Hormones, BMPs, Activins and TGF-βs: how do they cooperate in the testis?

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ABBREVIATIONS

Act	**Activin**
Amp	**Ampicillin**
APS	**Ammonium persulfate**
BMP	**Bone morphogenetic protein**
Bp	**Base pair**
BSA	**Bovine serum albumin**
°C	**Degree Celsius**
cDNA	**ComplementaryDNA**
CO_2	**Carbon dioxide**
Ctrl	**Control**
DMEM	**Dulbecco's modified Eagle's medium**
DMSO	**Dimethylsulfoxide**
DNA	**Deoxyribonucleicacid**
DNase	**Deoxyribonuclease**
dNTPs	**2'-deoxynucleoside-5'-triphosphate**
DTT	**Dithiothreitol**
et al.	**And others**
ECM	**Extracellular matrix**
EDTA	**Ethylene diamine tetraacetic acid**
ELISA	**Enzyme-linked immunosorbent assay**
ERK1/2	**Extracellular signal regulated kinases**
FCS	**Fetal calf serum**
FGF	**Fibroblast growth factor**
Fig	**Figure**
FITC	**Fluorescein isothiocyanate**
g	**Gravitational force**
GAG	**Glycosaminoglycan**
HRP	**Horseradish peroxidase**
JNK	**c-Jun N-terminal kinase**
Kb	**Kilo base pair**
kDa	**Kilodalton**
LAP	**Latency associated peptide**

LLC	**Large latent complex**
LTBP	**Latent-TGF-β binding protein**
M	**Molar**
MAPK	**Mitogen activated protein kinase**
mg	**Milligram**
min	**Minute**
ml	**Milliliter**
MMP	**Matix mataloproteinase**
mRNA	**Messenger ribonucleic acid**
MW	**Molecular weight**
NaCl	**Sodium chloride**
NGS	**Normal goat serum**
NFκB	**Nuclear factor-κB**
nm	**Nanometer**
OD	**Optical density**
PAGE	**Polyacrylamide gel electrophoresis**
PAI-1	**Plasminogen activator inhibitor-1**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
pen/strep	**Penicillin/streptomycin**
PMSF	**Phenylmethylsulfonyl fluoride**
PVDF	**Polyvinylidene fluoride**
RNA	**Ribonucleic acid**
RNase	**Ribonuclease**
Rpm	**Revolutions per minute**
RT	**Room temperature**
SDS	**Sodium dodecylsulphate**
sBG	**Soluble betaglycan**
sec	**Second**
TAE	**Tris acetate EDTA**
TBE	**Tris borate EDTA**
TBST	**Tris buffered saline Tween-20**
TEMED	**Tetramethylethyelenediamine**
TGF-β1	**Transforming growth factor-β1**

TGF-β2	**Transforming growth factor-β2**
TIMP	**Tissue inhibitor of metalloproteinase**
Tris	**Tris (hydroxymethyl) amino methane**
T3	**TIMP3**
U	**Unit**
UV	**Ultraviolet**
V	**Volt**
v/v	**Volume per volume**
w/v	**Weight per volume**
wt	**Wild type**
µ	**Micro**
µg	**Microgram**
µl	**Microliter**
µM	**Micromolar**

1 INTRODUCTION

1.1 The male reproductive system

The reproductive system in males has different components, namely the penis, urethra, seminal vesicles, vas deferens, epididymis, testis and accessory glands such as the prostate gland, coagulating glands, ampullary glands and preputial glands (Fig.1). The proper function of all these components is to ensure normal fertility of the male individual. The function of the male reproductive system is to produce, maintain, and transport sperm and the sythesis of sex hormones (androgens) (Bilinska, 2006).

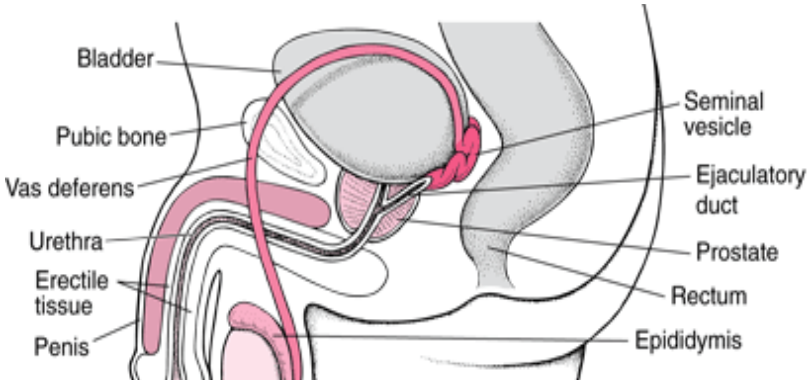


Figure 1. Anatomy of the male reproduction system (Hirsch, 2017)

Testes are oval, paired and descended outside of the abdominal cavity and play a central role in the male reproductive system. The testis and epididymis are located inside the scrotum, as it is outside of the body; the temperature is slightly lower from the core temperature. The testis has two major functions: i) the generation of male gametes, i.e. spermatozoa and ii) the synthesis and secretion of male sexual hormones, mainly testosterone. Sperm produced by the testis is transported through the rete testis to the epididymis (Cooper, 2007, Nieschlag et al., 2010).

The epididymis is a single, narrow, tightly coiled long whitish mass of tissue and extends along one margin of the testis (Cosentino & Cockett, 1986). The epididymis is mainly divided into three regions: i) the caput, ii) the corpus and iii) the cauda. Testicular fluid and spermatozoa released by the testis is absorbed by the proximal part of the epididymis, the caput. Maturation of sperm takes place during passage through the epididymal duct and

spermatozoa stored in the distal end of the epididymis, the cauda, before ejaculation. The cauda is attached to the vas deferens (Cosentino & Cockett, 1986).

1.1.1 Morphology of the testis

In human, the sex-determining region Y (SRY) protein, which is a transcription factor and it is encoded by the SRY gene located on the Y chromosome. It leads to male sex differentiation, however, if not; then the gonad develops into an ovary (Berta et al., 1990). The testis is surrounded by two layers; the *tunica vaginalis* and the *tunica albuginea* (Amann, 1989). Both layers are referred to as the testicular capsule. The t*unica albuginea* extends into the testes, creating partitions between seminiferous tubules where sperms are produced. In different species, each seminiferous tubule is surrounded by single or multiple layers of mesenchymal peritubular or myoid cells. The wall of the seminiferous tubules contains the spermatogonia, which will multiply and differentiate to produce spermatocytes, which develop into spermatozoa. The seminiferous tubules are connected to the rete testis (these are the network of delicate tubules), via a series of efferent ducts, to the epididymis (Amann, 1989). The products secreted from these tubules are collected by the rete testis and transferred to the adjacent epididymis. By the inferior and superior ligaments, the epididymis is linked to the anterior and caudal pole of the testis. In the testis, the interstitial compartment contains a loose connective tissue, capillaries, arterioles, macrophages, venules, lymphocytes, mast cells, dendritic cells, Leydig cells, and natural killer cells (Fijak & Meinhardt 2006). Macrophages are commonly observed in the interstitium and they are one fourth of the number of Leydig cells. During development and after experimental depletion they play a crucial role in the repopulation of Leydig cells (Gaytan et al., 1994a, b). The function of the testis is to perform reproductive (exocrine) and endocrine functions (Shalet, 2009). The bi-compartmental and bi-functional organization of the testis is highly conserved in testicular evolution (Pilsworth et al., 1981). The Sertoli cell is the main structural component of the seminiferous tubules, which supports the germ cells structurally, nutritionally and with growth factors (Gerard et al., 1991). The interstitial and semiferous tubules compartment is shown in Figure 2.

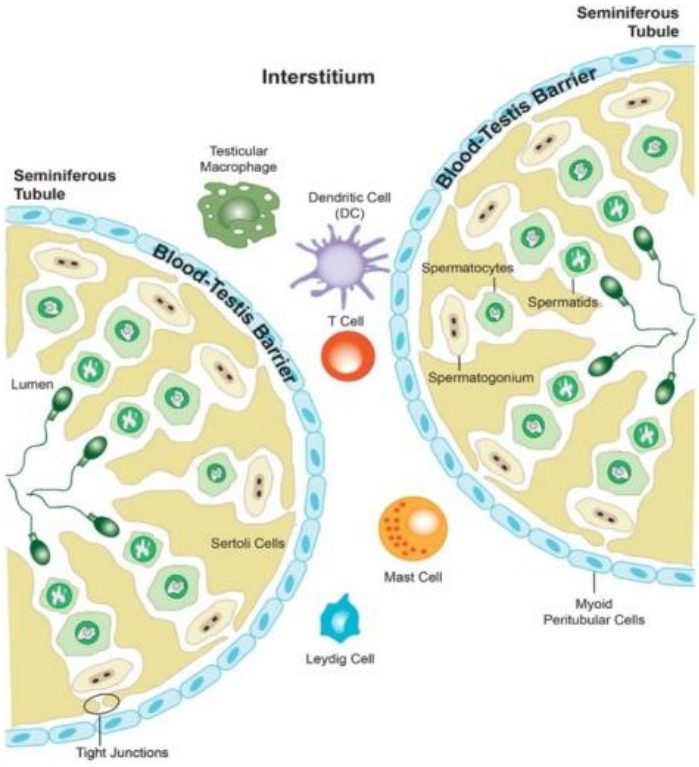


Figure 2. Scheme of the human testis (Redgrove & McLaughlin, 2014)

1.2 Fetal development of the testis

In mouse at 10.5 days post-coitum (dpc), primordial germ cells (PGCs) colonize the genital ridge, which gives rise to the bipotential gonad. Under the control of the Y chromosome between 12.0 and 12.5 dpc, male-specific structures are emerging in testis (Brennan et al. 2002). At this stage, Sertoli cells arise in the seminiferous cords and support the primordial germ cells (PGC; Karl & Capel, 1998). The establishment of the basement membrane takes place when an adjacent mesonephros forms with peritubular cells, which cooperate with the Sertoli cells (Skinner et al., 1985; Nishino et al., 2001). The testis is composed of somatic and germ cells. The somatic Sertoli cells provide the proper micro-environment and support the developing germ cells. During development, the somatic cells undergo rapid proliferation in the testis. However, at 13.5 dpc germ cells undergo a mitotic arrest and do not resume their proliferation until birth. After birth, they migrate to the seminiferous basement membrane and begin to differentiate (Phillips et al., 2010).

Normal spermatogenesis requires a variety of cell-cell interactions mediated by paracrine growth factors and maturation of testicular somatic and germ cell lineages (Shalet, 2009). In mice, at approximately embryonic day (E) 10.5 PGCs in response to their respective environments differentiate along the spermatogenic pathways (McLaren, 2001; Kocer et al.,

2009). The endocrine system, hormones and growth factors produced within the testis, tightly regulates testis development and the maintenance of adult spermatogenesis. However, the molecular pathways directing male germ line development are poorly understood, even though these processes are crucial for later fertility and for preventing germ cell tumors.

1.2.1 The TGF-β family with a special focus on TGF-β and activin A

The transforming growth factor β (TGF-β) signalling pathway is a critical regulator of many cellular processes, including proliferation, differentiation, migration, invasion, and angiogenesis (Gordon & Blobe, 2008). The TGF-βs and their receptors are members of a large superfamily, which includes activins, inhibins, nodal, bone morphogenetic proteins (BMP), lefty A and B, growth and differentiation factors (GDFs) and anti-Mullerian hormones (Chang et al., 2002; Peng, 2003). The TGF-βs comprise three isoforms, namely TGF-β1, TGF-β2, and TGF-β3.

Activin A is a member of the pleiotropic TGF-β superfamily (Vale et al., 1986). It affects many aspects of cellular development, between E12.5 to birth (0 dpp), activin A transcript levels rise and stimulate the deveoplment of the testes (Mendis et al., 2011). Activin is composed of two β disulphide-linked subunits of the inhibin β_A, β_B and β_C dimer (Vale et al., 1986). Which determine to form three activin proteins, activin A ($\beta_A\beta_A$), activin B ($\beta_B\beta_B$), and activin AB ($\beta_A\beta_B$) (Hotten et al., 1995; Loveland et al., 1996). Inhibins antagonize activin by binding to the activin receptor and block the access for activin ligands to bind (de Kretser & Robertson, 1989). Inhibin share structural similarities with the activins, have a subunit inhibin A ($\alpha\beta_A$) or inhibin B ($\alpha\beta_B$) (de Kretser & Robertson, 1989). Activin A and inhibin play opposite roles: activin A stimulates FSH secretion from the anterior pituitary (Ling et al., 1987), whereas inhibin suppresses it (de Kretser et al., 2002). Mice lacking inhibin (*Inha*$^{-/-}$) develop Sertoli cell tumors due to uncontrolled activin signalling which leads to unrestrained proliferation and failure to mature (Matzuk et al., 1992).

In cells TGF-βs and pro-activin A are produced in an inactive form (Wang et al., 2016); TGF-βs interact with a latency associated peptide (LAP) forming a complex called small latent complex (SLC). Inside the cells this complex remains until it is bound by another protein called latent TGF-β-binding protein (LTBP), which forms a complex called large latent complex (LLC; Rifkin, 2005).

After its secretion, TGF-βs remain in the extracellular matrix (ECM) as an inactivated complex, the LCC, which needs to be further, processed to be released as active TGF-β

(Annes et al., 2003). TGF-β is covalently linked to LTBP, thus maintaining TGF-β latency but also TGF-β activation by integrin (Robertson et al., 2015).

