Role of Nodal signalling in testis development and initiation of testicular cancer

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Abstract

Testicular development from the initially bipotential gonad is a tightly regulated process involving a complex signalling cascade to ensure proper sequential expression of signalling factors and secretion of steroid hormones. Initially, Sertoli cell specification facilitates differentiation of the steroidogenic fetal Leydig cells and establishment of the somatic niche, which is critical in supporting the germ cell population. Impairment of the somatic niche during fetal life may lead to development of male reproductive disorders, including arrest of gonocyte differentiation, which is considered the first step in the testicular cancer pathogenesis. In this review, we will outline the signalling pathways involved in fetal testis development focusing on the Nodal pathway, which has recently been implicated in several aspects of testicular differentiation in both mouse and human studies. Nodal signalling plays important roles in germ cell development, including regulation of pluripotency factor expression, proliferation and survival. Moreover, the Nodal pathway is involved in establishment of the somatic niche, including formation of seminiferous cords, steroidogenesis and Sertoli cell function. In our outline of fetal testis development, important differences between human and mouse models will be highlighted to emphasise that information obtained from mouse studies cannot always be directly translated to humans. Finally, the implications of dysregulated Nodal signalling in development of the testicular cancer precursor, germ cell neoplasia *in situ*, and testicular dysgenesis will be discussed – none of which arise in rodents, emphasising the importance of human models in the effort to increase our understanding of origin and early development of these disorders.

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Introduction

Testicular differentiation involves a tightly regulated and complex signalling cascade that is currently not completely characterised, although progress is continuously made in our understanding of the specific roles of signalling factors involved. Recently, several studies have implicated Nodal (and Activin) signalling as important pathways in the signalling cascade directing fetal testis development, with distinct roles in the regulation of germ cell development as well as in the establishment and function of the somatic niche (Souquet *et al.* 2012, Spiller *et al.* 2012, Miles *et al.* 2013, Wu *et al.* 2013, Tian-Zhong *et al.* 2016, Jørgensen *et al.* 2018). Understanding the mechanisms by which these pathways are involved and interact is important as even small alterations in signalling at this sensitive time point may result in failure of normal testicular development and/or function, which is associated with a number of male reproductive disorders later in life. The regulation of germ cell survival, proliferation and differentiation required to ensure the establishment of a normal germ cell population depends upon a complex

combination of signalling factors and the interaction between germ cells and the surrounding somatic cells that together form the microenvironment known as the somatic niche (reviewed in Rotgers *et al.* 2018). It is hypothesised that even slightly altered signalling between germ cells and the somatic niche during fetal development can impair differentiation of a sub-population of gonocytes that instead persist in their undifferentiated state with continued expression of pluripotency factors (reviewed in Rajpert-De Meyts 2006, Rajpert-De Meyts *et al.* 2016). This is considered to be the first step in the development of the testicular cancer precursor, germ cell neoplasia *in situ* (GCNIS), which later in life develops into testicular germ cell tumours (TGCTs) (Rajpert-De Meyts 2006, Rajpert-De Meyts *et al.* 2016). Altered signalling or timing of the expression of key factors in the testicular differentiation pathway may also impair the establishment of the somatic niche, which involves specification of Sertoli, fetal Leydig and peritubular myoid cell lineages as well as organisation and growth of the testis, including formation of the seminiferous cords. The effects of subtle alterations in signalling pathways during testicular

development is considered the basis of the testicular dysgenesis syndrome (TDS), which comprise disorders of male reproductive health that manifest either at birth (cryptorchidism, hypospadias) or in young adulthood (reduced sperm counts, testicular germ cell cancer, primary hypogonadism), with a common origin that is a consequence of impaired testicular differentiation and/or function during fetal life (Skakkebaek *et al.* 2001, 2016).

Fetal testis development

During embryonic development, bipotential gonads (genital ridges) arise from thickening and proliferation of the coelomic epithelium, overlying the ventral-medial surface of the mesonephros. In humans, they appear around gestational week (GW) 4 and in mice they emerge around 10 days post coitum (dpc) (Wilhelm *et al.* 2013). Around GW 5–6 in humans and 10.5 dpc in mice, the genital ridges become colonised by primordial germ cells (PGCs), the precursor cells of spermatozoa and oocytes, which have migrated from the proximal epiblast via the hindgut to the genital ridges (Fujimoto *et al.* 1977, Molyneaux *et al.* 2001, Wilhelm *et al.* 2013).

