Focus on TGF- β SignallingAll in the family: TGF- β family action in testis development

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Abstract

To achieve and maintain fertility, the adult mammalian testis produces many generations of sperm. While testicular integrity is established in the fetus and develops further in juvenile life, sperm production does not ensue until much later in life, following the onset of puberty. Signals from the transforming growth factor- β superfamily of proteins are vital for governance of testis development and spermatogenesis, and this review discusses our current understanding of the mechanisms and processes in which they have been implicated with a focus on the fetal and juvenile testis.

Reproduction (2006) **132** 233–246The canonical TGF- β superfamily signalling pathway

The transforming growth factor β (TGF- β) superfamily is defined as a group of over 40 ligands that characteristically form dimers for signalling, due to the presence of seven conserved cysteines in each subunit that form three intramolecular and one intersubunit disulphide bond. Some of these ligands are known by multiple names, due to the circumstances of their discovery. Those best studied in mammalian reproduction, to be discussed here, include: the TGF- β s 1–3 (each the product of a separate gene), the inhibins (inhibin α subunit linked with an inhibin β subunit) and activins (formed as dimers of activin β subunits; these subunits are also termed inhibin β subunits), the bone morphogenetic proteins (BMPs), Müllerian inhibiting substance (MIS; also known as anti-Müllerian hormone (AMH)), the growth and differentiation factors (GDFs) and the distantly related glial cell line-derived neurotrophic factor (GDNF; this signals through an atypical receptor complex). A review of this family and its mechanisms of function are presented by Lin *et al.* (2006).

The prototypical signalling receptors for this family are heterotetramers comprised of two type I and type II receptors, each with Ser/Thr kinase activity. The type II receptors are constitutively active, while the type I receptors are most commonly recruited and activated by the ligand-bound form of the type II receptor. Each ligand uses a distinct receptor subset, but many ligands share receptor subunits, so that competition amongst

the superfamily for signalling molecules occurs at the level of receptor binding (summarised in Table 1). Three type I receptors (ALK2, ALK3 and ALK6) and three type II receptors (BMPRII, ActRIIA and ActRIIB) are known to bind BMPs and signal, while activin uses ALK2, ALK4, ActRIIA and ActRIIB (reviewed in Zhao & Garbers 2002, Shi & Massague 2003). As a further example of the complexity of these interactions, activin B (a dimer of activin β B subunits), activin AB (a dimer of activin β A and B subunits) and nodal preferentially utilise the ALK7 type I receptor, with nodal binding being particularly enhanced in the presence of the nodal co-receptor, cripto (Reismann *et al.* 2001). The TGF- β ligands use a distinct set of type I (ALK1 and ALK5) and type II (T β RII) receptors in addition to a third receptor, termed type III or betaglycan, which appears required for transduction of TGF- β 2 signals, but not for those of TGF- β 1 or TGF- β 3. Thus, the synthesis or lack of betaglycan provides another level for regulating the response of a cell to a set of complex incoming signals, as when several TGF- β ligands are present simultaneously.

Extracellular and transmembrane inhibitors are a key feature of modulating cellular responses to TGF- β superfamily signalling. Numerous soluble inhibitors act to prevent ligand binding. Inhibin is understood to block activin binding to its type II receptors when inhibin is bound to betaglycan (Lewis *et al.* 2000), while as stated above, the presence of betaglycan facilitates signalling by TGF- β 2. Some controversy exists regarding whether

Table 1 Activin receptors, their ligands and inhibitors.

Type I receptor(other names)	Type II receptor	Ligand	Inhibitor	R-Smad
ALK 1 (Acvrlk1, SKR3)	TGF-βRII	TGF-βs, BMPs	decorin	1,5,8
ALK2 (ActR1A, SKR1)	ActRIIA/IIBBMPRII	GDF, BMPsAMH, activin	follistatincerberus	1,5,8
ALK3 (BmpR1A, SKR5)	ActRIIA/IIBBMPRII	BMPs, GDF		1,5,8
ALK4 (ActRIB, SKR2)	ActRIIA/IIB	TGF-βs, activin, nodal, BMP	decorin, inhibin, lefty1	2,3
ALK5	TGF-βRII, ActRIIB	TGF-β, activins	–	2,3
ALK6 (BmpRIB, SKR6)	ActRIIA/IIBBMPRII	BMPs, GDF, AMH	–	1,5,8
ALK7	ActRIIA/IIBTGF-βRII	nodal, TGF-β, activins	lefty, decorin, inhibin	1,5,8
Inhibitory receptors		Ligand		
betaglycan (TGF-βRIII)	–	Inhibin, TGF-β, FS	–	
BAMBI (Nma in mammals)	–	BMPs, activin, TGF-β	–	

Data collated from Hill 1996, Gu *et al.* 1999, Miyazono *et al.* 2001, Miyazawa *et al.* 2002, ten Dijke *et al.* 2003, Valdimarsdottir & Mummery 2005.

or not inhibin has its own signalling receptor (Bernard *et al.* 2002, discussed in Robertson *et al.* 2004), but to date no surface receptor has been validated. The GPI-linked protein cripto blocks activin interactions with the type II and ALK4 receptor subunits, but this appears to occur only when the alternative ligand for these receptors, nodal, is present and in these circumstances, cripto enables nodal signalling through these receptors (Yan *et al.* 2002, Gray *et al.* 2003). As a final example, the transmembrane receptor BMP and activin membrane-bound inhibitor (BAMBI; Onichtchouk *et al.* 1999) serves as a dominant-negative receptor for several TGF-β ligands (TGF-β, BMPs and activins). Its structural

relatedness to the type I receptors in the extracellular domain allows ligand binding, and its lack of an intracellular Ser/Thr kinase domain abrogates signal transduction.

The Smad pathway is a well-characterised intracellular response to TGF-β ligand binding. The R-Smads, Smad1, Smad5 and Smad8 are signalling molecules for BMPs, while Smad2 and Smad3 propagate TGF-β and activin signals (reviewed in Shi & Massagué 2003). Smad4 is recruited by all activated R-Smads, and it is this heteromeric complex, which translocates into the nucleus to effect changes in gene expression (Fig. 1). Smad6 and Smad7 are each inhibitors of this pathway, with several

Figure 1 Cartoon depicting TGF-β superfamily signaling by Smads. TGF-β superfamily ligand binds to and activates cell surface receptors. Smad2 and Smad3 (activin and TGF-β) or Smad1, Smad5 and Smad8 (BMPs) are phosphorylated by activated receptors, complex with Co-Smad4 and translocate to the nucleus to regulate gene transcription. Inhibitory Smads, Smad6 and Smad7, antagonise TGF-β superfamily signalling.

modes of action, including prevention of signalling complex formation and transcriptional regulation of elements of the signalling pathway.