The mechanism of activation of TGF-β is differently mediated by matrix metalloproteinases (MMPs) and integrins. MMP2 and MMP9 can directly cleave the LAP of TGF-β and release the active TGF-β isoform (Yu & Stamenkovic, 2000; Ge & Greenspan, 2006). However, other enzymes, like thrombin, or MMP14 interact with integrins to mediate TGF-β activation (Jenkins, 2008).

Active TGF-β1 regulates different functions such as inflammatory responses, wound healing, embryogenesis, and immunity (Johnston et al., 2016). However, dysregulation of TGF-β1 activation will lead to numerous diseases including Marfan's Syndrome (Neptune et al., 2003), organ fibrosis (Sheppard, 2006), autoimmunity, and malignant diseases (Wrzesinsk et al., 2007).

The TGF-β receptors consist of type I (TβRI; ALK5; activin receptor-like kinase), type II (TβRII) and type III receptors (TβRIII; Chang et al., 2002) and activin A signalling is predominantly via type II receptors ActRIIA and ActRIIB and type I (ALK-4 and ALK7; Pangas & Woodruff, 2000). TGF-β and activin A receptors type I and type II are transmembrane serine/threonine kinases. TGF-β/activin A initiate the intracellular signalling pathway by binding to cell surface type II receptors, which then transphosphorylate type I receptors. Then the activated type-I receptors phosphorylate Smad2 or Smad3, which bind to Smad4 in the cytoplasm or the nucleus forming a Smad complex (Massagué, 1998). The Smad complex interacts with transcription factors in the nucleus to regulate TGF-β/activin A responsive genes (Chen et al., 2003; Di Guglielmo et al., 2003; Biondi et al., 2007; Wrighton et al., 2009), which are responsible for biological functions such as proliferation and differentiation (Hedger et al., 2011).

Regulation of TGF-β and activin A signalling is achieved through the induction of the inhibitory Smads (I-Smads), Smad6 and Smad7, which block the activation of R-Smads and co-Smads (Moustakas et al., 2001). They also act on the type I receptor and trigger its degradation (Kariyawasam et al., 2009). I-Smads control the TGF-β signalling pathway by suppressing the overactivation, which might lead to tumor development due to uncontrolled active TGF-β signalling (Principe et al., 2014). Specifically, Smad7 appears to block TGF-β ligand signalling (Imamura et al., 1997; ten Dijke et al., 2000, 2002; Shi & Massagué, 2003), whereas activin A is further regulated by a potent physiological inhibitor, follistatin (Hashimoto et al., 1997).

Follistatin - another antagonist to activin signalling - can bind activin A and B with high affinity (Nakamura et al., 1990, 1991). Follistatin binds to activin and forms a complex, which undergoes endocytosis and lysosomal degradation and ultimately causes an irreversible inhibition of activin signalling (Hashimoto et al., 1997; Phillips & de Kretser, 1998).

TGF-β activates Smad-dependent (canonical) and Smad-independent (non-canonical) pathways. TGF-β induces the non-canonical pathway by activating MAPK-ERK1/2, P38, or JNK (Moustakas et al., 2001). The Smad-dependent signalling pathway activated by TGF-βs is shown in Figure 3.

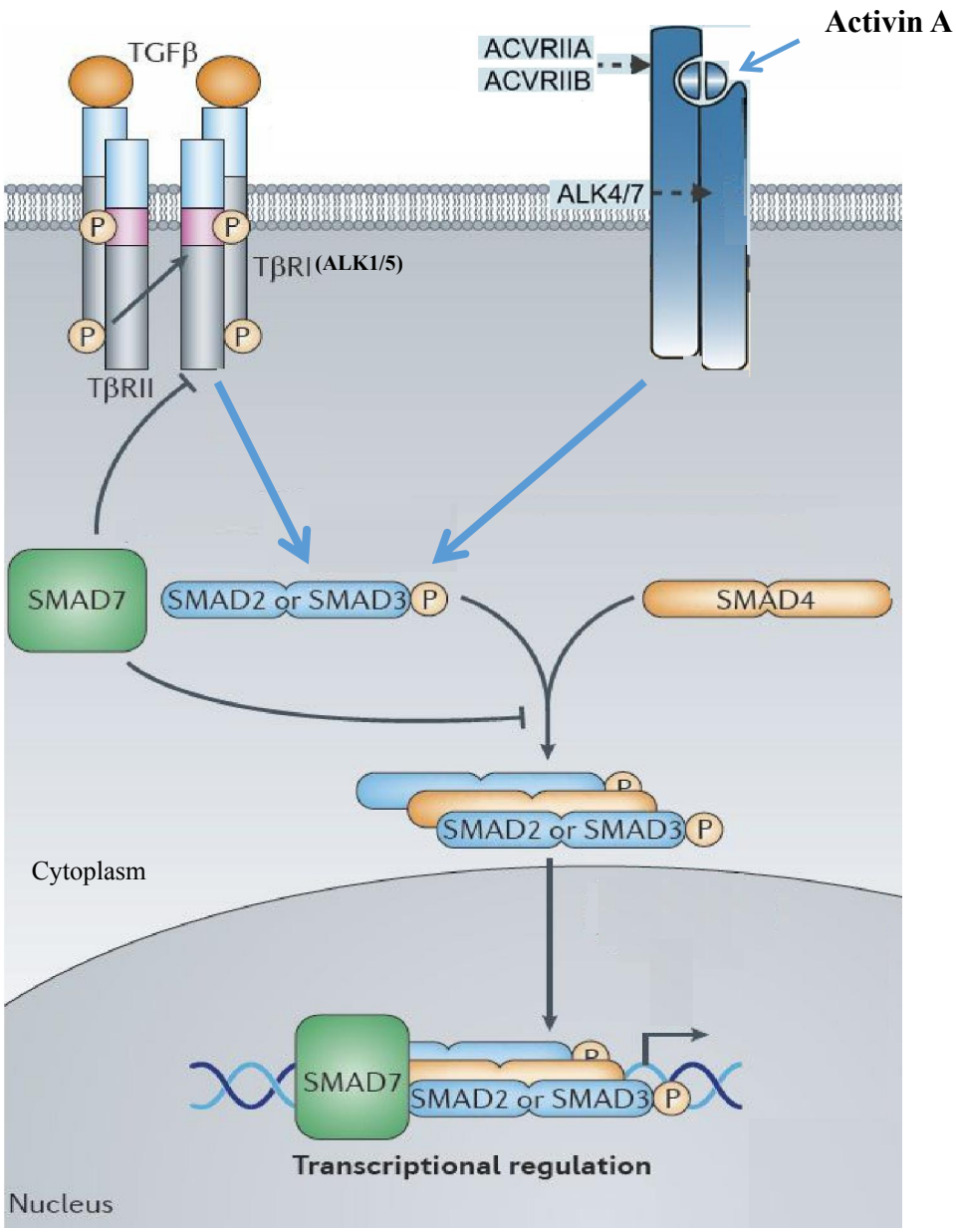


Figure 3. Signalling of the transforming growth factor-β (TGFβ) and activin A

Upon activation of TGFβ and activin A both bind to their receptors and form a heteromeric complex with specific type II and type I receptors (Massagué, 1998). TGFβ type-I receptors are ALK1 and ALK5, whereas activin A type-I receptors are ALK4 and ALK7. Type II receptors then transphosphorylate type I receptors, which then phosphorylate specific Smads (R-Smads: Smad2 and Smad3). They form heteromeric complexes with the common mediator Smad4 and translocate to the nucleus. Once in the nucleus the R-Smad-co-Smad complex preferentially associates with DNA at a specific domain called Smad-binding element (SBE) in a sequence-specific manner. Smad7 inhibits the TGF-β pathway degradation of the type I receptor, by inhibiting phosphorylation of R-Smads by the type I receptor kinase or by inhibiting the formation of the R-Smad-co-Smad complex (Massagué, 1998).

1.2.2 TGF-βs and their effects in testis

Members of the TGF-β superfamily play key roles in establishing fetal testis structure as indicated by testicular defects in several TGF-β superfamily knockout mice (Uzumcu et al., 2004; Memon et al., 2008). In general, the tight regulation and production of TGF-βs is important as they are also involved in tumorigenesis and inflammation (Padua & Massagué, 2009; Santibañez et al., 2011). In contrast, a decrease in the number of germ cells was noted in the TGF-β1 null mice at the day of birth, causeing infertility in male mice due to testosterone deficiency and sexual dysfunction (Ingman & Robertson, 2007; Memon et al., 2008). These data are in broad agreement with *in vitro* studies, showing that TGF-β1 reduces migration and growth of peritubular cells (Skinner & Moses, 1989) and in vivo cord formation (Cupp et al., 1999). TGF-β1 knockout mice die at gestation due to a defective vasculature (Shull & Doetschman, 1994). TGF-β2 knockout mice exhibit perinatal mortality and a wide range of developmental defects such as testicular agenesis etc (Sanford et al., 1997), and a decrease in the number of seminiferous cords at 15 dpc (Memon et al., 2008). In mature testis TGF-β3 is the major isoform expressed (Miller et al., 1989; Watrin et al., 1991). *In vivo* studies showed that TGF-β3 regulates the Sertoli cell tight junctions, which form the blood testis barrier (BTB; Lui et al., 2003). TGF-β3 knockout mice showed a delayed pulmonary and defective palate development (Kaartinen et al., 1995; Proetzel et al., 1995; Shi et al., 1999). All TGF-βs isoform are present in the immature testis. Peritubular and Sertoli cells express each isoform of the TGF-βs during the development of the testis (Mullaney &

Skinner, 1993). The TGF-β superfamily members have been shown to be locally produced within specific cell types of the developing rodent testis (Itman et al., 2011).

In vitro studies showed that rat germ and Sertoli cells released bioactive TGF-β (Haagmans et al., 2003). Sertoli cell expression of TGF-β1 increased slightly and reached a plateau during pubertal development, whereas TGF-β2 mRNA expression was evident in immature Sertoli cells (Mullaney & Skinner, 1993). Specifically in rat Sertoli cells at 14.5 dpc TGF-β1 and TGF-β2 protein expression appeared limited, and it was undetectable in gonocytes (Cupp et al., 1999). However, induction of TGF-β1 or TGF-β2 has been shown to cause apoptosis of rat gonocytes at 14 dpc (Olaso et al., 1998). This shows that TGF-β signalling directly influences the survival of gonocytes and Sertoli cells. At 13.5 dpc the proliferation of both gonocytes and Sertoli cells did not vary with the addition of either TGF-β1 or TGF-β2. However, a marked increase in DNA fragmentation and apoptosis in gonocytes was observed at this stage (Olaso et al., 1998). This phenomenon was not observed with 17.5 dpc explants (when gonocytes are quiescent) indicating that the influence of TGF-β1 is reduced during the period of germ cell quiescence. It also been stated that Sertoli cells alone expressed TGF-β1 at the mRNA level but not at the protein level, however, when the Sertoli and germ cells where co-cultured TGF-β1 was expressed at both the mRNA and protein level (Avallet et al., 1997). This observation demonstrates that Sertoli cell-germ cell interactions regulate TGF-β1 expression and secretion.

In vitro experiments showed that addition of exogenous TGF-β2 enhances aggregation of Sertoli cells in co-cultures with peritubular myoid cells from 19-day-old rats but not in monocultures (Konrad et al., 2000). Collectively, these data suggest that TGF-β1 and TGF-β2 secretion by somatic cells may act on both somatic and germ cells of the pubertal testis to regulate cord formation.

1.2.3 Activins and their effects in testis

Activin presence and actions in testes have been documented in numerous publications. It is shown that, Activin A levels were elevated in many acute and chronic inflammatory conditions (Zhang et al., 2009; Wu et al., 2013). Activin subunits A & B are highly expressed during testis development (Wijayarathna & de Kretser, 2016). In murine testis activin type II receptors are mainly localized in Sertoli cells (Mendis et al., 2011). Buzzard et al. (2004) showed that spermatogonia and primary spermatocytes also express activin A and B.

Furthermore, activin A and B are produced in mouse, rat and human Leydig cells (Lee et al., 1989; Anderson et al., 1998; Marchetti et al., 2003). Activin A expression varies in testis development: in early post-natal development activin A expression increases (Barakat et al., 2008; Mithraprabhu et al., 2010), which induces germ and Sertoli cell proliferation, but after reaching puberty activin A levels decrease (Buzzard et al., 2004). Knockdown of activin A in mice showed a significant reduction in testis weight, lower numbers of Sertoli cells, but increased numbers of gonocytes. Thus, activin A is important for the germ cell and Sertoli cell ratio (Archambeault & Yao, 2010). Activin A knockout mice had delayed fertility (Brown et al., 2016), whereas activin B knockout mice still remain fertile (Vassalli et al., 1994). All these observations suggest that activin A plays a pivotal role in male fertility.
In adult rat testis during the release of sperms into lumen at stage VIII of spermatogenesis activin A production increases in seminiferous tubules (Okuma et al., 2006). Moreover, at this stage activin A disrupts the Sertoli cell tight junctions (Nicholls et al., 2012). These data suggest that activin A might be involved in the process of opening up of the blood-testis barrier, which enables the transit of preleptotene spermatocytes to the intraluminal side of the tubule. Taken together, these studies suggest that activin A have multiple roles in the testis.