The sex-specific development of the bipotential gonads into testes or ovaries is determined by the presence or absence of the Y chromosome, respectively. The Y chromosome gene *sex-determining region of chromosome Y (SRY)* initiates the signalling cascade that results in testicular development. *Sry* is transiently upregulated around 10.5 dpc in mice and in humans the expression of *SRY* is initiated around GW 6 (Berta *et al.* 1990, Koopman *et al.* 1990, Sinclair *et al.* 1990, Hacker *et al.* 1995, Hanley *et al.* 2000). Expression of *SRY* induces upregulation of male-specific genes in the gonad, including *SOX9* (*Sry-box containing gene 9*), which promotes differentiation of pre-Sertoli cells. *Sox9* expression triggers the expression of *Fgf9* and *Amh* in Sertoli cells, which act to suppress female fate pathways and ensure regression of the female Müllerian ducts, respectively, thereby promoting male differentiation (reviewed in Rotgers *et al.* 2018). Upon arrival of PGCs in the developing gonads, the germ cells are termed gonocytes in XY individuals. As a result of the initiation and progression of the male signalling pathway, the gonocytes and Sertoli cells become enclosed within the forming seminiferous cords, dividing the gonads into two compartments – the seminiferous cords containing the Sertoli cells and germ cells, and the interstitial space consisting of fetal Leydig cells, progenitors of adult Leydig cells and peritubular myeloid cells (Wilhelm *et al.* 2013). In mice, the seminiferous cords become evident at 12.5 dpc and in human testes they are distinguishable from around GW 8–9 (Brennan & Capel 2004, Ostrer *et al.* 2007).

Germ cells within the fetal gonads are highly proliferating during first trimester in both sexes, but with a substantially higher number of germ cells present in fetal

ovaries (approximately 250,000 in GW 9) compared to fetal testes (30,000 germ cells in GW 9) (Bendsen *et al.* 2003, 2006). Another important distinction between male and female germ cells around this developmental time-point is the initiation of the first meiotic division in a sub-population of oogonia within fetal ovaries, while in fetal testes, initiation of meiosis is actively prevented. In mice, female germ cells enter meiosis at 13.5 dpc, whereas in humans, meiosis is initiated in an asynchronous manner starting from around GW 10–11 (Gondos *et al.* 1986, Bullejos & Koopman 2004, Stoop *et al.* 2005, Bendsen *et al.* 2006). Initiation of meiosis is mediated at least in part by actions of retinoic acid (RA), which promote upregulation of the pre-meiosis marker *Stra8* (*stimulated by retinoic acid 8*). Expression of additional factors involved in meiosis are subsequently initiated, resulting in meiotic entry and first meiotic division (Gaemers *et al.* 1996, Koubova *et al.* 2006, Le Bouffant *et al.* 2010, Jørgensen *et al.* 2012, Griswold 2016). In contrast, the initiation of meiosis is inhibited in fetal testes until puberty and in mice the germ cells enter a quiescent state. The primary mechanism by which meiosis is inhibited in mouse testes involves the actions of *Cyp26b1* (*cytochrome P450, family 26, subfamily b, polypeptide 1*), which is a RA-metabolising enzyme expressed by the Sertoli cells (Bowles *et al.* 2006, Koubova *et al.* 2006). Other factors, such as *Fgf9*, *Nanos2*, *Dmrt1* and *Nodal* have also been implicated in preventing initiation of meiosis in male germ cells in mice (Suzuki & Saga 2008, Bowles *et al.* 2010, Matson *et al.* 2010, Souquet *et al.* 2012, Wu *et al.* 2013). In human fetal testes, the mechanisms responsible for preventing meiosis are not completely understood (reviewed in Jørgensen & Rajpert-De Meyts 2014). However, several studies have found that CYP26B1 is expressed in both human fetal ovaries and testes, suggesting that, in contrast to mice, this is not a male-specific meiosis inhibitor (Childs *et al.* 2011, Jørgensen *et al.* 2012, Frydman *et al.* 2017). Conversely, the roles of other meiosis-inhibiting factors e.g. NANOS2 and DMRT1 appear to be more conserved between mice and humans based on their sexually dimorphic expression patterns in human gonads (Childs *et al.* 2011, Jørgensen *et al.* 2012). Interestingly, a recent study suggested that in humans, male and female gonads share several meiosis-preventing pathways, which likely explains the simultaneous presence of pre- and post-meiotic germ cells in human fetal ovaries (Frydman *et al.* 2017).

In the human fetal testis, gonocytes can be recognised by their expression of pluripotency factors, including OCT4, NANOG, C-KIT and AP2γ (Gaskell *et al.* 2004, Hoei-Hansen *et al.* 2004, Rajpert-De Meyts *et al.* 2004, Pauls *et al.* 2006). These factors are gradually downregulated starting from around GW 14–16 with simultaneous upregulation of MAGE-A4 expression, thereby marking the transition to pre-spermatogonia. Pre-spermatogonia are in addition to the expression of

MAGE-A4 characterised by their distinct morphology and localisation adjacent to the basement membrane. In human fetal testes, the transition from gonocytes to pre-spermatogonia constitutes an important differentiation step during testis development as failure of gonocyte differentiation is considered to be the first step in the malignant transformation of germ cells, resulting in the development of GCNIS and subsequently in TGCTs (Skakkebaek 1987, Rajpert-De Meyts *et al.* 2016).