TGF- β superfamily signalling in the embryo and developing testis

BMP and Smad signalling in germline specification

In the mammalian fetus, the gonad and the germ cells are originally bipotential, with the ability to develop into either an ovary with oogonia or a testis with spermatogonia. The cells that will form gametes are termed primordial germ cells (PGCs), and they are first visible by 7.25–7.5 days *post coitum* (dpc) in the mouse (Tam & Snow 1981, Zhao & Garbers 2002) due to their striking rounded morphology and the presence of surface alkaline phosphatase (Fig. 2). They arise within the proximal epiblast of the mouse embryo in response to extra-embryonic signals that occur approximately 2 days earlier (5.25–5.5 dpc), and as discussed below, have a clear dependence on BMP signalling (Lawson *et al.* 1999, de Sousa Lopes 2004, Drummond 2005).

The BMPs are grouped into several classes based on their degree of sequence identity or homology in the mature carboxyl domain (Hogan 1996, Zhao 2002). *Bmp4*, a member of the decapentaplegic (DPP) class and *Bmp8b* of the 60A class are each expressed in the extra-embryonic ectoderm of pregastrula and gastrula murine embryos at around 5.5–7.5 dpc (Zhao & Garbers 2002, de Sousa Lopes 2004). Targeted disruption of either gene results in failure to form PGCs (Lawson *et al.* 1999, Ying *et al.* 2000) and defects in allantois development, both components that arise from common precursors located in the proximal epiblast before gastrulation (Lawson *et al.* 1999). A close relative of *Bmp4*, *Bmp2* (also in the DPP class) is expressed mainly in the visceral endoderm at a slightly earlier (5.0–7.5 dpc) embryonic age (Coucouvanis & Martin 1999). Its targeted inactivation also significantly reduces germ cell numbers (Zhao & Garbers 2002).

Analysis of multiple lines of *Bmp* heterozygous animals has illuminated their functional interrelationships. Mice heterozygous for both *Bmp2* and 4 (*Bmp2*^{+/-}, *Bmp4*^{+/-}) have reduced PGC numbers compared with either single heterozygote, implying that these two proteins have additive effects (Ying & Zhao 2001). However, *BMP4* and *Bmp8b* double heterozygotes (*Bmp4*^{+/-}, *Bmp8b*^{+/-}) have the same PGC number as *Bmp4* single heterozygotes, while the number of PGCs in *Bmp2* and *Bmp8b* double heterozygote animals (*Bmp2*^{+/-}, *Bmp8b*^{+/-}) is similar to their respective single heterozygotes. This illustration that *Bmp8b* does not have additive effects with either *Bmp2* or *Bmp4* indicates that, while DPP class members signal through receptor complexes distinct from those used by 60A class members during PGC formation

(Ying *et al.* 2000, Ying & Zhao 2001), they influence the same fundamental processes (Fig. 2)

During PGC induction in epiblast cells, it is unclear, which specific receptor complexes transduce BMP2, BMP4 and BMP8b signals. A comprehensive study by de Sousa Lopes *et al.* (2004) aimed to elucidate the key surface receptors associated with this process. They showed that BMP4 produced in the extra-embryonic ectoderm signals through ALK2 in the visceral endoderm to induce PGC formation from the epiblast. Interestingly, ALK2 deficient embryos are devoid of PGCs, and their numbers are also reduced in heterozygous animals. This phenotype mimics that seen in the aforementioned *Bmp4* knockout animal, which consequently, can be rescued by the constitutive expression of active ALK2 in the visceral endoderm (de Sousa Lopes 2004). Targeted disruption of the *ALK4* gene in mouse embryos leads to a disorganised epiblast and extra-embryonic ectoderm, followed by aberrant development of the egg cylinder prior to gastrulation (Gu *et al.* 1998).

The Smads are also vital for embryonic development. Smad1 is localised to the epiblast and visceral endoderm during gastrulation but is not expressed immediately after, at 7.5 dpc in the wild-type mouse, suggestive of a role in PGC specification rather than proliferation and maintenance (Hayashi *et al.* 2002). Smad1 knockout mice die between 10.5 and 11.5 dpc (Tremblay *et al.* 2001). These mice produce only a few PGCs, if any supporting the concept that Smad1 is crucial for PGC formation (Tremblay *et al.* 2001, Hayashi *et al.* 2002).

Smad5 knockout mice also die *in utero*. Their embryonic lethal phenotype is seen between 9.5 and 11.5 dpc (Chang *et al.* 1999) and similar to Smad1 knockouts, they exhibit either greatly reduced numbers of PGCs or none at all. Smad5 may be important for determining, which cells of the epiblast become PGCs, as it is detected in the epiblast of normal mice at 6.5 dpc (Chang *et al.* 1999, Chang & Matzuk 2001) and is expressed by all three germ layers 1 day later in embryonic development. Smad1 and Smad5 are expressed in the epiblast at the onset of gastrulation, but Smad8 is detected only in the allantois (Chang *et al.* 1999, Yang *et al.* 1999, Chang & Matzuk 2001, Tremblay *et al.* 2001).

During fetal mouse development, the Smad2 gene appears to be ubiquitously expressed (Weinstein *et al.* 1998, Heyer *et al.* 1999). Mice with a targeted disruption of this gene die between 7.5 and 8.5 dpc, yet most Smad2 knockouts develop PGCs (Tremblay *et al.* 2001), suggesting that upstream signals that activate Smad2, such as activins, TGF- β s or nodal, may not be vital for PGC development.