Higher activin A levels cause differentiated adult Sertoli cells to revert back to an immature, proliferating phenotype (Wijayarathna & de Krester, 2016), highlighting the importance of activin A signalling in testis development and fertility. In clinical studies, men with Sertoli cell-only syndrome, which cause non-obstructive azoospermia, showed highly elevated Smad2 signalling activated by activin A (Sun et al., 2008). Human seminoma expresses elevated amount of activin type I and type II receptor mRNA (Dias et al., 2009). In a good correlation with these findings, activin A levels are higher in human testicular cancer, where the activin A levels were higher (Marino et al., 2014).
Activin A regulates phosphorylation of Smad2/3 differentially in testis. During dpp 6 activin A phosphorylates both Smad2/3, but only Smad3 accumulates in the nucleus and this differs in pubertal Sertoli cells (day 15), where both Smad2/3 accumulate in the nucleus (Itman & Loveland, 2013). Previous studies showed that Smad3 knockout mice live to adulthood and are fertile, but they had delayed Sertoli cell maturation and reduced expression of the androgen receptor (AR; Itman et al., 2011). In contrast, Smad2-null embryos dye around gastrulation (Waldrip et al., 1998). These data suggest that Smad2 and Smad3 are differentially regulated during development and both, activin and TGF-βs, use similar Smad proteins to activate signalling.

Smad4 is a co-Smad that binds to activated p-Smads and is an essential mediator of proliferative signals in Sertoli cells to activin; blocking Smad4 in Sertoli cells resulted in impaired Sertoli cell proliferation and shorter cords (Archambeault & Yao, 2010).

1.2.4 Effects of the BMPs and their cross-talks in testis

Bone morphogenetic proteins (BMP) belong to the decapentaplegic-Vg-related (DVR) family which is a member of a large, highly conserved family related to TGF-βs. The BMP superfamily is divided into DPP (decapentaplegic; BMP2 and BMP4) and 60A classes (BMP5, BMP6, BMP7, BMP8a and BMP8b) based on their similarity to each other and their Drosophila counterparts (Jain et al., 2013). These superfamilies play major roles during embryonic development and differentiation (Kingsley et al., 1994; Hogan, 1996; Massagué & Chen, 1994; 2000). BMPs null mice die due to defects in the eye, kidney and limb (Dudley et al., 1995; Luo et al., 1995). Zhao et al. (1996, 2001) showed that in mouse BMP8a plays a role in maintenance of spermatogenesis and placental development. During early puberty in mice BMP7 transcripts were detected in stage-7 to -15 spermatids in spermatogonia and early primary spermatocytes (Ross et al., 2007).

BMPs activate signalling through type I receptors (ALK2, ALK3 and ALK6) and type II receptors (ActRIIA, ActRIIB; Suzuki et al., 1994; Dewulf et al., 1995; Mishina et al., 1999). Upon BMP binding to their type II receptors, type I receptors are recruited which in turn cause phosphorylation and activation of BMP-specific Smads (Smad1, Smad5, and Smad8). Phosphorylated Smads1/5/8 associate with Smad4 and translocate into the nucleus which activates the transcription of BMP-target genes (ten Dijke et al., 2003; Zwijsen et al., 2003). I-Smads block the signal transduction activated by BMP and act through different mechanisms to prevent translocation of phosphorylated Smads into the nucleus. In the case of BMPs Smad6 preferentially inhibits signalling (ten Dijke et al., 2003). The BMP signalling cascade is shown in Figure 4.

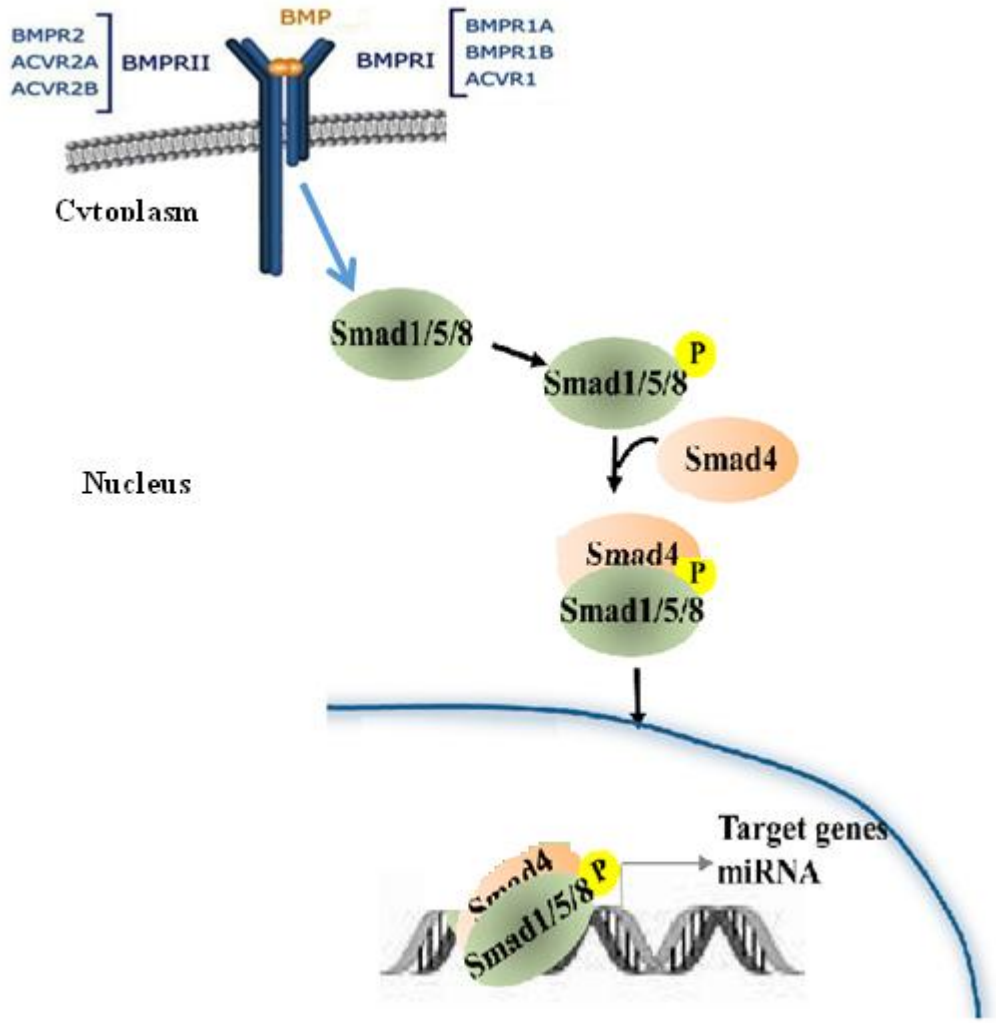


Figure 4. Scheme of BMP signalling-In the canonical pathway BMP activate constitutively a signal transduction cascade by binding to type II receptors. Active type II receptors then transphosphorylate the type 1 receptors and form a heterotetrameric complex. This complex phosphorylates Smad1/5/8. Phosphorylated Smad1/5/8 associate with co-Smad4 and are altogether translocated into the nucleus with the assistance of translocator proteins. Translocated Smads bind to coactivators or corepressors in order to regulate gene expression.

Previously published data have shown that BMP differentially activates Smad1, Smad5, and Smad8 during testis development and osteoblast differentiation (Itman & Loveland, 2008; Aoki et al., 2001). BMP4 leads to nuclear accumulation of Smad1/5/8, whereas BMP7 only activates Smad1 and Smad5 (Aoki et al., 2001). In chondrocyte cell lines, BMP2 signalling is mediated by Smad1 and Smad8 (Valcourt et al., 2002). Hence, Smad activation by BMPs leads to activation of distinct target genes (Valcourt et al., 2002; Hatakeyama et al., 2003). In mice Smad1$^{-/-}$ and Smad5$^{-/-}$ mice, germ cell numbers are remarkably reduced or even absent

(Chang & Matzuk 2001; Tremblay et al., 2001). In spermatocytes and round spermatids Smad1 mRNA and protein have been detected in postnatal mouse testis (Zhao & Hogan, 1997), whereas in the prepubertal mouse testis only Smad5 and Smad8 have been reported (Pellegrini et al., 2003).

BMP2, BMP4, BMP7, BMP8a, and the BMP receptors are highly expressed in mouse testis at all stages of development (Ciller et al., 2016). Recently published data showed that migration of osteoblast progenitors can be stimulated by BMP2 and BMP4 (Fiedler et al., 2002). Of note, during germ cell migration PGCs and somatic cells express BMP receptors and Smad1/5/8 (Dudley et al., 2007). Furthermore, BMPs also induce germ cell maturation and maintain sperm quality (Zhao et al., 1996, Hu et al., 2004). Compromised fertility or infertility was observed in BMP7 and BMP8a mutant male mice (Zhao et al., 1998, 2001). The precise development in mammalian spermatogenesis depends upon the functional coordination between the Sertoli cells and germ cells (Griswold, 1995). During early postnatal testis development the final size of the Sertoli cell population is very important for the maintenance of germ cells to undergo differentiation (Orth et al., 1988). Puglisi et al., (2004) showed that during this period BMP2 and BMP7 act as proliferative signals upon Sertoli cells and spermatogonia. Furthermore, in mice spermatogonial differentiation can be regulated also by BMP4 (Pellegrini et al., 2003; Baleato et al., 2005).

Effect of BMP on the blood-testis barrier due to the observation that the BTB integrity depends on peptidylprolyl cis/trans isomerase, NIMA-interacting 1 (Pin1; Islam et al., 2017). Pin1 is a peptidylprolyl cis/trans isomerase (PPIase) that specifically catalyzes the cis/trans isomerization of peptidyl-prolyl peptide bonds, preceded by a phosphorylated serine or threonine residue. It is highly expressed in Sertoli cells and can be also regulated by BMPs (Yoon et al., 2015; Islam et al., 2017).

1.2.5 Cross-talk between the TGF-β superfamily members TGF-βs, activin A and BMPs

The cross-talk between signalling pathways is very important for the maintenance of many functions including male fertility. Cross-talk among receptors can be exemplified by the interaction between the TβRII cytoplasmic domain and ALK2 (Chen & Derynck, 1994; Feng & Derynck, 1996). Furthermore, TGF-β-induced phosphorylation of intracellular mediators Smad1/5 acts via type I receptors ALK1; it was also shown that ALK1 opposes ALK5-mediated Smad3 phosphorylation (Finnson et al., 2008). Signalling cross-talk is a common

theme of TGF-β research, the cross-talk between TGF-β and other pathways have a dynamic role in the development of testis since Smads directly or indirectly bind numerous other proteins and activate different transcription factors (Itman & Loveland, 2008). In this thesis, we focus on the TGF-β receptor cross-talks and its effects on Sertoli and germ cell functions.

Previous publication showed that activin A binds to ALK2 and BMPR2, and stimulate follicle-stimulating hormone β (Fshb) subunit transcription in immortalized murine gonadotrope-like cells (Attisano et al., 1993, Rejon et al., 2013). In myeloma cell lines, activin A inhibited signalling of BMP6 and BMP9 by binding to BMP type 2 receptors, whereas BMP2 and BMP4 were not affected (Olsen et al., 2015). Furthermore, in hepatocytes activin B acts via BMP type I receptors and induces non-canonical Smad1/5/8 signalling (Canali et al., 2016).

Cross-talks between other signalling ligands are also possible. Recently it was shown that TGF-β and BMP receptors interact (Poorgholi et al., 2012; Ehrlich, 2012). Besides the canonical pathway of the TGF-βs, Smad1 or Smad5 can be activated via ALK1 (Shen et al., 2007; Grönroos et al., 2012; Flanders et al., 2014; Chan et al., 2017). Of note, TGF-β-induced phosphorylation of Smad1/5 is necessary for the protection against pulmonary artery endothelial cell apoptosis and anchorage-independent growth of epithelial cells (Lu, 2008). BMPs were also found to activate TGF-β-dependent Smad2/3 as evidenced by BMP2 stimulation of Smad3 via ALK3 (Holtzhausen et al., 2014; Wang et al., 2014) and also BMP2 upregulates plasminogen activator inhibitor-1 (PAI-1), which is a TGF-β-regulated gene (Murakami, 2009).

Chan et al. (2017) reported that in rat testis tubules TGF-βs signal via BMP receptors and phosphorylated Smad1. Specifically, TGF-β1 mainly used ALK2, whereas TGF-β2 preferred ALK3 and ALK6. This cross-talk also enhanced PAI-1 secretion. In current study, we also determined the possible cross-talks between BMP and activin A on Smad1 signalling.

1.3 Role of betaglycan, TIMPs and MMPs in testis

Beyond the classical TGF-β receptors, a third TGF-β receptor, known as betaglycan (TβRIII). It is membrane-bound, but it does only very negligible signalling on its own (Blobe et al., 2001). Although betaglycan is capable to bind all three TGF-β isoforms, it predominantly binds to TGF-β2 with high affinity as it facilitates signalling of TGF-β2 by presenting it to TβRII (Lopez-Casillas et al., 1994). Soluble betaglycan affinities for the TGF-β isoforms are

TGF-β2 > TGF-β3 > TGF-β1 and the antagonistic effects of soluble betaglycan were 10-fold higher for TGF-β2 than for TGF-β1 *in vitro* (Vilchis-Landeros et al., 2001).
Membrane-bound betaglycan binds TGF-βs and presents the ligands to their receptors and enhances the signalling cascades (Lopez-Casillas et al., 1994). However, betaglycan can also undergo ectodomain shedding, which results in releasing a soluble part called soluble betaglycan (sBG); a process known as shedding. The sBG binds TGF-β ligands and blocks ligand binding to their receptors, thus inhibiting the downstream signalling of the TGF-βs (Lopez-Casillas et al., 1994, Elderbroom et al., 2014). However, it depends on the ratio of soluble to membrane-bound betaglycan to determine the outcome of the TGF-β signalling cascade (Mendoza et al., 2009). However, in some cases (glycosaminoglycan chain) shedding-independent inhibition has also been demonstrated (Eickelberg et al., 2002). Betaglycan can also inhibit Smad2/3 signalling by binding to TGF-β type I and type II receptors and thus blocking signalling (Tazat et al., 2015).