The Nodal signalling pathway

There is increasing evidence to suggest that TGF β signalling plays important roles during fetal testis development, in particular a number of recent studies have implicated the Nodal pathway in germ cell development and establishment of the somatic niche (Souquet *et al.* 2012, Spiller *et al.* 2012, Miles *et al.* 2013, Wu *et al.* 2013, Tian-Zhong *et al.* 2016). The Nodal signalling pathway is part of the TGF β superfamily which is involved in the regulation of numerous cellular processes, including cell differentiation, proliferation and apoptosis (Schier 2009). The TGF β superfamily is generally divided into two branches based on which downstream effectors are activated. The first branch includes bone morphogenic proteins (BMPs) and the second branch consists of cytokines, growth differentiation factors (GDFs), Nodal, Activins and Inhibins. Since Nodal and Activin signal through essentially the same receptor complexes, redundancy between these signalling pathways is expected. This receptor complex consists of two serine/threonine receptors; the type I receptor (Alk4/7) and type

II receptor (ActRIIA/B). Nodal and Activin bind to the extracellular domains of the type II receptors, leading to a conformational change that allows its dimerisation with the type I receptor. Dimerisation of the receptors results in the activation via phosphorylation of their intracellular domains. This in turn leads to phosphorylation of the signalling transducer Smad2/3 that together with Smad4 forms a ternary complex and translocate to the nucleus. Here, the Smad complex activates transcription of target genes (reviewed in Schier 2009, Papanayotou & Collignon 2014) (Fig. 1).

In addition to ActRIIA/B and Alk4/7, signal transduction by Nodal requires the presence of the co-receptor Cripto (also known as teratocarcinoma derived growth factor 1; Tdgf1) that associates with the type I receptor and Nodal. Signalling by Nodal results in transcriptional activation of the *Nodal* gene itself as well as the inhibitor of the Nodal pathway *Lefty1* and *Lefty2*. Lefty acts as an endogenous inhibitor of Nodal by either binding to the obligate co-receptor Cripto, thus preventing the assembly of the required receptor complex or by direct binding to Nodal itself (Cheng *et al.* 2004). As Nodal stimulates both its own expression and the expression of the inhibitor Lefty1/2, the direct positive and negative feedback act to fine-tune signalling through this pathway (Sakuma *et al.* 2002, Schier 2009).

Role of Nodal signalling in fetal testis development

The majority of knowledge about the involvement of Nodal/Activin signalling in fetal testis development is obtained from mouse studies (Souquet *et al.* 2012, Spiller

Figure 1 Overview of the Nodal and Activin signalling pathways. The two ligands, Activin and Nodal, mediate signals through a receptor complex consisting of Alk4/7 (type I receptor) and ActRIIA/B (type II receptor), with Nodal requiring the co-receptor Cripto. Upon binding of Nodal or Activin to the receptor complex, the receptors become activated by phosphorylation of their intracellular domains. This in turn leads to phosphorylation of the transcription factors Smad2/3, which then forms a ternary complex with Smad4. The complex translocates to the nucleus and acts to regulate gene transcription together with co-activators and co-repressors. Signalling by Nodal leads to transcription of *Nodal* itself and its endogenous inhibitor *Lefty1/2*. Lefty binds to either Nodal itself or the co-receptor Cripto to exert its inhibitory effect. Follistatin is the endogenous inhibitor of Activin signalling. The effect of the Alk4/5/7 inhibitor SB431542, which is used in several referenced studies, is indicated on the figure. SB431542 inhibits the activity of the Alk4/7 receptors, thereby preventing activation of Smad2/3 and thus signalling transduction by Nodal and Activin.

et al. 2012, Miles *et al.* 2013, Wu *et al.* 2013, Tian-Zhong *et al.* 2016). However, we recently investigated the involvement of Nodal and Activin signalling in human fetal testes using a combination of *ex vivo* culture and xenografting approaches (Jørgensen *et al.* 2018). Below we will summarise and compare the currently available information regarding the role of Nodal signalling in mouse and human testicular development.

Expression pattern of Nodal and Activin signalling factors in fetal testes

In mouse fetal testes, Nodal signalling factors are predominantly expressed in the germ cells, including the co-receptor *Cripto*, *Nodal* itself and the endogenous inhibitors *Lefty1* and *Lefty2* (Souquet *et al.* 2012, Spiller *et al.* 2012, Miles *et al.* 2013, Wu *et al.* 2013). Consistently, the receptors, *Alk4/7* and *Acvr2a/b* (encoding *ActRIIA/B*), are expressed in male germ cells, indicating a role for this signalling pathway during mouse testis development. In contrast, Activin signalling factors appear to be the primary pathway in somatic cells (OCT4-negative cell population) in fetal mouse testes based on the expression of *Inhba*, *Inhbb*, *Alk4/7* and *Acvr2a/b* (Souquet *et al.* 2012, Spiller *et al.* 2012, Miles *et al.* 2013, Wu *et al.* 2013). Moreover, active Nodal/Activin signalling has been demonstrated by the presence of phosphorylated SMAD2 in both male germ cells and somatic cells at 12.5–14.5 dpc (Spiller *et al.* 2012, Wu *et al.* 2013). In accordance, *ALK4*, *ALK5*, *ACRV2B*, *NODAL*, *CRIPTO*, *LEFTY*, *INHBA* and *INHBB* are expressed in the first and second trimester human fetal testis samples (Jørgensen *et al.* 2018), with *NODAL* expression found primarily in the germ cells (Li *et al.* 2017). Thus, Nodal and Activin signalling also appear to be involved in human fetal testis development.