As expected, for a protein essential to such a multifaceted signalling pathway, expression of Smad4 is also ubiquitous. Smad4 knockouts display defects

Figure 2 Diagrammatic representation of key events in establishment of male fertility in the mouse. Primordial germ cells (PGCs) are first specified in the epiblast around 6.5 dpc. PGCs proliferate as they migrate to the site of the embryonic gonad, between 10.5 and 12.5 dpc, wherein they form the gonocyte population. Within the embryonic testis, gonocytes enter mitotic arrest around 13.5–14.5 dpc. After birth, the gonocytes re-enter the cell cycle and, upon migrating to the basement of the seminiferous epithelium, differentiate into A-type spermatogonia. These spermatogonia form the spermatogonial stem cell population and, by 5 dpp, the first spermatogonia enter the spermatogenic pathway. Spermatogonia continue to proliferate throughout the life of the individual, maintaining the stem cell component of the testis and spermatogonia committed to differentiation. As early as 6 dpp, some differentiating spermatogonia are present, as detected by the differentiation-marker c-kit on their surface. By 9 dpp, a significant proportion of differentiated B-type, c-kit positive, spermatogonia are present. B-type spermatogonia undergo a final DNA synthesis around 10–11 dpp, making the transition from $2n$ spermatogonia to $4n$ spermatocytes. The spermatocyte period concludes with two reductive meiotic divisions, to produce haploid (n) spermatids. Spermiogenesis involves differentiation of a round spermatid into an elongating spermatid, to eventually produce the mature spermatozoon, released into the lumen during spermiation. As described in the text, various TGF- β superfamily ligands have been implicated in regulating spermatogenesis. Ligands known to regulate specific events of germ cell specification and development are illustrated under the diagram. Events associated with establishment of the Sertoli cell population are depicted above the diagram.

in development of the visceral endoderm and are embryonic lethal between 6.5 and 8.5 dpc (Sirard *et al.* 1998, Yang *et al.* 1998). The phenotype of Smad4 knockouts is similar to most BMP knockouts (Sirard *et al.* 1998), highlighting the crucial role Smad4 plays in propagating signals from the numerous interacting TGF- β pathways.

TGF- β s and activins in proliferation and apoptosis

Mouse PGCs move passively to the developing genital ridge between 8.5 and 10.5 dpc, and then migrate actively during 10.5–11.5 dpc. PGCs are proliferating during this period and continue to do so when they arrive at the sexually indifferent gonad. TGF- β s and activins have been implicated in the migration, survival and proliferation of cells during this period of development. Among the TGF- β isoforms identified, three are

expressed in mammals (Cupp *et al.* 1999). In a very general sense, the primary actions of the TGF- β s are to enhance formation of the extracellular matrix and inhibit proliferation of most cells (Lawrence 1996), inhibiting progression into late G1 of the cell cycle. In the fetal testis, TGF- β s have been shown to exhibit multiple developmentally regulated effects.

TGF- β 1 and TGF- β 2 protein expression appeared limited to Sertoli cells of the 14.5 dpc rat testis, but was undetectable in gonocytes, whilst TGF- β 3 was found in cells surrounding the seminiferous cords at the interface between the testis and mesonephros (Cupp *et al.* 1999). In the rat, the actions of TGF- β during embryonic testis development at 14 dpc are considered to be mainly on gonocytes, and by 16.5 dpc on both Leydig cells and gonocytes (Olaso *et al.* 1998). Both TGF- β 1 and TGF- β 2 have been shown to cause apoptosis of rat gonocytes at 14 dpc (Olaso *et al.* 1998). TGF- β 1 also reduced the

size of rat testis organ cultures at both 13 and 14 dpc, probably by regulating first germ cell and then somatic cell numbers (Cupp *et al.* 1999).

Additional studies illustrate the changing responsiveness to TGF- β that is a feature of fetal testis development. Experiments using single cell suspensions produced from PGC-containing fragments of 8.5 dpc mouse embryos showed that TGF- β 1 decreases the number of PGCs (Godin & Wylie 1991), but has no effect on PGC number at 10.5 dpc (de Felici *et al.* 1992). Another study revealed that TGF- β 1 and activin inhibit proliferation of both isolated pre-migratory and post-migratory mouse PGCs *in vitro*, and these cells contain mRNAs encoding appropriate receptor subunits (Richards *et al.* 1999). Chemotropic substances released by the genital ridges at around 10.5 dpc can attract PGCs; in particular TGF- β 1 mimics the chemotropic effect of genital ridges in culture (Godin & Wylie 1991). In this experiment, TGF- β 1 inhibited proliferation of PGCs from 8.5 dpc mouse embryos cultured on embryonic fibroblast feeder layers. While these experimental systems have unveiled roles in proliferation, apoptosis and migration for this signalling pathway, the *in vivo* consequences of multiple inputs from this signalling pathway will arise from a more complex set of signals that have been reproduced in these experiments.

Between 11.5 and 12.5 dpc, the PGCs become encased in Sertoli cells and surrounded by peritubular myoid cells and are thus sequestered inside testicular cords. Signals from the Sertoli cells direct PGCs into the male lineage, and they are now termed gonocytes, the precursors of the spermatogonial stem cells. At present, it is understood that TGF- β signalling directly influences the survival of these cells. When 13.5 dpc fetal rat gonads were cultured as explants with TGF- β 1, a concentration-dependant decrease in the number of gonocytes was observed after 2 days in culture (Olaso *et al.* 1998). This phenomenon was also seen using 3 day *post partum* (dpp) testis explants (when the gonocytes are re-entering the cell cycle) but not with 17.5 dpc explants (when gonocytes are quiescent), indicating the influence of TGF- β 1 is reduced during the period of germ cell quiescence. Bromodeoxyuridine labelling indices of both gonocytes and Sertoli cells at 13.5 dpc did not vary with either TGF- β 1 or TGF- β 2 addition. However, a marked increase in the number of TUNEL-positive gonocytes was recorded when these factors were added, indicating an increase in DNA fragmentation and apoptosis in these cells rather than a lack of proliferation (Olaso *et al.* 1998). Rat gonocytes begin to arrest in G1 of the mitotic cell cycle around 13.5 dpc and are fully arrested by 16.5 dpc. They remain quiescent until shortly after birth, as in the mouse, when they begin the process of spermatogenesis, which will ultimately give rise to mature spermatozoa in the adult (Yao *et al.* 2003).