Betaglycan regulates cell proliferation and migration by activating Cdc42 (Cell division control protein 42; Mythreye & Blobe, 2009). Furthermore, betaglycan suppresses the progression of multiple types of cancer, including breast cancer (Dong et al., 2007). TGF-β1 decreases betaglycan expression on both mRNA and protein level in ovarian and breast cancer through effects on the ALK5/Smad2/3 pathway (Hempel et al., 2008). Embryonic lethality was observed in betaglycan knockout mice at 16.6 dpc due to developmental defects in the liver and heart (Stenvers et al., 2003). This observation implies the high importance of betaglycan in development.

Cleavage of membrane-anchored betaglycan into its soluble form is carried out by MMPs and A Disintegrin and Metalloprotease (ADAMs; Velasco-Loyden et al., 2004). The mechanisms of regulating ectodomain shedding and generation sBG remain undefined, making it more difficult to delineate the function of cell-surface betaglycan or the relative contribution of cell-surface betaglycan and soluble betaglycan to signalling. The MMPs which mediate shedding are the membrane-type MMPs: MT1-MMP, MT2-MMP, and MT3-MMP (Velasco-Loyden et al., 2004). Betaglycan shedding can be reduced, but not blocked with the pan-metalloproteinase inhibitor tumor necrosis factor alpha protease inhibitor 2 (TAPI-2), as well as with more specific inhibitors against MT1-MMP and MT3-MMP (Velasco-Loyden et al., 2004).

Matrix metalloproteinases are members of the metzincin superfamily; they play an essential role in cell biological processes such as tissue remodeling and wound healing (Page-McCaw et al., 2007). Dysregulated MMP activity results in pathological conditions such as arthritis, inflammation, and cancer (Löffek et al., 2010). In testis, MMPs dysregulations affect the integrity of the BTB (Barone et al., 2017). Of note, MMP2 and MMP9 can be activated by activin A and TGF-β1 (Ogawa et al., 2000; Kim et al., 2004).

MMP activation is tightly controlled by tissue inhibitors of metalloproteinases (TIMPs). TIMPs are specific inhibitors of MMP activity, which bind MMPs non-covalently in a 1:1 ratio (Baker et al., 2002; Brew & Nagase, 2010). Generally, there are four types of TIMP (1-4) and they differ in their ability to block proteases (Baker et al., 2002). However, TIMP3 is the major regulator of MMP activity *in vivo* because in mice TIMP3 ablation cause lung emphysema-like alveolar damage and after weaning faster apoptosis of mammary epithelial cells (Fata et al., 2001; Leco et al., 2001; Nagase et al., 2006). TIMP1-3 is present in undifferentiated gonads (11.5 dpc) and neonatal testis (Guyot et al., 2003). Of note, TIMPs can be upregulated by TGF-βs (Leco et al., 1994; Leivonen et al., 2013) and they have complex regulatory interactions. TGF-βs induce TIMP3 synthesis via Smad3 and Smad4 (Leivonen et al., 2013).

Betaglycan has cell surface heparan sulfate/chondroitin sulfate proteoglycan chains that bind to all TGF-β isoforms (Prydz, 2015). Betaglycan also binds BMP and inhibin ligands via its core protein with high affinity and modulates the association of these ligands to their receptors (Wang et al., 1991; Bernabeu et al., 2009). Previously, it was shown that fibroblast growth factor-basic (bFGF) binds to betaglycan via its heparan sulfate chains (Fiore et al., 2001). TGF-β2, which is well-known to bind to betaglycan several hundred-fold more strongly than TGF-β1 or TGF-β3 (Villarreal et al., 2016), activate the TGF-β2 signalling cascade mainly via betaglycan. Cells lacking betaglycan expression are less responsive to TGF-β2 compared to TGF-β1 or TGF-β3 (de Crescenzo et al., 2006; Baardsnes et al., 2009).

Betaglycan sequence analysis showed an extracellular region containing potential sites for glycosaminoglycan (GAG) attachment (Prydz, 2015). The role of these GAG chains in the function of betaglycan as a growth-factor-binding proteoglycan is unclear. Betaglycan GAG chains consist of heparan sulfate (HS), chondroitin sulfate (CS); HS and CS chains are characterized by repeated disaccharide units containing glucuronic acid and either N-acetylgalactosamine for HS or N-acetylglucosamine for CS (Prydz, 2015). Consequently,

shed/soluble betaglycan and their attached GAG chains can affect ligand availability, and downstream signalling. It might promote interactions between GAG chains and ligands.

The GAG chains of betaglycan are not necessary for the expression of betaglycan at the cell surface, the binding of TGF-β to the betaglycan core protein, and the inhibitory effect of TGF-β (Cheifetz & Massagué, 1989). But it was shown that GAG chains influence the binding ability of TGF-β1 to their receptors (Eickelberg et al., 2002). GAG chain function in testis is still unknown; thus we investigated the effects of GAG chain influence in TGF-βs signalling and effects on the downstream Smads in Sertoli cells. The betaglycan structure is explained in details in Figure 5.

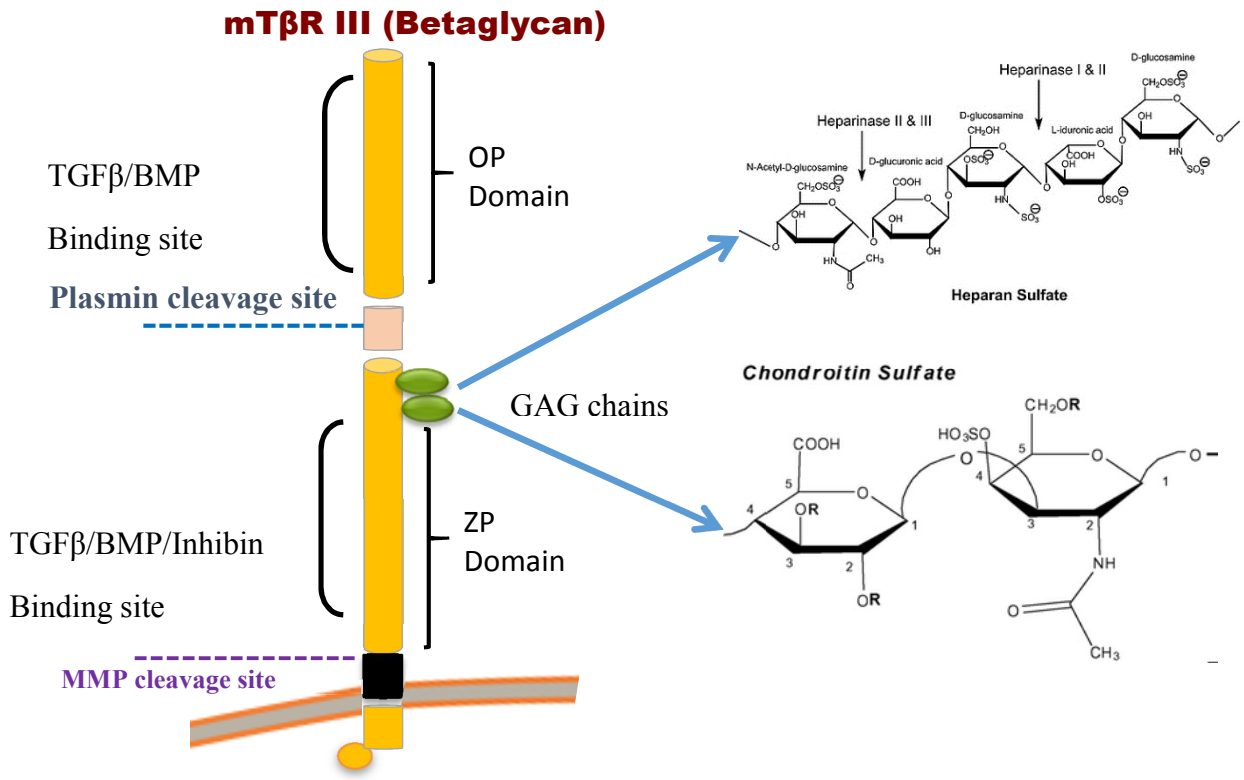


Figure 5. Scheme of the betaglycan structure: Betaglycan has a large extracellular domain comprised of two subdomains, a membrane distal orphan domain (OP) and a membrane proximal zona pellucida (ZP) domain (López-Casillas et al., 1991). The ZP domain can bind inhibin, BMPs and TGF-βs, while OP binds only BMPs and TGF-βs (Fukushima et al., 1993; Pepin et al., 1994; Makanji et al., 2007). GAG chains, heparan and chondroitin sulfate are attached to two residues in the ZP domain. This is shown schematically as bead on a string. Plasmin cleaves betaglycan at a specific site and thus reduces the TGF-βs ability to bind the receptor (Lamarre et al., 1994). R groups as sulfate groups.

1.3.1 Cross-talks of betaglycan with other ligands and receptors

Betaglycan and the type II activin receptor form a complex that binds inhibin A with high affinity and prevents the formation of the functional activin type I and II receptor complex; it has been proposed that this accounts for the anti-activin effect of inhibin (Lewis et al., 2000). It has also been shown that TGF-β blocks inhibin binding to different target cell types via betaglycan and vice versa (Farnworth et al., 2007; Looyenga et al., 2010). The binding affinities of different ligands to betaglycan is TGF-β2 > inhibin A > TGF-β1 (Esparza-Lopez et al., 2000).

Betaglycan binds multiple BMPs (BMP2, 4, 7) and functions as a BMP co-receptor, by enhancing their ligand binding ability to the BMP type I receptors, ALK3 and ALK6 (Gatza et al., 2010). In contrast, betaglycan can also inhibit BMP signalling by promoting inhibin, which blocks the BMP binding to betaglycan (Wiater & Vale, 2003). Furthermore the balance between the soluble and the membrane-bound betaglycan influences BMP signalling in epithelial cells (Gatza et al., 2014). The soluble form can bind BMPs ligands, thus preventing binding to the receptor and finally blocking signalling (Gatza et al., 2014).

1.3.2 Betaglycan in testis

Betaglycan is essential for normal seminiferous cord and Leydig cell development in the fetal mouse testis (Sarraj et al., 2007). Betaglycan has been associated with the testicular dysgenesis syndrome in men (Sarraj et al., 2013). However, the mechanisms underlying betaglycan-regulated testis development are unclear. In the 11.5-12.5 dpc testis, the betaglycan ligands, the TGF-βs, and inhibins are more prevalently expressed (Sarraj et al., 2013). Betaglycan knockout male mice survive to sexual maturity but exhibit poor fertility (Stenvers et al., 2003). During 12.5 dpc and 14.5 dpc the expression of betaglycan is increased in mouse testis (Sarraj et al., 2007). Specifically, betaglycan is highly expressed in Leydig and peritubular cells at 12.5 dpc upto 5 dpp, whereas in Sertoli cells it is observed only after birth (Sarraj et al., 2007). Although betaglycan and TGF-β signalling contribute to many testicular functions, they have not been analyzed in Sertoli cells in detail.

1.4 Role of hormones

Follicle-stimulating hormone (FSH) is a member of the glycoprotein hormone family and mediates its signals via binding to the FSH receptor (Walker & Cheng, 2005).

FSH and testosterone are required to obtain the full reproductive potential in males (Walker & Cheng, 2005). In testis, fertility and regulation of spermatogenesis depend upon Sertoli cells, to produce factors such as TGF-α and TGF-β, insulin-like growth factor-I (IGF-I), fibroblast growth factor (FGF) epidermal growth factor (EGF), and hormones that regulate development. All these are required by developing germ cells during differentiation into spermatozoa (Sharpe, 1994, Griswold et al., 1998, Skinner, 2005).

In testis, Sertoli cells build the BTB that protects germ cells from cells of the immune system. In the early stages of meiosis, germ cells pass through the BTB (Mruk & Cheng, 2004). In seminiferous tubules, Sertoli cells express FSH receptors. FSH binding to its receptors catalyses an exchange of GDP (Guanosine diphosphate) for GTP (Guanosine triphosphate) leading to the dissociation of Gα and Gβ subunits. GTP-Gα activates membrane bound adenylate cyclases that catalyze the production of cAMP. cAMP was found to be at its highest level in stage XIV-VI of spermatogenesis (Kangasniemi et al., 1990). cAMP can activate different signalling pathways in Sertoli cells, for example the cAMP-PKA pathway, which stimulates the transcription factor CRE binding protein (CREB; Walker et al., 1995). Mutations of CREB in Sertoli cells lead to apoptosis of spermatocytes and subsequent loss of spermatids (Scobey et al., 2001). CREB is activated during Sertoli cell proliferation (Fix et al., 2004).
The other pathway which is induced by FSH is the MAP kinase pathway; in early stages of testis development it was found that FSH triggers phosphorylation of ERK in Sertoli cells and Stimulates their proliferation (Crepieux et al., 2001).