Nodal/Activin signalling has been shown to act downstream of the *Fgf9* pathway, which plays an important role in the early reinforcement of male fate by promoting Sertoli cell differentiation and antagonising female fate through inhibition of *Wnt4* expression (Jameson *et al.* 2012). It has been suggested that *FGF9* expression precedes and triggers the expression of *Cripto* (Spiller *et al.* 2012, Wu *et al.* 2013), which subsequently initiate expression of *Nodal* and the endogenous inhibitors *Lefty1* and *Lefty2* as a response to the increased *Cripto* expression. The onset of *Cripto* expression has been reported to be 12.5 dpc followed by *Nodal*, *Lefty1* and *Lefty2* at 13.5 dpc (Spiller *et al.* 2012). Similarly, *Nodal* expression was shown to be highest from 12.5 to 13.5 dpc, with a subsequent decline in the expression at 13.5–15.5 dpc (Miles *et al.* 2013). In contrast, Souquet *et al.* found a peak in expression of all three Nodal pathway genes at 11.5 dpc (Souquet *et al.* 2012). Despite these minor discrepancies, the studies have all described a transient expression pattern of the Nodal signalling factors during the critical

developmental window of sex differentiation in mice. In human fetal testes, a similar transient expression pattern was not observed, as expression of *NODAL*, *CRIPTO*, *LEFTY1* and *LEFTY2* was found throughout the first and second trimester (Jørgensen *et al.* 2018). This difference highlights that the process of germ cell differentiation in mice takes place in a synchronous manner at 12.5–13.5 dpc, while gonocyte differentiation in humans is an asynchronous process spanning a much longer period of time starting from around GW 14–16 and continues throughout fetal life (Mitchell *et al.* 2008).

Role of Nodal and Activin signalling in male fetal germ cells

Several studies have examined the role of Nodal and Activin during mouse testis development by utilising either pharmaceutical inhibition of the pathways or gene-knockdown approaches (Souquet *et al.* 2012, Spiller *et al.* 2012, Miles *et al.* 2013, Wu *et al.* 2013, Tian-Zhong *et al.* 2016). Similarly, in the study using human fetal testes, pharmaceutical inhibition of Nodal and Activin signalling was used (Jørgensen *et al.* 2018). Simultaneous inhibition of Nodal and Activin signalling with SB431542 (*ALK4/5/7* inhibitor) (Inman 2002) in *ex vivo* cultures of the first trimester human fetal testes resulted in almost complete loss of the gonocyte population. The observed effect of the inhibitor treatment was due to a combination of reduced proliferation and increased apoptosis and not a consequence of premature gonocyte differentiation. In line with the results from the first trimester testes, the number of both gonocytes and pre-spermatogonia was significantly reduced in the second trimester samples after simultaneous inhibition of Nodal and Activin signalling. Since treatment with SB431542 not only inhibits Nodal signalling, the effects of Nodal and Activin signalling were separated by treatments with recombinant Nodal, *Lefty*, *Activin A* and *Follistatin*, respectively, in *ex vivo* cultures of the first trimester fetal testes (Jørgensen *et al.* 2018). Treatment with the endogenous Nodal pathway inhibitor *Lefty* resulted in a lower number of OCT4⁺ gonocytes, while stimulation with Nodal significantly increased the number of OCT4⁺ gonocytes, indicating that the Nodal pathway is directly involved in the regulation of pluripotency factor expression in human fetal gonocytes (Jørgensen *et al.* 2018). Interestingly, when Nodal signalling was initially stimulated in *ex vivo* cultures of the second trimester fetal testes followed by subsequent xenografting, an increase in the ratio of OCT4⁺ gonocytes to the total number of germ cells was reported, suggesting prolonged expression of OCT4 in the gonocytes. This directly implicates Nodal signalling in the regulation of the gonocyte to pre-spermatogonia transition in human fetal testes (Jørgensen *et al.* 2018). In mouse fetal testes, Nodal signalling has also been shown to regulate the expression of pluripotency factors