Müllerian inhibiting substance

Testis fate is determined by the expression of *Sry*, a gene present on the Y chromosome, which initiates the differentiation of Sertoli cells and their structural organisation into testicular cords (Koopman *et al.* 1990, Yao *et al.* 2003). This is the point in the mouse between 10.5 and 11.5 dpc where the male and female pathways of germ cell differentiation diverge; in the absence of the *Sry* gene, ovarian fate proceeds and the germ cells spontaneously enter meiosis by 13.5 dpc in females (Yao *et al.* 2003). MIS (also known as AMH) is secreted by pre-Sertoli cells of the embryonic XY gonad and, as its name suggests, induces the regression of the Müllerian duct of the female reproductive tract (Donahoe *et al.* 1987, Ross *et al.* 2003). Male mice lacking the MIS gene are sterile due to blockage of the efferent ducts; retention of female ducts in mature males (pseudo-hermaphroditism) blocks sperm efflux during ejaculation (Behringer *et al.* 1994). MIS exerts its actions on somatic cells of the fetal testis via ALK2 and ALK6 receptor complexes, which had been previously shown to function as MIS type I receptors (Clarke *et al.* 2001). Mice lacking the genes encoding either ALK2 or ALK6 have been generated. ALK2 knockouts die before 9.5 dpc, well before Müllerian duct regression takes place, whilst ALK6 knockouts survive to adulthood and do not exhibit any defects in Müllerian duct regression, suggesting this receptor may not be essential for this event (Clarke *et al.* 2001). *AMHRII* (also termed *MISRII*), the gene encoding the MIS type II receptor is expressed in fetal Sertoli cells and mesenchymal cells of the Müllerian ducts, fetal ovaries and testes and in granulosa cells of the post-natal ovary (di Clemente *et al.* 1994, Lee *et al.* 1999). Intercrossing female mice with a floxed *ALK3* gene and heterozygote males carrying the Cre recombinase under regulation of the *AMHRII* promoter resulted in the conditional knockout of *ALK3* in the developing Müllerian ducts. The resulting males developed as pseudohermaphrodites, containing uteri and oviducts (Jamin *et al.* 2002) a similar phenotype to what has been observed with the MIS knockout males. This finding suggests that *ALK3* inactivation was enough to block Müllerian duct regression and that this receptor subunit can physically interact with the *MISRII* to transduce MIS signals (Jamin *et al.* 2002). Thus, MIS signalling can be effected through the same type I receptors used by the BMPs.

The enzyme prostaglandin D₂ (PGD₂) is more highly expressed in the testis relative to the ovary at 12.5 dpc (Adams and McLaren 2002). This signalling molecule can partially masculinise female embryonic gonads when added in organ cultures, as indicated by induction of MIS expression in female somatic cells gonads at the age of sex determination (Adams & McLaren 2002). Therefore, the local synthesis of PGD₂

by both somatic and germ cells of the testis may provide a key feedback loop ensuring that male gonadal differentiation follows.

Gene expression profiles in the embryonic gonad

The migratory phase of mouse germ cell development lasts for approximately 4 days (8.5–12.5 dpc; Buehr 1997). At the end of this period, PGCs within the gonad undergo sex determination upon exposure to gender-specific signals, continue to proliferate, and then become quiescent at around 14.5 dpc. During this dynamic period of cellular progression, PGC numbers increase from less than 100 at 8.5 dpc to approximately 25 000 at 13.5 dpc, the result of numerous division cycles (Tam & Snow 1981, Zhao & Garbers 2002). Concomitant with this growing PGC population in the testis are changes in the expression of many genes, including those related to TGF- β superfamily signalling. The cues that govern these steps in PGC development are likely to result from a complex interaction between several TGF- β superfamily ligands and inputs from other signalling pathways.

Changes in the level and sites of expression of TGF- β signalling-related genes during testis development have been documented using *in situ* hybridisation and microarray analyses. A large body of data relating to the mouse testis and to specific testis cell types has been collected using Affymetrix arrays (see Small *et al.* 2005, gene expression omnibus (GEO) data repository, NCBI, available at: www.ncbi.gov/geo/). This information can be interrogated to gain insights concerning the key regulatory changes that occur in the TGF- β signalling pathway as the testis develops. For example, the *ActRIIB* mRNA level does not change remarkably between the time of sex determination (11.5 dpc) and germ cell quiescence (16.5 dpc) in either the male or female gonad (Fig. 3). In contrast, the *activin* β A subunit mRNA shows a continuous male-specific elevation up to the day of birth. Similarly, the *inhibin* α subunit shows a profile of male-specific increase up to birth. Note that, while the expression profiles for these ligands follow a similar pattern, the *inhibin* α numerical data indicates a tenfold higher signal than that recorded for *activin* β A subunit mRNA. These data, while requiring verification using independent means (see Loveland *et al.* 2006), provide a clear indication that the level of ligand, not receptor subunit, is likely to be regulated during these developmental processes. The profile for Smad signalling does not show distinct sex differences during fetal life. However, in both embryonic testis and ovary Smad4, the common Smad, appears to be more highly expressed in comparison to the other Smads, based on the data of Small *et al.* (2005).

Figure 3 Affymetrix data illustrate relative expression levels of TGF- β signaling components across fetal development and between male and female gonads. Data compiled from Small *et al.* 2005, available at: www.ncbi.gov/geo/. Solid lines indicate testis levels; dotted lines in the same color show levels for the same gene in ovary. (A) Activin receptor subunit mRNA expression levels in the embryonic gonad. Levels of receptor subunits ALK-2, ALK-4 and ActRIIA subunit mRNAs detected by Affymetrix microarray analyses. These did not appear to differ between genders or with embryonic age. Relative expression levels for these receptors were relatively quite low compared with that of ActRIIB levels. (B) Activin β A and inhibin α mRNA expression levels in the embryonic gonad. Differential expression of activin β A is observed between male and female gonads. Ovarian levels of activin β A were constant throughout embryonic development but increase in the testis after 12.5 dpc. Inhibin α mRNA levels in the ovary show an overall decline during ovarian development. In the testis, inhibin α mRNA dipped slightly at 12.5 dpc then increased sharply to day 0. Both activin β A and inhibin α share a similar gene expression profile, but note levels of inhibin α mRNA levels are recorded as approximately tenfold higher than activin β A. (C) Smad signalling mRNA expression levels in the embryonic gonad. Expression levels of mRNAs encoding Smads that mediate activin signalling do not appear to vary with either embryonic age or sex.

TGF- β superfamily signalling in post-natal spermatogenesis

Cellular differentiation events in post-natal spermatogenesis

It is in post-natal life that spermatogenesis is established as the process by which a spermatogonial stem cell divides and develops into a mature spermatozoon in the seminiferous tubules of the testis. In the mouse, spermatogenesis begins within a day after birth, when the gonocytes resume mitosis and migrate from their central position within the seminiferous cord to contact the peripheral basement membrane (Orth 1993). This forms the first generation of spermatogonia seen in the 6 dpp mouse testes, and any remaining gonocytes subsequently die by apoptosis. The spermatogonia undergo incomplete cytokinesis during successive mitotic divisions to produce A_{paired}, A_{aligned}, type A₁ through A₄ spermatogonia, intermediate (In) spermatogonia and finally, the type B spermatogonia first observed in the 8 dpp testis (reviewed in McCarrey 1993). The final mitotic division by type B spermatogonia produces the first cell of the meiotic phase, the pre-leptotene spermatocyte (emerging at around 10 dpp in mouse testes), which loses contact with the basement membrane and crosses through the tight junctions between adjacent Sertoli cells.