During the fetal days, FSH levels in rats increase at birth and are almost doubled by day five after birth, reaching their peak steady-state levels after 50 days (Chowdhury & Steinberger, 1976; Ketelslegers et al., 1978). FSH acts directly to stimulate Sertoli cell proliferation and indirectly on Leydig cells to increase androgen production (O'Shaughnessy et al., 2010). The function of Sertoli cells and spermatogenesis depend on FSH. This has been already shown in hypogonadal (hpg) mice, which lack circulating FSH and are infertile due to the failure of the germ cells to progress beyond early meiosis (Cattanach et al., 1977). Oduwole et al. (2018) showed that strong activation of FSH receptor can induce testosterone-independent spermatogenesis and their mechanism underlying for this behaviour is might be due to maintainance of Sertoli cell genes expression, which are considered androgen dependent by FSHR activation.

FSH interacts with other growth factors like activin A and BMPs to increase Sertoli cell proliferation (Meehan et al., 2000; Puglisi et al., 2004). Previously published data by Konrad et al. (2000) showed that FSH reduced TGF-β2 production in Sertoli cells. As FSH induced Sertoli cell proliferation and reduced TGF-βs secretion.

1.5 Objectives and aims of the study

Despite of the many investigation concerning TGF-β superfamily has been demonstrated to play important roles in testis development, but many questions remains to be answered. Of note, a cross-talk with other signalling pathways has been reported, but the exact effect on the somatic cells has not been fully established. Thus, it is interesting to examine the cross-talk among some members of the TGF-β superfamily and their effects on testicular cells in as much as Smads and receptors are potentially involved.

One protein at the crossroad of several pathways of TGF-β family members is betaglycan. It has been found to regulate TGF-βs, activin, and BMP signalling in many cells, thus it will be of great importance to elucidate how TGF-βs regulate the expression of betaglycan and other pathways utilized for its regulation.

In a reductionist approach, normally only one substance and its effects is studied. However, in the case of TGF-β family members, TGF-βs, BMPs and activin A there is often an overlap in the use of receptors, such as with betaglycan. Futhermore, also an overlap between the Smad signalling cascade (Smad2/3) for TGF-βs and activin A can occur. Thus, our main focus was to elucidate how testicular cells react to the stimulation of TGF-βs alone or together with either activin A or BMPs. In summary, we asked how the cells can discriminate between the different ligands. Is there a hierarchy of signalling or are there inhibitory or synergistic effects of the distinct ligands on testicular cellular functions?

1.5.1 Aims of the study

TGF-β, activin A and BMP play an important role in the development of the testis and spermatogenesis. Because very few studies have addressed betaglycan in testis and it is known that betaglycan plays a crucial role in proper function of testes. Furthermore, the molecular mechanism of betaglycan shedding has not yet been elucidated. In our study, we investigated the effect of betaglycan shedding on signalling of distinct members of the TGF-β family. We addressed the following aims:

- Regulation of betaglycan shedding by TGF-βs, activin A and BMPs

- Effect of TIMPs in regulating shedding of betaglycan and on TGF-β signalling
- Activin cross-talks with BMPs signalling cascade and their effects on Sertoli cells
- Involvement of MMPs in betaglycan shedding by TGF-βs, activin A and BMPs
- Cross-talk between TGF-βs and activin A, and their effects on cell proliferation
- Influence of BMPs on Sertoli and germ cell migration and proliferation
- The effect of BMP2 on the blood-testis barrier integrity
- Investigating hormone effects on Sertoli cell proliferation and on secretion of TGF-βs
- Role of betaglycan GAG chains in the modulation of the signalling cascade of TGF-βs and BMPs

This study will elucidate the roles of TGF-β, activin A and BMPs in maintaining proper testicular functions.

2 MATERIALS AND METHODS

2.1 Materials and Chemicals

Acrylamide 30% (w/v)	Roth	Karlsruhe
Agarose	Invitrogen	Karlsruhe
Aprotinin	Tocris	Wiesbaden
Auxcillary Reagent Duoset Kit	R & D Systems	Wiesbaden
β-Mercaptoethanol	AppliChem	Darmstadt
Bromophenol blue sodium salt	Sigma-Aldrich	Steinheim
Calcium chloride ($CaCl_2$)	Merck	Darmstadt
Chloroform	Merck	Darmstadt
DNA ladder (100 bp)	Promega	Mannheim
Dimethyl sulfoxide (DMSO)	Merck	Darmstadt
1,4-Dithiothreitol (DTT)	Roche	Mannheim
Ethanol	Sigma-Aldrich	Steinheim
Ethidium bromide	Roth	Karlsruhe
Ethylene diaminetetraacetic acid disodium salt (EDTA)	Merck	Darmstadt
Formamide	Merck	Darmstadt
Glycerol	Merck	Darmstadt
Glycine	Sigma-Aldrich	Steinheim
Isofluran	Baxter	Germany
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Roth	Karlsruhe

Magnesium chloride ($MgCl_2$)	Merck	Darmstadt
Magnesium sulfate ($MgSO_4$)	Sigma-Aldrich	Steinheim
Methanol	Sigma-Aldrich	Steinheim
Non-fat dry milk	Bio-Rad	München
Paraformaldehyde	Merck	Darmstadt
Pepstatin A	Tocris	Wiesbaden
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich	Steinheim
Plasmocin Prophylactic	Invitrogen	USA
Ponceau S	Roth	Karlsruhe
Potassium di hydrogen phosphate (KH_2PO_4)	Merck	Darmstadt
Reagent diluent	R & D Systems	Wiesbaden
Sample activation kit	R & D System	Wiesbaden
Sodium acetate	Roth	Karlsruhe
Sodium azide	Merck	Darmstadt
Sodium chloride	Sigma-Aldrich	Steinheim
Sodium di hydrogen phosphate (NaH_2PO_4)	Merck	Darmstadt
Sodium dodecyl sulfate (SDS)	Merck	Darmstadt
Stop solution	R & D Systems	Wiesbaden
N, N, N', N'-Tetramethylethylenediamine	Roth	Karlsruhe
Tris (hydroxymethyl) aminomethane (TEMED)	Roth	Karlsruhe
Triton X-100	Sigma-Aldrich	Steinheim
Trypan Blue Dye, 0.4%	Biorad	Munchen
Tween-20	Roth	Karlsruhe

2.1.1 PCR Reagents

DNase I	Invitrogen	Karlsruhe
dNTPs	Promega	Mannheim
MMLV RT	Promega	Mannheim
Oligo dT	Promega	Mannheim
SYBR green	Bio-Rad	München
Taq polymerase	Promega	Mannheim

2.1.2 Antibodies

Table 1: Primary antibodies used for Western blots

Primary Antibody (Species reactivity)	Clone	Manufacturer	Catalogue No.	Dilution
p-Smad1/5 (Mouse/Rat)	Monoclonal	Cell Signalling	9516	1:1000
Vinculin (Human/Mouse/Rat)	Polyclonal	Cell Signalling	13901	1:5000

Table 2: Secondary antibodies

Secondary antibody	Manufacturer	Catalogue No.	Dilution
Goat anti rabbit IgG -HRP	Cell Signalling Technology, Massachusetts, USA	7074	1:10,000
Horse anti mouse -HRP	Cell Signalling Technology, Massachusetts, USA	7676	1:10000

2.1.3 Inhibitors

Table 3: Inhibitors

Name	Inhibition of	Manufacturer	Catalogue No.
GM6001	Broad collagenase and spectrum inhibitor of MMPs	US biological	G8237
TIMP3 recombinant human	MMPs, TACE (ADAM-17)	R & D Systems	973-TM-010
LY364947	TGF-β Type I receptor kinase (TGF-β RI, ALK5)	Sigma-Aldrich	L6293

2.1.4 Growth factors

Table 4: Ligands

Name	Manufacturer	Catalogue No.
Activin A, recombinant human/mouse/rat	Promokine	C-60057
FSH (Follicle stimulating hormone) recombinant human	Promokine	C-69102
TGF-β1, recombinant human	Promokine	C-63503
TGF-β2, recombinant human	Promokine	C-63498
Retinoic acid	Sigma	R 2625
BMP-2 recombinant human	Promokine	C-67309
BMP-7 recombinant human	Promokine	C-67318
BMP-8a recombinant human	R & D Systems	1073-BP-010

2.1.5 ELISA kits

Table 5: ELISA kits

Name	Species	Manufacturer	Catalogue No.
BrdU cell proliferation (Colorimetric)	-	Abcam	Ab126556
TGF-β1 duoset	Human	R & D systems	DY240
Fluoroprofile protein quantification kit	-	Sigma-Aldrich	FP0010
TGF-β2 duoset	Human	R & D systems	DY302
Betaglycan	Mouse	Mybiosource	MBS2883897-96
Cytoselect 24-well cell migration assay (8 µm, Fluorometeric format)	-	Cell Biolabs	CBA-101
Smad1 (pS463/S465)+total Smad1	Mouse and Rat	Abcam	Ab186035
Smad3 (pS423/S425)	Mouse and Rat	Abcam	Ab186038
TIMP1	Rat	Mybiosource	MBS263032
TIMP2	Rat	Mybiosource	MBS2880823
TIMP3	Human and Rat	R & D systems	DY973
MMP-3	Mouse	Raybiotech	ELM-MMP3
MMP-13	Mouse	Aviva systems Biology	OKEH00175
MMP-14	Mouse	Aviva systems Biology	OKAG00883
MMP-15	Mouse	Aviva systems Biology	OKAG00884
MMP-16	Mouse	Aviva systems Biology	OKAG00885

2.1.6 Enzymes

Table 6: Enzymes used for GAG chain digestion

Name	Manufacturer	Catalogue No.
Heparinase I	Sigma-Aldrich	H2519
Heparinase III	Sigma-Aldrich	H8891
Chondroitinase ABC	Sigma-Aldrich	C3667

2.1.7 Primers

All primers were designed from the primer blast software http://www.ncbi.nlm.nih.gov/tools/primer-blast available online and were exon-exon junction spanned. Forward and backward sequence, annealing temperature, size and accession number of each gene is described in Table 7.

Table 7: Primers

Gene (Species)	Sequence (5′⟶ 3′)	AT	Size (bp)	Accession no:
ALK2 (Mouse)	F: CGCTGGACCAGAGGAACAAA R: GGGCCGTGATGTTCCTGTTA	59°C	432	NM_001110205.1
ALK3 (Mouse)	F: CAGTGAGACAGCAGGACCAG R: GGCTTTCGGTGAATCCTTGC	59°C	409	NM_009758.4
ALK4 (Mouse)	F: TTGTGGGCACTCAAGGGAAG R: TCGGAGGGCACTAAGTCGTA	59°C	346	NM_007395.3
ALK5 (Mouse)	F: GGCCGGGCCACAAACA R:TTTCTGAAAAAGGTCCTGTAGTTG	59°C	376	NM_009370.3
ALK6 (Mouse)	F: CCTCGCCGGAGTATTACCAC R: GAGGAATGGGAGTGTCACGA	59°C	598	NM_007560.4
ALK7 (Mouse)	F: CGGCAGGACTGAAGTGTGT R: CAAGAGAGGCAGACCAGAGC	59°C	491	NM_001111030.1
Betaglycan (Mouse)	F: CGGCTTGAGAACAACGAGGA R: TGGAGCCTGCACCACAATAG	59°C	516	NM_011578.4
MMP2	F: CCCCATGAAGCCTTGTTTACC	59°C	540	NM_008610.3

(Mouse)	R: GTCAGTATCAGCATCGGGGG			
MMP3 (Mouse)	F: GTCCCTCTATGGAACTCCCAC R: GGAGACCCAGGGTGTGAATG	59°C	365	NM_010809.2
MMP7 (Mouse)	F: AACAATCGCCTGTTGATGGC R: AGCTGTCTCCATGATCTCTCC	59°C	523	NM_010810.5
MMP8 (Mouse)	F: CTTGCCCATGCCTTTCAACC R: GCCCAGTACTGTCTGCCTTTA	59°C	515	NM_008611.4
MMP9 (Mouse)	F: CAGCCGACTTTTGTGGTCTTC R: GGGGATCACGACGCCTTT	59°C	585	NM_013599.4
MMP10 (Mouse)	F: AATCCCTGTATGGAGCCGGT R: GAGTGTGGATCCCCTTTGGG	59°C	354	NM_019471.3
MMP11 (Mouse)	F: TCTCCTCCGCGCGATTTC R: ATGCCAGTACCTGGCGAAG	59°C	487	NM_008606.3
MMP12 (Mouse)	F: AACCGTGTGCAATGCTTGTG R: GAAGTCTCCGTGAGCTCCAA	59°C	441	NM_001320076.1
MMP13 (Mouse)	F: GAGTGCCTGATGTGGGTGAA R: GTCTTCATCGCCTGGACCATAA	59°C	527	NM_008607.2
MMP14 (Mouse)	F: TGTCTTCAAGGAGCGATGGT R: TCACTGCCCATGAATGACCC	59°C	411	NM_008608.4
MMP15 (Mouse)	F: AAGCTGGGCTGGTACAACTC R: GAGGCTGATTCCATGCAGGT	59°C	300	NM_008609.4
MMP16 (Mouse)	F: AGGATGGTGAACACTTGCCG R:TGTAACCAAACCTCCACATTGAAA	59°C	373	NM_019724.4
MMP19 (Mouse)	F: AGCAAAGACCTGGAGGATTACC R: AATGTCAGCCCAACCAGCTT	59°C	390	NM_001164197.2
B-actin (Mouse)	F: GCCTTCCTTCTTGGGTATGGAA R: CAGCTCAGTAACAGTCCGCC	59°C	359	NM_007393.5
B-2 microglobulin (Mouse)	F: GACCGGCCTGTATGCTATCC R: GTCTCGATCCCAGTAGACGG	59°C	310	NM_009735.3
GAPDH (Mouse)	F: GGTCCCAGCTTAGGTTCATCA R: CTGTGGTCATGAGCCCTTCC	59°C	586	NM_001289726.1