(Spiller *et al.* 2012, Miles *et al.* 2013, Tian-Zhong *et al.* 2016). In a hypomorphic *Nodal* mouse model, the expression of pluripotency factors (*Nanog*, *Oct4* and *Sox2*) were downregulated, while markers of male germ cell differentiation were upregulated (*Nanos2*, *p15* and *Dnmt3l*), indicating that Nodal functions to maintain pluripotency and delay differentiation in male germ cells (Spiller *et al.* 2012). In line with these findings, an independent study reported high expression of *Fgf9* and *Nodal* signalling factors as well as persistence of *Oct4*⁺/*Nanog*⁺ gonocytes and prevention of germ cell differentiation in a conditional *Emx2*-knockdown mouse model (Tian-Zhong *et al.* 2016). Simultaneous inhibition of *Fgf9* and Nodal signalling using SU5402 (FGFR tyrosine kinase inhibitor) (Mohammadi *et al.* 1997) and SB431542, respectively, reversed the pattern of pluripotency factor expression and differentiation in testes from *Emx2*-knockdown mice, indicating that *EMX2* plays a role in male germ cell differentiation through regulation of *Fgf9* and Nodal signalling. Moreover, reduced expression of *Nanog* was found in mouse testes cultured with SB431542, supporting the notion that Nodal signalling is involved in maintaining the expression of pluripotency factors in male germ cells (Miles *et al.* 2013). The finding that Nodal signalling is involved in the regulation of pluripotency factor expression in fetal male germ cells is in accordance with previous findings in mouse epiblast stem cells and human embryonic stem cells, where Nodal/Activin signalling has been shown to maintain the expression of pluripotency factors (Vallier *et al.* 2004, Camus *et al.* 2006).

In human fetal testes, both simultaneous inhibition of Nodal and Activin signalling and inhibition of the two pathways separately reduced the number of germ cells, suggesting overlapping functions of Nodal and Activin signalling in germ cell survival (Jørgensen *et al.* 2018). Correspondingly, Nodal and Activin signalling have also been suggested to be involved in germ cell survival in mouse fetal testes (Wu *et al.* 2013). To gain insight into the long-term consequences of reduced Nodal and Activin signalling, human fetal testis tissue initially treated with SB431542 in *ex vivo* culture was xenografted into nude mice (without additional treatment) (Jørgensen *et al.* 2018). The xenografting period did not rescue the effects on the germ cell population in either the first or second trimester testis samples, consistent with an initial loss of the germ cells upon *ex vivo* exposure to SB431542. Accordingly, stimulation of Nodal signalling in *ex vivo* cultures of the first trimester fetal testes increased the number of OCT4⁺ gonocytes.

In mouse fetal testes, Nodal/Activin signalling has been implicated in preventing premature meiotic entry of germ cells (Souquet *et al.* 2012, Wu *et al.* 2013). In the study by Souquet and colleagues, treatment with

SB431542 in *ex vivo* cultures of mouse fetal testes resulted in upregulation of the meiosis markers γ H2AX, SYCP3 and *Dmc1* (Souquet *et al.* 2012). Conversely, in cultured mouse fetal ovaries stimulated with recombinant Nodal, expression of the pre-meiosis marker *Stra8* decreased, thus suggesting that a reduced number of germ cells initiated meiosis. In accordance, an independent study found increased expression of the meiosis markers *Stra8*, *Dmc1* and *Rec8* following SB431542 treatment in *ex vivo* cultures of mouse fetal testes (Wu *et al.* 2013), while the study by Spiller and colleagues did not detect increased expression of *Stra8* in their *Nodal*-knockdown model (Spiller *et al.* 2012). However, in this model the expression of *Nodal* was only reduced to approximately 30% of wild-type *Nodal* expression, and therefore, it is possible that the remaining expression of *Nodal* maintained the inhibition of premature initiation of meiosis and promotion of male fate. In human fetal testes, inhibition of Nodal and Activin signalling with SB431542 did not result in the initiation of meiosis as judged by immunohistochemical analysis of SCP3 expression, but since the majority of germ cells were lost this was difficult to evaluate (Jørgensen *et al.* 2018).

The Nodal/Activin signalling pathway has also been implicated in promoting male differentiation, including mitotic arrest of germ cells in mouse fetal testes (Souquet *et al.* 2012, Miles *et al.* 2013, Wu *et al.* 2013). The reduced expression of *Nodal* upon SB431542 treatment in mouse testes also resulted in decreased expression of male germ cell differentiation markers *Nanos2* and *Dnmt3l* (Souquet *et al.* 2012, Wu *et al.* 2013). Reduced *Nanos2* expression was confirmed at 14.5 dpc in the conditional *Nodal*-knockout mouse model, but the expression of *Nanos2* was normal at 16.5 dpc, indicating that this was a transient effect (Wu *et al.* 2013). In the same model, expression levels of *Lefty1/2* and phosphorylated SMAD2 were unaffected, despite the fact that *Nodal* expression was demonstrated to be successfully abolished, indicating that compensatory signalling by, for example, Activin might be involved (Wu *et al.* 2013). In an independent study, it was reported that treatment with SB431542 reduced the expression of the male-specific marker DPPA4 in a subset of male germ cells (Miles *et al.* 2013). The reduced DPPA4 expression was accompanied by an increased expression of the mitosis marker KI-67, consistent with increased mitotic activity of germ cells isolated from SB431542-treated testes. Accordingly, in *ex vivo* cultures of mouse fetal testes treated with SB431542, increased expression of mitosis markers was observed, suggesting a role for Nodal/Activin signalling in the regulation of mitotic arrest of male germ cells. In contrast, Spiller *et al.* reported increased expression of male germ cell differentiation markers *Nanos2*, *p15* and *Dnmt3l* in their hypomorphic *Nodal* mouse model (Spiller *et al.* 2012).

