ligands may result in different cellular response. For example, although both GDF-9 and Nodal activate Smad2, the former promotes folliculogenesis while the later induces follicular atresia. This suggests that, in addition to the activation of Smads, other signalling pathways may also be involved in the action of these TGFβ family members. Their identity and the nature of the interactions with the Smads signalling pathway also need to be further investigated.

Acknowledgements

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