ALK2 (Rat)	F: CTGTACGCTGTCAGGCTCTC R: GATTTCCCTTTAGTGGGCAGC	59°C	454	NM_024486.1
ALK3 (Rat)	F: TGATGAATGTCTTCGAGCCGT R: GTCCAGGCTGTTTACAGAGACA	59°C	578	NM_030849.1
ALK4 (Rat)	F: GGTTACTATGGCGGAGTCGG R: GCTCCTTGAGGTGTCCACTG	59°C	353	NM_199230.1
ALK5 (Rat)	F: TGCCTGCTTCTCATCGTGTT R: TGCTTTTCTGTAGTTGGGAGTTCT	59°C	302	NM_012775.2
ALK6 (Rat)	F: CCTCGCCGGAGTATTACCAC R: GAGGAATGGGAGTGTCACGA	59°C	598	NM_007560.4
ALK7 (Rat)	F: GCGGCCGACAACAAAGATAA R: TCAAGCATTTCGGGAGCCAT	59°C	365	NM_139090.1
Betaglycan (Rat)	F: CTGCGAGGCAAGTTGAACAG R: GGAGTTGAGCAGGAACACGA	59°C	424	NM_017256.1
TIMP3 (Rat)	F: CAGACAGACGCCAGAGTCTCC R: CGGGTAGGAGGGAGGCTGAT	59°C	139	NM_012886.2
B-actin (Rat)	F: CAGCCTTCCTTCCTGGGTATG R: AGGGTGTAAAACGCAGCTCA	59°C	374	NM_031144.3
B-2 microglobulin (Rat)	F: ATTCACACCCACCGAGACCG R: GGTCCAGATGATTCAGAGCTCCA	59°C	127	NM_012512.2
GAPDH (Rat)	F: CAGTGCCAGCCTCGTCTCAT R: TCAGCCTTGACTGTGCCGTT	59°C	209	NM_017008.4

F-Forward primer, **R-**Reverse Primer, **AT-** Annealing temperature

2.1.8 Cell culture reagents

Bovine serum albumin (endotoxin free) (BSA)	Invitrogen	Karlsruhe
Dulbeccos PBS (1 ×) w/o Ca^{2+} & Mg^{2+}	PAA Laboratories	Cölbe

Penicillin/Streptomycin (100x) (Pen-Strep)	PAA Laboratories	Cölbe
DMEM/F12 1:1 GlutaMAX™	Invitrogen	Karlsruhe
FCS	Invitrogen	Karlsruhe
ITS (insulin transferrin selenium)	Thermo scientific	Germany
Accutase	Thermo scientific	Germany

2.2 Equipment

Cell culture CO_2 incubator	Thermo scientific	Germany
Desktop centrifuge Biofuge Fresco	Hettich	Germany
Electronic balance SPB50	Sartorius	Göttingen
Heat block DB-2A	Techne	Cambridge
Horizontal mini electrophoresis system	PEQLAB	Erlangen
Microwave oven	Samsung	Schwalbach
Mini centrifuge Galaxy	Heathrow Scientific	USA
Mini-rocker shaker MR-1	PEQLAB	Erlangen
NANODROP ND 2000	Promega	Mannheim
PCR thermo cycler	Biozyme	Oldendor
Power supply units	Consurs	Reiskirchen
SDS gel electrophoresis chambers	BioRad	München
Semi-dry-electroblotter	Bio rad	München
Vertical electrophoresis system	Bio rad	München
Ultrasonic homogenizer Bandelin Sonopuls	Bandelin	Berlin
TC 10 (Automatic cell counter)	BioRad	München

M200 microplate reader	BioRad	München

2.2.1 Miscellaneous

Enhanced chemiluminescence (ECL) reagents	Amersham	Freiburg
Protein size markers	Invitrogen	Karlsruhe
Sterile plastic ware for cell culture	Sarstedt	Nümbrecht
RNeasy Micro Kit	Qiagen	Hilden
10X Cell lysis buffer	Cell signalling	USA
Protease Inhibitor	Cell signalling	USA
Phosphatase Inhibitor	Cell signalling	USA

2.3 Buffer solutions and reagents used for Western blots

2.3.1 Cell Lysis buffer: For 2 ml: 200 µL 10x Lysis buffer
1750 µL of distilled water
1 mM PMSF
25 µL of Protease Inhibitor,
25 µL of Phosphatase Inhibitor,

*: prepare fresh every time before cell lysis

2.3.2 Protein gel sample loading buffer

Table 8: Western blot buffers

10 × Phosphate buffered saline (PBS)	4 g KCl 4 g KH_2PO_4 160 g NaCl 23 g Na_2HPO_4 * H_2O Dissolved in 1L H_2O, pH to 7.4 with HCl
10 × Tris base buffered saline (TBS)	24.2 g Tris base 80 g NaCl Dissolved in 1L H_2O, pH to 7.4 with HCl

Washing buffer TBST	1 × TBS 0.1% (v/v) Tween-20
Blocking buffer (100 ml)	100 ml 1 × TBS 0.1 ml Tween-20 5 g Non-fat dry milk
10 × Electrophoresis buffer	30.3 g Tris base 144 g Glycine 10 g SDS Dissolved in 1L distilled water
Stripping buffer	6.25 ml 1 M Tris HCl 2 ml 10% SDS 700 µl β-mercaptoethanol* Make volume up to 100 ml with water * added freshly just before stripping of membrane

2.4 Cell culture

2.4.1 93RS2 Sertoli cells

The prepubertal rat Sertoli cell line 93RS2 (Jiang et al., 1997) was cultured in DMEM/Ham's F-12 (1/1 by vol.) suplimented with L-glutamine, 10% FCS, 1% penicillin/streptomycin (Pen/Strep) and 1% ITS (Insulin transferase selenium) in a humidified incubator (37 °C, 5% CO_2). The media were changed every two days. After the medium was removed by aspiration, cells were washed with 1 × PBS without Ca^{2+} and Mg^{2+} and subsequently harvested by incubation with 0.25% accutase for 4 min at 37 °C. Cell culture reagents were from Life Technologies (Darmstadt, Germany).

2.4.2 GC-2 germ cells

The murine spermatogenic cell line GC-2 spd (ts) (Hofmann et al., 1995; hereafter referred to as GC-2) was cultured in 1 × DMEM high glucose containing 1% L-glutamine supplemented with 10% fetal calf serum (FCS) and 1% Pen/Strep (100 U/ml each). Cells were incubated in a humidified incubator at 32 °C under 5% CO_2. The medium was renewed every two days. Cells were washed with 1 × PBS without Ca^{2+} and Mg^{2+} and subsequently harvested by incubation with accutase (0.25%) for 4 min at 37 °C.

2.4.3 TN4A5 Sertoli cells

Immortalized mouse Sk11 TN4A5 Sertoli cells (Strothmann et al., 2004) were cultured in DMEM (high glucose, +L-glutamine) (CellGro, Fisher Scientific, Schwerte, Germany)

supplemented with 10% FCS and 1% Pen/Strep (100 U/ml each) in a humidified incubator at 37 °C under 5% CO_2. The media were changed every two days; cultures were grown to 90-95% confluence. Then the medium was removed and cells were washed with 1 × PBS without Ca^{2+} and Mg^{2+} and subsequently harvested by incubation with 0.25% accutase for 4 min at 37 °C.

2.5 Treatments and sample preparations

2.5.1 Preparation of cell lysates

Cell lysates were prepared as described in the supplier protocol (Abcam, Cambridge, UK). Briefly, 8×10^4 cells were grown in 12-well plates for 24 h as described above. Serum starvation (1% FCS + 1% P/S in DMEM/F12 or DMEM (1×) media) was for 24 h prior to the addition of rhTIMP3, TGF-β1 & TGF-β2. The cells were washed once with 1 ml ice cold 1 × PBS and lysed in 300 µl cell lysis buffer (Abcam, Cambridge, UK) containing 1 × protease inhibitor cocktail. All lysis steps were carried out on ice. The cells were detached from the culture plate with cell scrapers and transferred into reaction vials. The lysates were centrifuged at 13,000 × *g* for 10 min at 4 °C. The protein concentration in the supernatants was measured using the Fluoroprofile protein quantification kit. Aliquots of the supernatant were stored at -20 °C until further processing.

2.5.2 ELISAs sample collection

1×10^5 93RS2 & TN4A5 cells/ml were grown in 12-well plates for 24 h as described above and serum starved (1% FCS) for additional 24 h. Then the cells were stimulated with 10 ng/ml of recombinant human TGFβ1 and TGFβ2 (Promocell) or 10 nM of recombinant human TIMP3 (R & D Systems) for 24 h - 48 h. The supernatants were transferred into 1.5 ml Eppendorf tubes with 1 x protease inhibitor cocktail and stored at -20°C until further use. The cells were washed once with PBS and detached with 0.25% accutase for 4 min at 37 °C. 20 µl of the cell suspension were then stained with Trypan Blue and the total cell number determined with the automated cell counter system (TC 10, Bio Rad).

2.5.3 p-Smad 1, 2 & 3 ELISAs

TN4A5 & 93RS2 Sertoli cells grown under the standardized conditions in 12-well plates were pre-treated with 5 µM of the specific inhibitor LY364947 (diluted in DMSO) for 2 h,

followed by treatment in duplicate wells with rhTIMP3 (10 nM), rhTGF-β1 (10 ng/ml) or rhTGF-β2 (10 ng/ml) for 2 h. Untreated samples without the recombinant proteins were used as negative controls. After washing with washing buffer cell lysates were prepared and equal amount of protein samples and the anti p-Smad3 antibody cocktail (Table 5) was added to a 96-well plate. The plate was incubated for 1 h on a orbital shaker at 400 rpm, washed 3 times with 1 × wash buffer provided in the kit, and the TMB substrate was added. The absorbance of each sample was determined at 450/550 nm using the M200 microplate reader. Tecan values were normalized to the total protein content determined with the Fluoroprofile protein quantification kit.

2.5.4 ELISAs for TIMP3, TGFβ1/β2 & TβRIII

The levels of TIMP3, TGFβ1/β2 & TβRIII in the supernatants of TN4A5 & 93RS2 Sertoli cells were measured by ELISA according to the manufacturer's instructions. Briefly, 100 µl of supernatant per well were added to a 96-well microplate pre-coated with capture antibodies overnight at room temperature. The wells were emptied (tapping on sterile papers) and washed three times with 1× wash buffer followed by incubation with 100 µl detection antibody (Table 5) for 2 h at room temperature. After repeating the washing steps, an incubation with 100 µl/well substrate solution for 20 min at room temperature in the dark followed. The reaction was stopped after 20 min and the absorbance was determined immediately with the use of the microplate reader M200 microplate reader set to 450/550 nm. Data were standardised against $1x10^5$ viable cells.

2.5.5 ELISAs for MMP 14, 15 & 16

The relative amounts of MMP 14, 15 & 16 in cell cultures of 93RS2 & TN4A5 Sertoli cells were measured by colorimetric cell-based ELISAs according to the manufacturer's instructions. Briefly, 200 µl of 20,000 cells in culture medium were added to each well of a 96-well microplate. The cells were incubated overnight at 37 °C under 5% CO_2; thereafter the cells were incubated with or without growth factors for 24h and 48 h. Then, the wells were emptied (tapping on sterile papers) and washed twice with 1× TBS, followed by incubation with 100 µl fixing solution (4% formaldehyde) for 20 min at room temperature (RT). After washing the wells 3 times with 1 × washing buffer, the plates were incubated with 100 µl of quenching buffer/well for 20 min at room temperature. After repeating the washing steps, 200 µl of blocking buffer were added and incubated for 1 h at RT. After repeating the washing steps 50 µl of 1 × primary antibody (1:100) of anti-MMP-14, 15 & 16 and anti-GAPDH

antibody were added into the respective wells, thereafter the plate was incubated overnight for 16 h at 4°C.

After washing the plate 3 times with 1 × washing buffer, it was incubated for 1.5 h at room temperature with 50 µl 1 × secondary. The washing steps were repeated and the TMB substrate was added for 20 min at room temperature in the dark with gentle shaking. The reaction was stopped after 20 min and the absorbance of each well was determined immediately with the use of the microplate reader M200 microplate reader set at 450/550 nm. Data were normalized to the total GAPDH protein content.

2.5.6 BrdU proliferation assay

Cell proliferation was determined using the colorimetric BrdU Cell Proliferation ELISA Kit (Abcam, Cambridge, UK). Briefly, Sertoli and germ cells were cultured in 96-well plates at a density of $4x10^4$ cells/well in complete growth media (10 % FCS) for 24 h and serum starved for an additional 24 h. To determine the effects of rhTIMP3 on cell proliferation the cells were incubated with various concentrations of rhTIMP3 for 48 h. Four hours before the end of the incubation period the cells were labelled with 1 × BrdU (10 µl/well). The cellular DNA was subsequently denaturated with the supplied fixing solution (200 µl/well) followed by incubation with the primary anti-BrdU detector antibody (100 µl/well) for 1 h at room temperature. Then, the wells were emptied and washed twice with 1 × wash buffer, followed by adding 1 × peroxidase goat anti mouse IgG conjugate and incubating for 30 min at room temperature. After removal of the antibody conjugate, the wells were washed with 1 × wash buffer and the substrate solution was added. The reaction product was quantified by measuring the absorbance at dual wavelength of 450/550 nm using the microplate reader M200.