Effects of Nodal and Activin signalling on the testicular somatic niche

In addition to the effects on germ cells, Nodal and Activin signalling were also reported to affect somatic cell function in human fetal testes (Jørgensen *et al.* 2018). Especially, the Nodal pathway was shown to play an important role in the establishment of the seminiferous cords. Treatment with both SB431542 and recombinant Lefty in *ex vivo* cultures of the first trimester samples resulted in the disruption of the seminiferous cord formation. However, no effects on seminiferous cords were observed after SB431542 treatment in the second trimester fetal testes, suggesting that Nodal (and Activin) signalling is not involved in the maintenance of seminiferous cords. Interestingly, the seminiferous cord structure was re-established in SB431542-treated first trimester testis samples after xenografting, indicating that the initial disruptive effects of simultaneous inhibition of Nodal and Activin signalling can be rescued with time. In mice, two independent studies have also implicated Nodal/Activin signalling in the formation of seminiferous cords (Miles *et al.* 2013, Wu *et al.* 2013). The simultaneous inhibition of Nodal and Activin signalling in the first trimester human fetal testes did not affect the expression of the Sertoli cell marker SOX9 nor did it induce trans-differentiation of the Sertoli cells into granulosa like-cells (FOXL2⁺) despite disruption of the seminiferous cords (Jørgensen *et al.* 2018), consistent with results from mice (Souquet *et al.* 2012, Miles *et al.* 2013). This is to some extent in line with an independent study that did not find altered *Wnt4* or *Foxl2* expression upon SB431542 treatment of mouse fetal testes, although increased expression of *Bmp2* and *Follistatin* (considered possible targets of WNT4 and FOXL2) were observed, leading the authors to suggest that Nodal/Activin signalling may suppress female reprogramming of the Sertoli cells (Wu *et al.* 2013). Despite the continued expression of SOX9 in *ex vivo* cultures of the first trimester fetal testes exposed to SB431542, expression of COUP-TFII (marker of interstitial cells) and AMH (Sertoli cell marker) as well as secretion of AMH were reduced, suggesting additional effects on the function of the somatic niche (Jørgensen *et al.* 2018). Subsequent xenografting of the SB431542-treated first trimester fetal testes appeared to restore normal expression of COUP-TFII and AMH. Specific inhibition of the Nodal signalling pathway following treatment with recombinant Lefty in *ex vivo* cultures of first trimester fetal testes appeared to decrease the expression of AMH, but with no significant effect on AMH secretion (Jørgensen *et al.* 2018). In contrast, Nodal stimulation increased AMH secretion, while no apparent effect on the expression of COUP-TFII was observed after manipulating signalling by Nodal in either direction. Manipulation with the Activin signalling pathway did not alter expression or secretion of AMH. In mice, effects on Sertoli cells have also

been reported following SB431542 treatment in fetal testis cultures, including impaired arrangement of the Sertoli cells and a reduced proliferative capacity (Miles *et al.* 2013). Since the interaction between the somatic niche and germ cells is crucial for normal germ cell development, it is likely that some of the effects on germ cell observed after SB431542 and Lefty treatments are mediated via alterations to the somatic niche rather than being a direct effect of Nodal inhibition (Fig. 2).

The study in human fetal testes was the first to report reduced androgen production (testosterone and androstenedione) following simultaneous inhibition of Nodal and Activin signalling in *ex vivo* cultures of the first trimester samples (Jørgensen *et al.* 2018). Since the levels of steroidogenic precursors 17-hydroxyprogesterone (17-OHP) and progesterone were also elevated, this could indicate reduced CYP17A1 enzyme activity. The effect on androgen production was not completely rescued following xenografting as evidenced by significantly reduced host mouse seminal vesicle weight (readout of testosterone production by tissue grafts in castrated male mice) and serum testosterone levels (Jørgensen *et al.* 2018). Interestingly, stimulation of the Nodal pathway in *ex vivo* cultures of the first trimester samples affected the androgen production in the opposite direction, with increased levels of androstenedione and dehydroepiandrosterone (DHEAS) reported, while levels of the early precursors 17-OHP and cortisone were reduced (Jørgensen *et al.* 2018).

Involvement of dysregulated Nodal signalling in reproductive disorders?