The Sertoli cells are specialised 'nurse cells' that provide the nutritional and architectural support required for adult germ cell development. They proliferate continuously until around 14 dpp, when they undergo a significant morphological and functional transformation to their final, differentiated phenotype. The inter Sertoli tight junctions, which initially form between 7 and 14 dpp, separate the meiotic and post-meiotic germ cells from factors present in the lymph and blood circulation, and secretions from the apical surface of this seminiferous epithelium create a specialised environment for their development and a lumen for passage out of the testis by spermatozoa.

Meiotic metaphase begins with each spermatocyte producing two diploid secondary spermatocytes, which rapidly undergo a second meiotic division to yield four haploid spermatids, clonal siblings, which remain linked by their persistent cytoplasmic bridges. The round spermatids first appear at 20 dpp; they undergo further differentiation and morphological changes to first form mature sperm by 35 dpp. In the adult mouse testes, the process is continuously repeated, so that each type A spermatogonium, located at the basement membrane, undergoes six mitotic divisions, two meiotic divisions and more than 2 weeks of differentiation to become spermatozoa that are released into the lumen at the centre of the seminiferous tubule (reviewed in McCarrey 1993, de Rooij & Russell 2000, Kerr *et al.* 2005).

Initiation of spermatogenesis and the first wave

In vitro studies using both organ culture and dispersed cell preparations have furthered our understanding of TGF- β superfamily signalling in spermatogenesis, particularly at the time that undifferentiated spermatogonia first commit to differentiation at the onset of the first wave of spermatogenesis.

Activins

Activins and inhibins have long been identified as hormonal regulators of testicular development through their impact on pituitary secretion of follicle-stimulating hormone (FSH; reviewed in Cook *et al.* 2004, de Kretser *et al.* 2004). Their potential to act in a paracrine manner to influence spermatogonial proliferation was first identified over 15 years ago. In studies with cells obtained from adult animals, inhibin was reported to decrease the level of DNA synthesis in spermatogonia of the Chinese hamster, (Mather *et al.* 1990), while activin elevated proliferation rates of rat spermatogonia that were placed in co-culture with Sertoli cells purified from a 20 dpp animal (van Dissel-Emiliani *et al.* 1989). While these studies were effected without the benefit of the current suite of molecular probes that permit a refined assessment of somatic and germ cell maturation states, they firmly demonstrated the potential for inhibin and activin levels, and TGF- β superfamily signalling pathways, to influence male germ cell development.

The addition of activin to fragment cultures of 3 dpp rat testes led to a significant increase in the number of gonocytes and a decrease in the number of Sertoli cells after 3 days in culture, the timeframe, which represents the very onset of spermatogenesis. The addition of follistatin (a soluble inhibitor of activin) in the presence of FSH (which acts on receptors present only on Sertoli cells) resulted in an increase in the number of spermatogonia, with no significant effect on gonocyte numbers compared to unsupplemented media (Meehan *et al.* 2000). These data suggest that a reduction in the level of activin signalling may be required for gonocytes to differentiate into spermatogonia. This is further indicated by the fact that rodent gonocytes themselves produce *activin β A subunit* mRNA in fetal life and store protein until after birth. However, this protein becomes undetectable and both *follistatin* and *Bambi* mRNAs are switched on in the cells that have differentiated into spermatogonia (Meehan *et al.* 2000, Loveland *et al.* 2003, Loveland *et al.* 2005). Since BAMBI is a natural transmembrane dominant-negative receptor (Onichtchouk *et al.* 1999), the implication of its expression on spermatogonia is that it specifically downregulates activin and other TGF- β ligand signalling inputs into these cells. In contrast, production of the soluble secreted proteins, activin, inhibin or follistatin, could influence any neighbouring somatic or germ cells. A recent analysis of activin A levels in the developing rat

testes showed the βA subunit was at relatively high levels in homogenates of the 3 dpp testis and showed a peak at 6 dpp, the time window enveloping the onset of spermatogenesis (Buzzard *et al.* 2004). Unfortunately, this study did not examine earlier timepoints, such as 1 dpp, when the gonocytes are present but quiescent and non-motile in the rat testis, so the relative contribution from germ cells to the pool of activin βA subunit cannot be evaluated from these data.

The capacity for activin to enhance the FSH-mediated stimulation of Sertoli cell proliferation in the rat has been established through *in vitro* studies from two independent laboratories (Boitani *et al.* 1995, Buzzard *et al.* 2003). This effect has been linked to the discrete upregulation in the synthesis of the *ActRII* mRNA in Sertoli cells between 7 and 11 dpp, in accord with data that have identified the transient timing when activin addition stimulates their proliferation *in vitro* (Fragale *et al.* 2001).

The mechanisms that underpin production and secretion of activin and inhibin are complex and are discussed in many reviews (see Loveland & Robertson (2005) and references contained therein). The regulation of activin and inhibin secretion by Sertoli cells is not only modulated by FSH, but also by other factors synthesised in the juvenile and adult testis, such as the pro-inflammatory cytokine, interleukin-1 (IL-1). For example, addition of IL-1 α and IL-1 β to immature (20 dpp) and adult rat Sertoli cell cultures stimulates activin A protein release and suppresses inhibin B (Okuma *et al.* 2005a, b). The addition of FSH significantly modifies these responses by acting directly through the FSH receptors that reside exclusively on Sertoli cells. In contrast, IL-1 α and IL-1 β are constitutively produced by the Sertoli cells examined in these experiments, and their production (mRNA or protein) can be further stimulated by addition of developing germ cells and residual bodies to these cultures (Okuma *et al.* 2005a,b). Hence, there exists a complex autocrine and paracrine regulation on the production of activin and inhibin by Sertoli cells, which in turn act in an autocrine and paracrine manner to regulate testicular development and function.

GDNF

In vitro culture techniques have verified and exploited the role for GDNF as an essential maintenance factor for undifferentiated spermatogonia, first identified using *in vivo* mouse models (see 'Adult spermatogenesis and male fertility: insights from genetically modified animals'). The long-term cultures of germline stem cells derived from newborn mouse testes have been successful only when cultured in the presence of GDNF (Kanatsu-Shinohara *et al.* 2003, 2005, discussed further in 'Spermatogonial stem cells'). GDNF is produced and secreted by Sertoli cells, and it acts on undifferentiated

spermatogonia, which express the c-ret/GFR1 α receptor complex (Tadokoro *et al.* 2002).