2.5.7 Cell Migration Assay

93RS2 and GC-2 were plated at a density of 30,000 cells in inserts specially designed by CytoSelect cell migration assay kit (Cell Biolabs, San Diego, CA). Cell migration experiments were performed according to the manufacture's protocol. The cells were allowed to adhere overnight at 37°C in a humidified incubator with 5% CO_2. Then stimulated with and without BMP2, BMP7 or BMP8a (25 ng/ml each), the lower well was loaded with medium supplemented with 10% FCS and incubated for 48 hrs at 37°C in a humidified incubator. After incubation overnight, the cells that migrated through the pores to the underside of the insert were detached by placing the insert into the detachment solution. Detached cells were

incubated with the fluorescence dye for 20 min, and photographed under a microscope. The absorbance of each sample was determined at 450/550 nm using the M200 microplate reader.

2.5.8 Tracer diffusion assay

A total of 50,000 93RS2 cells were grown in 24-well inserts for 24 h at 37 °C with 5% CO_2. Then starvation medium was added and cells were incubated for another 24 h. Next, fresh medium was supplemented with TGF-β1 (10 ng/ml) and BMPs (25 ng/ml) and kept for 48 h. Thereafter 5 mg/ml of FITC-coupled Dextran (MW 4; FD4, Sigma-Aldrich) was added into the upper compartment of the insert and also in the blank insert with no cells (blank). FD4 has a radius of 14 Å and thus can pass through a Sertoli cell monolayer. After another 24 h of incubation and careful handling, a 100 µl sample was taken from the lower compartment and measured on a black 96-well chimney plate (Greiner) in a dilution series. Fluorescence intensity at the wavelength 490 nm/520 nm was measured by the ELISA reader M200.

2.5.9 RNA Isolation

Total RNA was isolated from TGF-β treated 93RS2 and TN4A5 cells by the RNeasy Micro kit (Qaigen, Hilden). Briefly, cells were lysed with 700 µl RLT lysis buffer after washing two times with ice-cold PBS. The lysed cells were transferred into a microcentrifuge tube, and homogenized by passing through a 24-gauge needle attached to a 1 ml plastic syringe for 4-5 times. These lysates were transferred into a new Eppendorf tube and mixed thoroughly with an equal volume of 70% ethanol. Samples up to 700 µl of mixture were transferred to an RNeasy spin column placed in a 2-ml collection tube and centrifuged for 15 s at 16000 × g (all centrifugation steps were performed at room temperature). The flowthrough was discarded and the RNeasy spin column was washed with 700 µl of RW1 buffer by centrifuging for 15 s at 16,000 × g. After centrifugation, the RNeasy spin column was carefully removed, transferred to a new collection tube and washed with 500 µl RPE buffer. This step was repeated one more time by centrifugation for 2 min at 10,000 × g. Any possible contamination of RPE buffer in the RNeasy spin column was removed by an additional centrifugation at full speed. To elute the total RNA, 30-50 µl of RNase-free water were added directly to the RNeasy spin column membrane and centrifuged at 10,000 × g for 1 min. The concentration and quality of RNA was measured spectrometrically by Nano drop (Promega, Mannheim).

2.5.10 DNase digestion

RNA preparations should be free of DNA contamination prior to RT-PCR. DNA contamination was removed by treating each RNA sample with DNase I (Invitrogen, Karlsruhe) at room temperature for 15 min in the reaction mixture given below.

2.5.11 DNase diestion reaction mix

Table 9: DNase digestion reaction mixture

Volume	Components
x µl	2 µg RNA
1 µl	DNase I (10 U/µl)
2 µl	10 × DNase I buffer
to 20 µl	RNase free water

After DNA digestion, DNase I was inactivated by adding 2 µl of 25 mM EDTA (pH 8.0) to each sample and subsequently heatinactivated at 65°C for 10 min.

2.5.12 cDNA synthesis

DNA-digested samples were reverse transcribed by using kit H-Minus (PeqLab/VWR, Erlangen, Germany). Briefly, oligo-dTs and dNTPs were mixed with RNA samples as given below in Table 10. The reaction mixture was heated at 65°C for 5 min and quickly chilled on ice.

2.5.13 Primer annealing and reverse transcription

Table 10: RNA mix for annealing

Volume	Components
21µl	2.5 µg of DNase I digested RNA
2 µl	Oligo dT
2 µl	dNTPs (A,C, G and T, each 10 mM)

The RT mix (Table 11), pre-heated for 2 min at 42°C and 1 µl of the reverse transcriptase enzyme was added to each sample. The reaction mixtures were incubated for 50 min at 42°C. Subsequently reactions were inactivated by heating samples at 75°C for 15 min. Samples were stored at -20°C for further analysis.

Table 11: RT mix for reverse transcription

Volume	Components
8 µl	5 × M-MLV RT buffer
2 µl	RNase-free water
4 µl	0.1 M DTT

2.5.14 RT-PCR

The other PCR reagents were purchased from Bio&Sell (Nürnberg, Germany). Semi-quantitative PCR was performed with 1 µg cDNA. GAPDH was used as a positive control. After an initial heating to 95°C for 4 min, each cycle consisted of denaturing at 95°C for 30 seconds, annealing at 58°C for 20 sec and elongation at 72°C for 40 sec except for the final extension which lasted 5 min. The program consisted of 35 cycles.

2.5.15 Quantitative real-time PCR

In the mixture of the qRT-PCR specific fluorescent reporter probes such as SYBR green are added. The reporter probes can bind to the dsDNA every time the DNA is polymerized, and emit fluorescence signals detected by a detector as shown in Figure 6. The increasing fluorescence signal is directly related to the exponential amplification of the DNA product and recorded in the real-time PCR thermocycler, which is used to determine the threshold cycle (Ct) value and facilitates quantification of gene expression. The expression of the gene is measured after reaching the *ct* value and relative gene expression is calculated using the ΔΔCt method (Livak & Schmittgen, 2001). The target genes expressions were normalized to the unregulated reference gene β2 microglobulin (β2M).

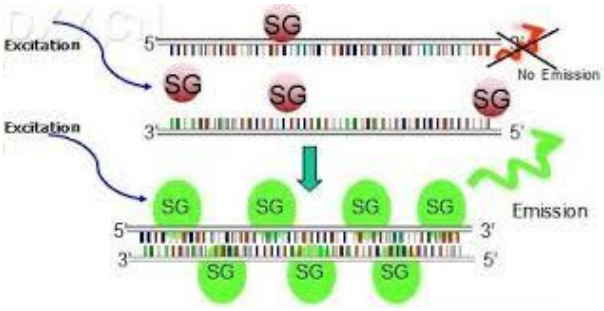


Figure 6. SYBR green qRT-PCR (adopted from www.pixgood.com/qrt-pcr-sybr-green)

The primers were designed by primer blast (annealing temperatures see Table 7). Gradient PCR was employed to determine the optimal annealing temperature for individual genes. Accordingly, the temperature of each gene was set. All primers' efficiencies were between 100 ± 15%.

The preparation of a standard 25 µl qRT-PCR reaction mix is given below.

Table 12: qRT-PCR

Components	**Volume per reaction**
cDNA	1 µl
2X iQ SYBR green super mix	12.5 µl
Forward and reverse primer mix (10 pM/µl)	1 µl
DNase/RNase free water	10.5 µl
Total volume	**25 µl**

Realtime PCR amplification with iQ™ SYBR® Green Supermix was performed in duplicate by using the iCycler iQ® System (Bio-Rad, München) according to manufacturer's procedure. Cycling conditions were described as below (Table 13).

Table 13: Real time PCR Program

Real time PCR program		
Cycle	**Temperature**	**Time**
1	95°C	8 min

40 cycles	94°C	15 s
	60°C	30 s
	72°C	15 s
1	50°C	10 s

2.5.16 SDS-Polyacrylamide gel electrophoresis

The SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) technique is used to separate proteins according to their size. The gels were prepared freshly, depending on the size of the investigated proteins; separating gels and stacking gels were made according to the pipetting scheme given below (Table 14).

Separating gel:

Table 14: SDS -PAGE gel preparation

Solutions	**7.5%**	**10%**	**12.5%**	**15%**
Water	4.85 ml	4.01 ml	3.17 ml	2.35 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml
10% (w/v) SDS	100 µl	100 µl	100 µl	100 µl
Acrylamide	2.5 ml	3.34 ml	4.17 ml	5 ml
10% (w/v) APS	50 µl	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl	5 µl
Total	10 ml	10 ml	10 ml	10 ml

Ammoniumpersulfate (APS) -prepared freshly every time.

Table 15: Stacking Gel

Solutions	4%
Water	3 ml
0.5 mM Tris-HCl pH 6.8	1.25 ml
10% (w/v) SDS	50 µl
Acrylamide	0.65 ml
10% (w/v) APS	25 µl
TEMED	5 µl
Total	**5 ml**

The gel percentage depends on the molecular weight of the protein of interest (based on 5:1 acrylamide/bisacrylamide ratio). 4-5% gel: > 250 kDa, 7.5% gel: 250-120 kDa, 10% gel: 120-40 kDa, 13% gel: 40-15 kDa, 15% gel: < 20 kDa.

2.5.17 Protein sample preparation and procedure

After treatment of the cells with 25 ng/ml BMPs for the indicated time points, TN4A5 Sertoli cells were washed two times with ice-cold PBS and lysed with 1 × lysis buffer (section 2.3.1). Lysed cells were gently sonicated on ice (10 s, 1 pulse, 50% power) and centrifuged for 10 min at 14,000 × g in a cold 4°C microfuge. Supernatants were heated at 90°C for 10 min. Equal amounts of protein of each sample were separated on SDS-PAGE. Separated proteins were electrophoretically transferred onto a 0.2 µm pore size PVDF membrane (HybondTM ECL^{TM}, GE Healthcare, UK) by using the Perfect BlueTM semidry electroblotter (Bio-Rad, München, Germany). The nonspecific binding of proteins was blocked by incubation with blocking buffer (5% BSA in 1 × TBST) for 1 h at room temperature. The primary antibodies (Table 1) were diluted in 5% BSA in 1xTBST. The membranes were probed with primary antibodies overnight at 4ºC. In a next step, the membranes were washed 3 times with 1 × TBST on a shaker, changing the buffer every 5 min. Membranes were probed for 1 h at room temperature with the respective HRP-conjugated secondary antibodies (Table 2). The membrane was washed 3 times on a shaker with 1 × TBST changing the buffer every 5 min. Immunoreactivity proteins were detected using the ECL Kit (ImmobilonTM Western Chemiluminescent HRP substrate, Millipore, Billeria, MA, USA), according to the manufacturer's instructions.

2.5.18 Reprobing of membranes

The membrane was washed 3 times for 5 min each in 1 × TBST buffer and incubated with the stripping buffer at 60°C for 3 min (section 2.3.2). Again the membrane was washed with 1 × TBST and blocked with 5 % milk buffer for 1 h at room temperature and subsequently the Western blot procedure was repeated with other primary antibodies as mentioned earlier.

2.5.19 Statistical analysis

All experiments were repeated at least three times in duplicates. Mean and the standard error of the mean (SEM) of all the experiments were used for analysis. The comparison of the means between groups was performed by one-way analysis of variance (ANOVA), Dunnett's test and for comparison between 2 groups is done by t-test using GraphPad prism software (Version 7.0, Graphpad Inc. La Jolla, CV, USA). P values of ≤ 0.05 were considered significant.

3 RESULTS

3 TGF-β family receptor mRNA expression in Sertoli and germ cells

TGF-β signalling pathways play very important roles during development of the testis to ensure proper spermatogenesis and fertility (Miles et al., 2013). TGF-βs activate the signalling cascade via type-I receptors (ALK5), which are differentially regulated during testis development (Itman et al., 2006). ALK4 and ALK7 (Activin Like Kinase) are activated by activin A, whereas BMPs activate signalling via ALK 2, ALK3 and ALK6 (Lin et al., 2016).

In this study, mRNA expression levels of the receptors mentioned above were assessed in Sertoli and germ cell lines. We analysed the expression pattern of the receptors by RT-PCR in 93RS2, TN4A5 and GC-2 cells were grown on 10 cm^2 dishes in 10% FCS for 24 h and serumstarved for another 24 h in 1% FCS. RNA isolation was done with the Qiagen RNA isolation kit, cDNA was made by using the Peq GOLD cDNA kit. Each PCR product was generated after 35 PCR cycles. We used GAPDH and β-2-microtubulin as housekeeping genes. All data are representative of three independent experiments (Table 16).

Table 16: Receptor expression in Sertoli and germ cells

	Receptors	93RS2 Sertoli cells	TN4A5 Sertoli cells	GC-2 germ cells
BMPR1	**ALK2**	++	++	++
	ALK3	+++	+++	+++
	ALK6	+	++	-
ACVR1	**ALK4**	++	+++	++
	ALK7	-	+	-
TβR1	**ALK5**	++	++	++
Betaglycan	**TβRIII**	+++	+++	+++

- = No expression, + = Moderate expression, ++ = High expression, +++ = Very high expression

Interestingly, we found that ALK2, ALK3, ALK4 and ALK5 are expressed in all cell lines, however, AKL6 and ALK7 are expressed only in one Sertoli cell line. Additionally betaglycan was expressed in all cell lines. These data suggest that TGF-β family receptors are expressed in Sertoli and germ cells.