Increasing the understanding of underlying mechanisms directing fetal testis development are important also in the context of the fetal origin of male reproductive disorders comprised within TDS. Since continued expression of pluripotency factors and failure of a sub-population of gonocytes to differentiate to pre-spermatogonia during fetal testis development are considered the key initial step in the testicular cancer pathogenesis, the recent finding of the Nodal pathway as a regulator of pluripotency factor expression in fetal male germ cells (Spiller *et al.* 2012, Miles *et al.* 2013, Tian-Zhong *et al.* 2016, Jørgensen *et al.* 2018) has important implications for the understanding of malignant transformation of germ cells. Importantly though, the regulation of pluripotency factor expression and transition from gonocyte to pre-spermatogonia are not well characterised, but are understood to constitute a multifactorial and polygenic process in which Nodal signalling is one among several pathways involved. However, the increased number of gonocytes and prolonged expression of pluripotency factors observed after stimulation of the Nodal pathway (Jørgensen *et al.* 2018) provides insight into a possible mechanism by

Figure 2 Summary of effects following manipulation of Nodal/Activin signalling in human fetal testes. The middle part of the figure (blue area) illustrates Nodal/Activin signalling in normal human fetal testes with formation of seminiferous cords, AMH secretion in Sertoli cells and steroidogenesis (including androgen production) in the fetal Leydig cells. Together, the somatic niche supports the survival and proliferation of OCT4⁺ gonocytes. The left part of the figure (red area) illustrates the situation where Nodal and Activin signalling have been simultaneously inhibited resulting in impaired formation of seminiferous cords, reduced expression and secretion of AMH as well as reduced production of androgens. This impaired somatic niche fails to sufficiently support the gonocyte population (OCT4⁺), which are most likely lost as consequence. The right part of the figure (green area) illustrates the situation where the Nodal pathway is stimulated (and Activin signalling is normal) resulting in increased AMH and androgen secretion in addition to an increased number of OCT4⁺ gonocytes. The effect on pluripotency factor expression in the gonocytes is most likely direct, but the prolonged 'early fetal stage' of the somatic niche probably contributes to their impaired differentiation to pre-spermatogonia, and the continued support of the arrested gonocytes.

which pluripotency factor expression can be maintained if this pathway is dysregulated (Fig. 3).

In line with the multifactorial process of testicular development and regulation of pluripotency factor expression in germ cells, it is known that the risk of developing GCNIS and subsequently TGCTs is increased in some patients with Differences of Sex Development (also known as Disorders of Sex Development, DSD), which covers a wide range of medical diagnoses related to atypical gonadal development due to a genetic defect. The high risk of germ cell malignancy is mainly related to DSD patients with defects in genes involved in the signalling cascade directing testis development, demonstrating that disruption of this tightly controlled process also affects germ cell differentiation. Moreover, development of GCNIS and TGCTs is included in TDS in which genetic susceptibility as well as lifestyle and environmental factors are considered to impact the risk of developing the male reproductive disorders included in the syndrome (Skakkebaek *et al.* 2001, 2016). Interestingly, despite the different aetiologies of DSD and TDS, the pathogenesis of GCNIS and TGCTs appears to be similar with failure of gonocyte differentiation and continuous expression of pluripotency factors as the initial steps (reviewed in Jørgensen *et al.* 2015).

Another important determinant of the risk for developing germ cell malignancies in DSD patients is the androgen responsiveness of the gonads, with a high

risk in DSD patients with partial androgen insensitivity syndrome (Jørgensen *et al.* 2015). This is in line with the understanding that timing and level of androgen production are important for masculinisation of the foetus, including correct male-specific differentiation of the fetal gonads (Sharpe 2006). Reduced androgen production and/or action from the fetal testis is included in TDS, and it has been suggested that reduced androgen secretion during fetal development (in rats during the specific time of the masculinisation programming window; Welsh *et al.* 2008) alters signalling between the germ cells and their somatic niche, resulting in arrested gonocyte differentiation and prolonged expression of pluripotency factors in these cells (Rajpert-De Meyts 2006, Rajpert-De Meyts *et al.* 2016). The observed reduction in androgen secretion in human fetal testes following simultaneous inhibition of the Nodal and Activin pathway with only partial rescue of the effect (Jørgensen *et al.* 2018), could suggest long-term effects on androgen production. This is in line with a previous study in rodents, where it was demonstrated that progenitor stem cells for adult Leydig cells were present in the fetal testis and that the number of these were severely reduced when the androgen production in the fetal testis was reduced (Kilcoyne *et al.* 2014).

Focal testicular dysgenesis is another important feature of both DSD and TDS, which is frequently observed in testicular biopsies from these patients (Skakkebaek *et al.* 2001, Hoei-Hansen *et al.* 2003).