BMPs

To date, proliferating Sertoli cells of the juvenile rodent testes have been shown to produce or secrete activin, inhibin and BMP4 subunits, all of which influence germ cell behaviour by signalling through common receptor molecules. Spermatogonia in the early post-natal rodent testis are known to express the *activin typeIIb*, *ALK2*, *ALK3* and *ALK4* receptor subunits (de Winter *et al.* 1992, Puglisi *et al.* 2004).

In enriched germ cell cultures from day 4 mouse testes, which contain only undifferentiated spermatogonia, BMP4 addition appeared to induce germ cell differentiation by increasing the level of functional c-kit receptor in preparations of isolated spermatogonia (Pellegrini *et al.* 2003). In a different experiment, fragment cultures from day 7, mouse testes exhibited increased spermatogonial proliferation in the presence of BMP2 and FSH (Puglisi *et al.* 2004).

TGF- β ligands can also influence germ cell survival before and around the time of spermatogenesis onset. TGF- β 2 protein is expressed in the gonocytes of the rodent neonatal testis along with TGF- β receptors (Olaso *et al.* 1997), and culture of 3 dpp rat testis fragments in the presence of TGF- β 1 or TGF- β 2 increases the rate of germ cell apoptosis (Olaso *et al.* 1998). TGF- β 3 protein is also detectable in gonocytes (Olaso *et al.* 1999) but its functional impact on these cells has not been reported. However, the downregulation of TGF- β 3 mRNA and protein appears to be required for formation of Sertoli cell tight junctions (Liu *et al.* 2003). The addition of TGF- β 3 to cultures of Sertoli cells isolated from 20 dpc rats perturbed their capacity to form tight junctions and increased the levels of phosphorylated p38 mitogen-activated protein (MAP) kinase, an alternative signalling pathway activated by the TGF- β superfamily, without affecting the level of Smad proteins. In contrast, the inhibition of p38 MAP kinase phosphorylation in these cultures allowed the tight junction formation to proceed, implicating TGF- β 3 action in the regulating the dynamics of Sertoli cell tight junctions (Liu *et al.* 2003).

Spermatogonial stem cells

The capacity of the male to continuously produce sperm during adult life depends on the establishment of a spermatogonial stem cell population early during testis development and its subsequent maintenance. Although two models have been proposed for maintenance of the spermatogonial stem cell population (discussed in de Rooij 1998), the mechanism essentially translates at a molecular level as a decision to balance spermatogonial stem cell self-renewal division vs unequal cell division

to yield one stem cell and one cell committed to differentiation.

Presently, the absence of a definitive spermatogonial stem cell marker restricts our understanding of stem cell biology to what is inferred from the results of transplantation of isolated spermatogonia into a recipient testis devoid of endogenous spermatogenesis. Analysis of the restoration of spermatogenesis usually facilitated by cells expressing a reporter gene is measured by the number and size of colonies derived following transplantation. This is interpreted as an indication of the number of cells injected that possessed stem cell capacity, or more precisely, the number of spermatogonial stem cells that were able to find a niche, and how efficiently they proliferated before they committed to spermatogenesis.

Transplantation analysis has been routinely used since the mid 1990s (Brinster & Nagano 1998), and has been developed as a particularly informative tool in the analysis of the effect of growth factors on spermatogonial stem cell potential following *in vitro* culture prior to transplantation.

Spermatogonial stem cell culture aims to establish conditions that support proliferation of stem cells, but not differentiation. As spermatogonia have proven difficult to maintain in culture for long periods of time, culture studies initially assessed the effect of growth factors on spermatogonial stem cells for short periods *in vitro*. Activin A was identified as a mitogenic factor for rat spermatogonia (Mather *et al.* 1990), and a transgenic mouse model (BK mouse) with reduced levels of bioactive activin shows a delay in the onset of spermatogenesis (Brown *et al.* 2000, discussed in 'Adult spermatogenesis and male fertility: insights from genetically modified animals'), however, a specific effect of activin on spermatogonial stem cells, rather than on spermatogonia at other stages of differentiation, had not been demonstrated. Nagano *et al.* (2003) addressed this by culturing an enriched population of spermatogonia from immature and cryptorchid mice on a STO cell feeder line for 7 days in the presence of activin A. They observed a significant reduction in the number of cells with spermatogonial stem cell capacity following transplantation. Culture with BMP4 had a similarly negative impact on the presence of spermatogonial stem cells, while conversely, culture with GDNF significantly increased repopulation following transplantation. These data are in excellent agreement with other *in vitro* and *in vivo* analyses, which clearly identify GDNF as the factor that is essential for maintenance of spermatogonial stem cells (discussed in 'Initiation of spermatogenesis and the first wave' and 'Adult spermatogenesis and male fertility: insights from genetically modified animals').

The use of feeder layers to maintain spermatogonia in culture gives the additional complication that such feeder layers themselves provide growth stimuli. Nagano

et al. (2003) aptly addressed this by looking at the effect of 13 different feeder layers. Not surprisingly, the ability to maintain spermatogonial stem cells varied with the feeder cells used. For example, when spermatogonia were cultured the Sertoli cell-derived TM4 feeder layer, transplantation potential was reduced. Interestingly, this TM4 cell line is likely to produce activin A, as the *activin* β_A subunit transcript is detected by RT-PCR in these cells (our unpublished data).

Addition of GDNF to serum-free culture media has permitted long-term culture of stem cell-competent spermatogonia (Kanatsu-Shinohara *et al.* 2003), although this result depended on the mouse strain tested. Maintenance in culture of spermatogonial stem cells derived from C57Bl/6 mouse strain requires addition of soluble GFR1- α (a GDNF receptor) as well as basic fibroblast growth factor, whereas GDNF alone is sufficient to maintain spermatogonial stem cells from DBA mice. Other studies have reported similar enhancement of stem cell maintenance when culture media is supplemented with both GDNF and GFR1- α (Kubota *et al.* 2004, Ryu *et al.* 2005), in accord with data from analysis of genetically modified mice (discussed further in 'Glial cell line-derived neurotrophic factor').

Spermatogonial stem cells isolated from immature pup testes have a better short-term survival rate than do adult spermatogonial stem cells (Nagano *et al.* 2003). A key difference between these populations is that the c-kit receptor tyrosine kinase, understood to be absent from the surface of mouse adult spermatogonial stem cells, appears present in a proportion of spermatogonia with repopulation potential isolated from immature testes (Ohbo *et al.* 2003). This finding is in accord with the observation that the first wave of spermatogenesis is shorter than adult waves (de Rooij 1998), and growth factor profiles within the testis differ between the immature and adult testis (Buzzard *et al.* 2004). In a recent report by Pellegrini *et al.* (2003), addition of BMP4 to preparations of spermatogonia from 4 dpp mouse testes upregulated c-kit expression, implicating BMP4 in the commitment of undifferentiated spermatogonia to spermatogenesis, characterised by the onset of c-kit expression. However, in the vitamin A-deficient (VAD) mouse testis, high levels of BMP4 correlated with arrested spermatogenesis due to VAD (Baleato *et al.* 2005). In this model, all spermatogonia are understood to be spermatogonial stem cells, and the provision of retinol to these VAD mice resulted in a concomitant reduction in BMP4 mRNA levels and alleviation of the spermatogenic arrest. The authors of this study suggested that in the VAD testis, that BMP4 maintains spermatogonia in an undifferentiated state.

It is promising that mechanisms of spermatogonial stem cell maintenance and proliferation appear to be conserved across species. Although most studies addressing the role of TGF- β superfamily regulation of spermatogonial stem cell potential focus on the mouse, a recent

report that rat spermatogonial stem cells are maintained and proliferate in culture in conditions that were optimised for mouse spermatogonial stem cells (which required GDNF supplementation (Ryu *et al.* 2005)) not only suggest that the role of TGF- β superfamily ligands in regulating spermatogonial stem cells may be conserved across species, but will also potentially expand possibilities for culturing spermatogonial stem cells of other species, with a view to developing agricultural and medical applications (Ryu *et al.* 2005).

Adult spermatogenesis and male fertility: insights from genetically modified animals

Several lines of genetically modified mice have been generated to examine the impact of TGF- β superfamily signalling components on normal physiology (Chang *et al.* 2002). In many cases, a reproductive phenotype has been identified, leading us to understand that specific family members have roles at precise stages of cellular development during spermatogenesis (Chang *et al.* 2001, 2002, Kulkarni *et al.* 2002). Selected examples are reviewed briefly here.

BMPs

BMP7, 8a and 8b are expressed in spermatogonia and early spermatocytes in the juvenile testis, with expression shifting to round spermatids of the adult testis. Targeted inactivation of these genes revealed that BMP8b is important for the onset of spermatogenesis in the juvenile testis, while BMP8a did not affect the onset, but is necessary for maintaining spermatogenesis in the adult. Deletion of BMP8a results in increased apoptosis of meiotic germ cells in 20–50% of the seminiferous tubules, compromising spermiogenesis and resulting in loss of fertility. When *BMP8a* null mice were crossed with BMP7 heterozygous mice, the proportion of tubules exhibiting the degenerative phenotype increased to 40–80%, indicating a supplementary role of BMP7 in maintaining spermatogenesis (Zhao *et al.* 1996, 1998, 2001). *BMP4* knockout mice die at birth and initial analysis of their testes showed there were no germ cells present (Lawson *et al.* 1999). On the other hand, heterozygous mice survive to adulthood and at 4 weeks of age display normal testicular phenotype. However, the adult heterozygotes have lower testis weights and display a decline in sperm number and motility due to the degeneration of meiotic germ cells, similar to the phenotype observed with *BMP8a* null mice (Hu *et al.* 2004). These knockout models also revealed the importance of BMP4, 7, 8a and 8b in maintaining epididymal function; the degeneration of the epididymis in null mice may be partly responsible for the observed defects in sperm motility.

Activin and inhibin

While deletion of the coding sequence of the mature β_A subunit (encoded by the *Inhba* gene) produces a mouse

that does not survive beyond birth (Matzuk *et al.* 1992), deletion of the mature coding domain of the activin β_B subunit (encoded by the *Inhbb* gene) yields fertile mice that do survive to adulthood and have an apparently normal testis phenotype (Vassali *et al.* 1994). In order to understand the impact of activin in the post-natal animal, transgenic mice were produced, termed BK mice that have reduced levels of bioactive activin through insertion of the mature activin β_B coding sequence into the *Inhba* locus. In this way, the activin β_B protein is produced with the β_A precursor and so is processed similar to the wild-type activin β_A protein. However, activin B protein has a lower biopotency than activin A due to its lower affinity for activin receptors and different physiological properties (Brown *et al.* 2000). The insertion of *Inhbb* coding region into the *Inhba* locus rescued the *Inhba* null mice from craniofacial malformations and neonatal lethality, but BK mice exhibit lower testes weights and volume than their wild-type siblings. In addition, the delayed onset of fertility experienced by the BK mice revealed the importance of activin A in establishing spermatogenesis in the mouse. A related transgenic mouse line, the *activin typeIIA receptor (ActRIIA)* knockout, with compromised signalling from activin and some BMPs, also has reduced testis size and delayed fertility onset. However, the BK male mice show a 50% increase in FSH levels, while the *ActRIIA* null mice show a reduction in FSH levels, suggesting that activin A alone, independent of FSH levels, can influence germ cell and Sertoli cell development and ultimately, fertility (for a detailed review see Chang *et al.* (2001)). However, the prospect for altered signalling by other ligands of this superfamily, in the absence of activin A, remains unexplored.

Further, evidence of the importance of regulating TGF- β superfamily signalling during germ cell development is derived from analysis of transgenic mice overexpressing the activin β_A subunit by placement of the *hInhba* subunit under the metallothionein promoter (Tanimoto *et al.* 1999). Preferential ectopic expression of activin β_A subunit in the pachytene spermatocytes and round spermatids was observed from 3 weeks of age. These animals were initially fertile and then became sterile by 10 weeks of age due to an inability to produce sperm. The testes were smaller in size and histology revealed the tubules were deficient in secondary spermatocytes and round spermatids and exhibited vacuolation.