Figure 3 Summary of effects following manipulation of the Nodal signalling pathway in human fetal testis development (grey area) and its implications for the development of the testicular cancer precursor, germ cell neoplasia *in situ* (GCNIS). Nodal signalling factors are expressed in human fetal gonocytes (indicated by colour code). In the central part of the figure normal testis development is illustrated, in which gonocytes undergo differentiation to pre-spermatogonia during fetal development and spermatogenesis is initiated after puberty. Stimulation of Nodal signalling (top part of figure) results in an increased number of OCT4⁺ gonocytes as well as increased production of AMH and androgen precursors in *ex vivo* cultures of human fetal testes. Subsequent xenografting maintains OCT4 expression in a sub-population of gonocytes, implicating Nodal signalling in the regulation of pluripotency factor expression and thereby in the development of GCNIS and subsequent malignant progression. Diminished Nodal signalling (bottom part of figure) results in loss of gonocytes, impaired seminiferous cord structure, reduced AMH and androgen production and lower expression of COUP-TFII in *ex vivo* cultures of human fetal testes. Subsequent xenografting into castrated nude mice to determine long-term effects of short-term inhibition of Nodal signalling, do not rescue the gonocyte population, but the effects on seminiferous cord structure and partially the androgen production is rescued with time in addition to the apparent rescue of AMH production and COUP-TFII expression. ESC, embryonic stem cell; GCNIS, germ cell neoplasia *in situ*; PGC, primordial germ cell.

This includes morphological alterations like abnormal-shaped seminiferous tubules, Leydig cell nodules and Sertoli-cell-only tubules in which the Sertoli cells are occasionally undifferentiated. The induced testicular dysgenesis observed after simultaneous inhibition of Nodal and Activin signalling in both human and mouse fetal testes (Miles *et al.* 2013, Wu *et al.* 2013, Jørgensen *et al.* 2018), could suggest that dysregulated Nodal and/or Activin signalling may be one of multiple signalling pathways involved when testicular dysgenesis develops. However, there is an important difference between the testicular phenotype observed in biopsies from patients, which is focal, and the complete dysgenesis observed in the fetal testes following inhibition of Nodal (and Activin) signalling, making direct comparison difficult. The simultaneous inhibition of Nodal/Activin signalling in human fetal testes, resulted in an almost complete loss of the gonocyte population (Jørgensen *et al.* 2018) rather than arrest of the gonocyte to pre-spermatogonia transition as may have been expected. This could be a consequence of the 'complete' testicular dysgenesis observed in these fetal testes (Fig. 2). In line with this notion, previous studies in which specific signalling pathways were manipulated resulting in testicular dysgenesis also substantially reduced the number of gonocytes without arrest of gonocyte differentiation being observed (Wu *et al.* 2013, Jørgensen *et al.* 2015).

Interestingly, a recent study in which DMRT1 expression was repressed in human fetal testes using a gene-editing approach, focal testicular dysgenesis was observed as well as increased expression of OCT4 (Macdonald *et al.* 2018). However, germ cells (especially gonocytes) were rarely observed in areas with focal testicular dysgenesis (Macdonald *et al.* 2018), indicating that these cells were lost most likely due to insufficient support from the somatic niche. These observations emphasise that it would be highly relevant to further examine the effects on gonocyte to pre-spermatogonia transition in a fetal testis model with focal testicular dysgenesis to better address this in future studies. Despite these limitations and the need for a cautious interpretation of the results, the finding that Nodal signalling is involved in the regulation of pluripotency factor expression (Spiller *et al.* 2012, Miles *et al.* 2013, Tian-Zhong *et al.* 2016, Jørgensen *et al.* 2018) and in particular the prolonged expression of pluripotency factors following stimulation of Nodal signalling (Jørgensen *et al.* 2018), highlight the importance of this pathway in normal testis development and germ cell differentiation. Importantly though, the signalling cascade ensuring normal fetal testis development is complex and dysregulation of any of the pathways involved may result in alterations consistent with impaired germ cell development, reduced androgen secretion and testicular dysgenesis

suggested to be involved in the initial development of germ cell malignancy.

The novel insight into the involvement of Nodal signalling in testis development obtained during recent years and the implications for a role in the development of GCNIS and subsequent TGCTs when the pathway is dysregulated should be addressed in future studies. There are already studies demonstrating that Nodal signalling factors are overexpressed in human samples containing GCNIS and TGCTs, supporting the notion that this pathway may also play a role in the continued malignant transformation of germ cells (Baldassarre *et al.* 1997, Spiller *et al.* 2012, Nettersheim *et al.* 2015). Summarising the current knowledge about Nodal signalling in the progression of germ cell malignancy is beyond the scope of this review and have been reviewed elsewhere (Spiller *et al.* 2013, 2017). Future studies examining in more detail the effects of manipulating Nodal signalling on fetal testis development, particularly on germ cell differentiation – possibly using gene editing approaches in fetal testis cultures – may provide important insight into the role of the Nodal pathway in the pathogenesis of testicular germ cell cancer and delineate important information relevant for the understanding of male reproductive disorders.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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