Mice that do not produce the inhibin α subunit (resulting from deletion of the *Inha* gene) develop testicular somatic cell tumours by 4 weeks of age and die by 12 weeks from cachexia, a wasting syndrome induced by elevated activin levels (Matzuk *et al.* 1994). These mice cannot make inhibin, illustrating that inhibin acts as a tumour suppressor, but its precise role in normal adult testis function cannot be determined from this model, as the tumours disrupt the architecture of the testis. However, the histology of

these testes prior to 4 weeks of age, when activin and presumably inhibin are governing developmental events, has not been reported. To elucidate, if inhibin plays a role in testicular development, inhibin A protein was overexpressed in the livers of mice using a GeneSwitch system (Pierson *et al.* 2000), which drives production of linked coding sequences of activin β_A and inhibin α protein subunits. The inhibin A protein produced was secreted and acted on the pituitary to reduce levels of circulating FSH. When inhibin A protein was induced in 3-week-old *Inha* knockout mice, the mice did not develop tumours and were fertile, displaying a normal testis phenotype and undetectable levels of activin A. When inhibin A protein was induced in 3-week-old wild-type mice for 8 weeks, it exhibited reduced testis weights, reduced seminiferous tubule volume and diameter and were fertile (Pierson *et al.* 2000). Transgenic mice, overexpressing the *inhibin* α subunit using the metallothionein-I (MT- α) promoter were also reported to be fertile and displayed reduced FSH levels. The transgene was expressed in the pituitary, liver, testis and kidney, however a testis phenotype was not recorded (Yomogida *et al.* 2003). The phenotypes observed in the inhibin overexpressing mice are similar to that of the *ActRIIA* null mice mentioned earlier. Hence, whether inhibin has a direct role in testis development or whether its role is in modulating activin activity cannot be deciphered in these transgenic models. It appears that the relationship between the gonadal hormones, inhibin, activin and FSH, is very sensitive, so that disruption of one affects the expression or action of other, hence the resulting phenotype cannot be attributed to the effect of a single gene product.

Glial cell line-derived neurotrophic factor

Genetically modified mice have also been informative in identifying a role for GDNF in spermatogonial stem cell biology. GDNF is secreted by Sertoli cells and its expression is regulated by FSH (Tadokoro *et al.* 2002). Mice with one allele of the *GDNF* gene exhibit depletion in stem cell reserves, revealing that a threshold level of GDNF is required for spermatogonial stem cell maintenance *in vivo* (Meng *et al.* 2000). The first wave of spermatogenesis is initiated, but testicular atrophy is observed in the testis of these mice at 5 weeks of age, with many tubules containing spermatocytes and spermatids but lacking spermatogonia. Subsequently, ongoing adult spermatogenesis failed and male mice were rendered infertile as the spermatogonial stem cell population became exhausted, and by 8 weeks, depletion of germ cells resulted in Sertoli cell only tubules.

Conversely, transgenic mice overexpressing GDNF in the testis are also infertile, with a testicular phenotype showing overpopulation of undifferentiated spermatogonia and a block in the progression into

differentiating spermatogonia followed by germ cell apoptosis (Meng *et al.* 2000). In this model, overexpression of hGDNF in mouse testes was driven using the testis-specific elongation factor-1 α (EF-1 α) promoter. The spermatogonial stem cells proliferated but did not appear to respond to normal differentiation cues, including retinoic acid, leading to disruption of the seminiferous epithelium and spermatogenic failure. At 2–3 weeks of age, the tubules displayed a chimeric phenotype with large cell clusters of type A spermatogonia. These mice exhibited lower testis weights at 4 and 8 weeks of age and did not become fertile as expected by 8 weeks. The germ cell clusters degenerated such that at 10 weeks of age, the tubules were occupied by a ring of spermatogonial cells with no spermatozoa observed. These transgenic mice ultimately fall subject to non-metastatic testicular tumours (Meng *et al.* 2000, Meng *et al.* 2001). Interestingly, overexpression of hGDNF in Sertoli cells of 12-day old mice by *in vivo* transfection using electroporation also resulted in spermatogonial cell clusters in the seminiferous tubules 4 weeks after transfection. These cells were confirmed to be spermatogonial stem cells as they were able to colonise the tubules of busulphan-treated mouse testes and progress through spermatogenesis (Yomogida *et al.* 2003).

Evidence that germ cells exposed to high levels of GDNF sustain their ability to differentiate was obtained through transplantation analysis. Spermatogonia transferred from a mouse in which Sertoli cells overexpress GDNF results in normal spermatogenesis in a normal recipient testis (Yomogida *et al.* 2003); once spermatogonia are removed from the hyper-stimulated environment, they are able to differentiate. As a method of *in vivo* spermatogonial stem cell amplification, this can potentially provide a highly efficient means of generating a large number numbers of stem cells for experimental application compared to the numbers obtained from methods currently available.

Conclusions: what is left to learn and why we need to know it

While the A_{single} (A_s) spermatogonium, the rare germ cell within the adult seminiferous epithelium which is an individual entity with a single nucleus in contrast to its differentiating 'offspring' that exist as a syncytium, is understood to be the stem cell, the markers, which distinguish it from other spermatogonial cells or from gonocytes are just now being defined (reviewed in McLean 2005). This has hampered our ability to study the effects of TGF- β superfamily ligands on the spermatogonial stem cell alone (Meachem *et al.* 2001). The limitations of existing germ cell lines, and the difficulty of transfecting primary isolates of germ cells are other limitations faced by reproductive biologists. However, primary germ cell cultures have indeed

provided insight into the function of some of the TGF- β family members during the onset of spermatogenesis. Cues for this work have been taken from the *in vivo* models available through genetic modification of mice, though in many cases, the early phenotypes of mice with TGF- β signalling defects precludes their use for analysis of post-natal or adult phenotypes. Cell-specific knock-outs are a promising avenue for addressing this need. Researchers are now coupling new methods for identification of cell-type specific 'fingerprints', such as Affymetrix arrays and proteomic analyses, with increasingly successful cell culture enabled by emerging knowledge of key regulatory factors.

As touched on earlier, additional difficulties in understanding the cues that govern testis development and germ cell maturation arise from increasing reports that mouse strain differences are noted when genetically modified mice are crossed onto different genetic backgrounds. During culture experiments, medium composition also has demonstrable effects on spermatogonial stem cell properties, leading to difficulties in comparison of data between laboratories. Spermatogonial stem cells isolated from the mouse strains C57Bl/6J and 129/Sv (Kubota *et al.* 2004) and ICR, C57Bl/6J X DBA/2 F1 (Kanatsu-Shinohara *et al.* 2003) mice fail to proliferate in culture, while those on a DBA/2Jx ROSA strain, when cultured with GDNF, will do so (Kubota *et al.* 2004). While this adds complexity, it also offers a valuable opportunity for using direct comparison of mouse strains to identify factors required for maintenance of stem cell properties and proliferation. As more functional data are gathered, and techniques and experimental systems are refined to detect outcomes of the set of integrated signals delivered within the TGF- β superfamily network, we can apply this knowledge to key outcomes in reproductive health, spanning from testicular cancer to fertility control.

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