3.1 TIMP3 influences betaglycan shedding and TGF-β signalling in rat 93RS2 Sertoli cells

3.1.1 Effects of TGF-βs on shedding of betaglycan

Since betaglycan is an important co-receptor especially for TGF-β2 (Fukushima et al., 1993; Mendoza et al., 2009), we first sought to determine if TGF-βs modulate betaglycan shedding in Sertoli cells. For this purpose, we isolated supernatants from 93RS2 Sertoli cells treated with TGF-β1 or TGF-β2 for 24 h or 48 h, and measured sBG (soluble Betaglycan) by ELISAs. We found that 10 ng/mL TGF-β1 and particularly 10 ng/mL TGF-β2 progressively and significantly reduced shedding of betaglycan after 48 h by ~50% and ~70%, respectively, compared to untreated controls (Fig. 7A, B).

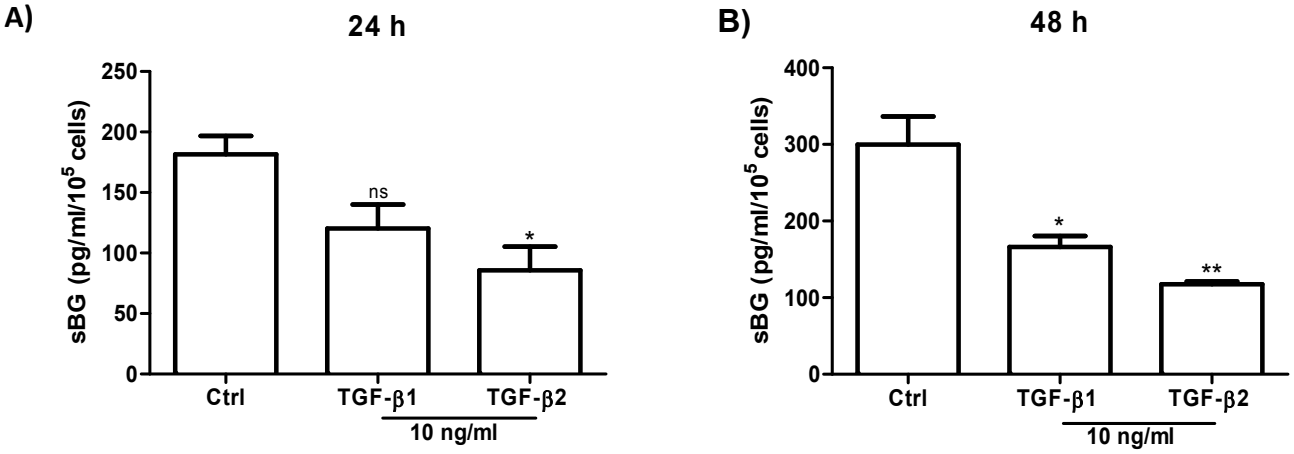


Figure 7. TGF-βs modulate shedding of betaglycan-7×10^4 93RS2 cells/well were incubated with 10 ng/ml of TGF-β1 or TGF-β2 for **(A)** 24 h and **(B)** 48 h and supernatants analysed by sBG ELISAs. Each bar represents mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *$P \leq 0.05$, **$P < 0.01$, **sBG**=soluble betaglycan; **ns**=not significant.

3.1.2 Diverse effects of TGF-βs on TIMP3 secretion and vice versa in Sertoli cells

Because TGF-βs reduced shedding of betaglycan, we analysed possible candidates for inhibition of proteases like MMPs which might be involved in shedding of betaglycan (Velasco-Loyden et al., 2004). Additionally, MMPs and TIMPs regulate shedding of betaglycan in rat muscle cells (Velasco-Loyden et al., 2004). Therefore, we investigated secretion of TIMPs in 93RS2 Sertoli cells cultured with or without TGF-βs.

Neither TIMP1 nor TIMP2 were detected in 48 h culture medium after stimulation with different doses of TGF-β1 or TGF-β2 within the detection limit (62.5 pg/ml) of the ELISA (data not shown). In contrast, TGF-β2 (Fig. 8), but not TGF-β1 (data not shown), induced secretion of TIMP3 in a dose-dependent and significant manner. The 48 h samples contained about ~30 times more TIMP3 than the 24 h samples.

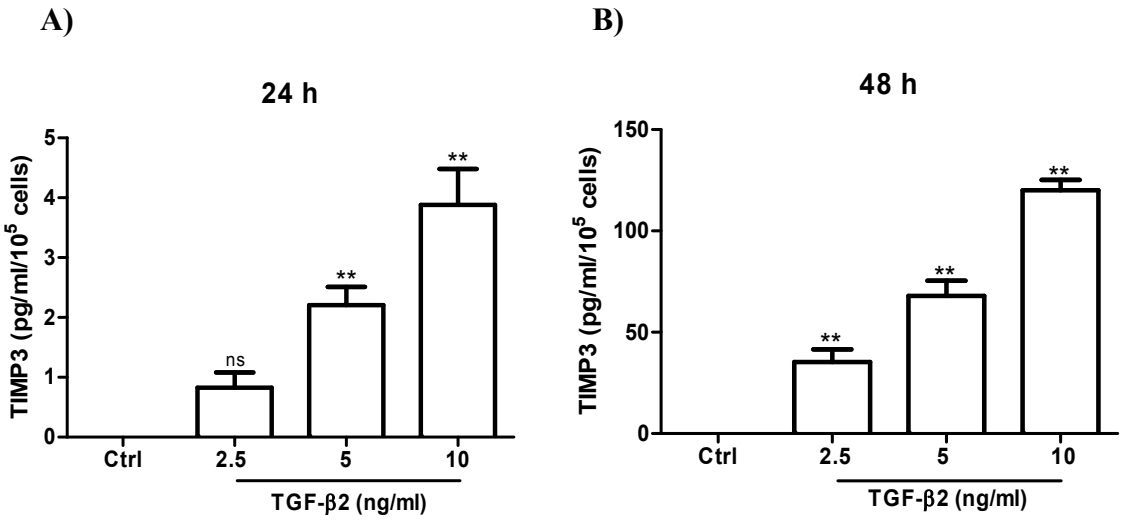


Figure 8. TGF-β2 treatment induces TIMP3 secretion-1 × 10^5 93RS2 cells/well were incubated with TGF-β2 for **(A)** 24 h or **(B)** 48 h and supernatants were analysed for TIMP3 by ELISAs. Each bar represents mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; **P<0.01; **ns**=not significant.

Next, we analyzed the influence of rhTIMP3 on the secretion of TGF-βs. Both TGF-β1 (~800 pg/mL/1x10^5 cells) and TGF-β2 (~300 pg/mL/1x10^5 cells) were detected in 48 h culture supernatants from 93RS2 cells (Fig. 9A, B). Treatment with rhTIMP3 caused a dose-dependent and significant decrease in secretion of TGF-β1 (~40% reduction with 10 nM and 20 nM) and of TGF-β2 (~70% reduction with 20 nM; Fig. 9).

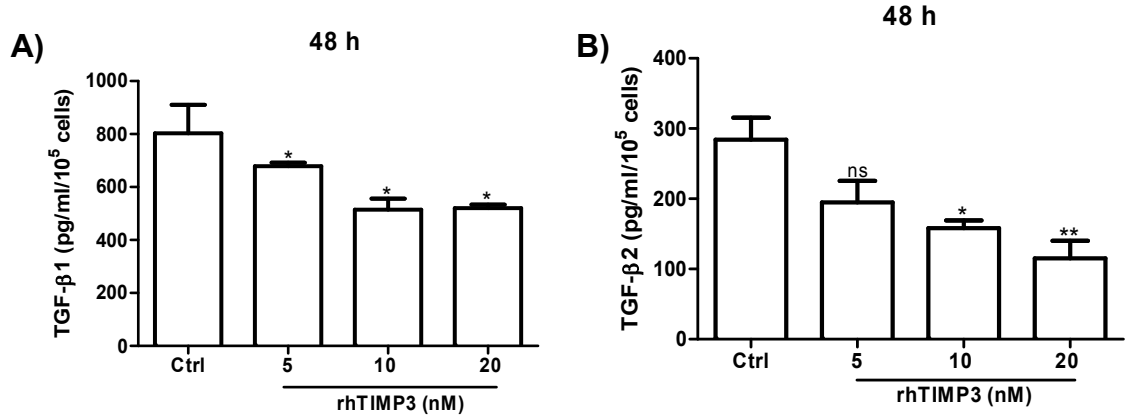
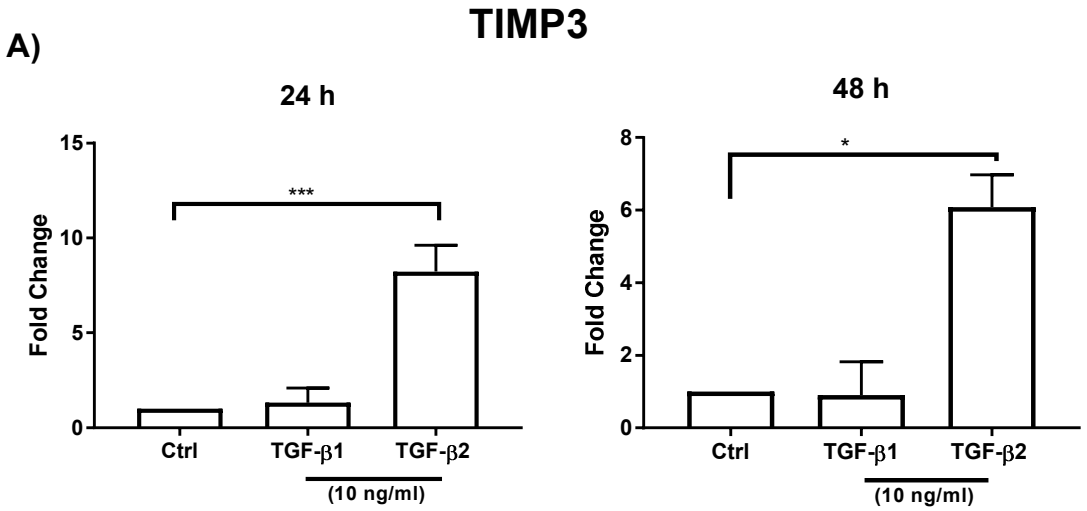


Figure 9. TIMP3 treatment reduces secretion of TGF-β1 and TGF-β2-1×10^5 93RS2 cells/well were incubated with rhTIMP3 for 48 h and supernatants analysed for **(A)** TGF-β1 and **(B)** TGF-β2 by ELISAs. Each bar represents mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *P≤ 0.05, **P<0.01. **rhTIMP3**=recombinant TIMP3, **ns**=not significant.

3.1.3 Expression of TIMP3 and betaglycan in Sertoli cells

Increased secretion of TIMP3 was observed by TGF-β2 (Fig. 8). To confirm these findings, we further analysed gene expression of TIMP3 and betaglycan in Sertoli cells by real-time PCR.

Treatment of 93RS2 cells with TGF-β1 or TGF-β2 for 24 h and 48 h resulted in a ~7-fold increase in TIMP3 mRNA expression in samples treated with TGF-β2 but not in those treated with TGF-β1 (Fig. 10A). A similar behaviour could be observed for betaglycan expression stimulated by TGF-β2 after 24 h and 48 h (Fig. 10B). These results confirmed that betaglycan and TIMP3 mRNA and protein expression is modulated by TGF-β2 in Sertoli cells.



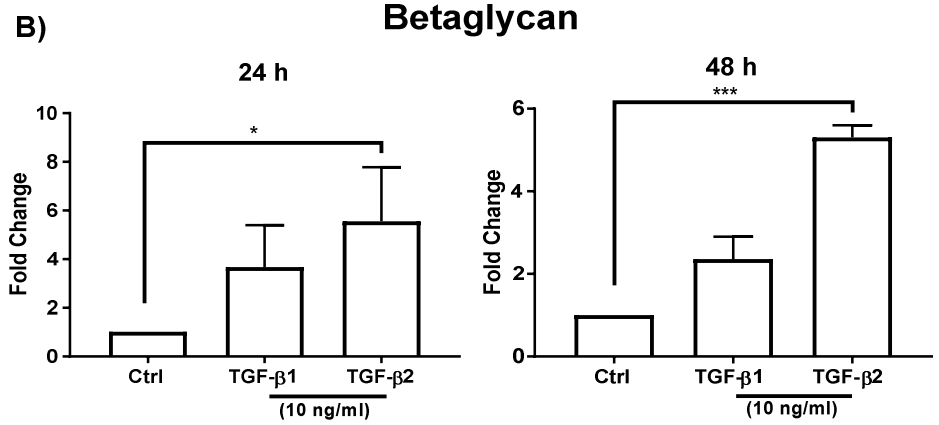


Figure 10. qRT-PCR analysis-TIMP3 and betaglycan mRNA expression is increased by TGF-β2-1 × 10^5 93RS2 cells/well were incubated with TGF-β1 or TGF-β2 (10 ng/ml each) for 24 h and 48 h. Measurement of **(A)** TIMP3 and **(B)** betaglycan gene expression. Data were normalized to expression levels of the β-microglobulin housekeeping gene and are presented as the mean ± SD of three independent experiments. Dunnett's test was employed for statistical analysis. P-values ≤ 0.05 *, <0.001***.

3.1.4 Effects of TIMP3 and MMPs on shedding of betaglycan

Next, we analysed the effect of TIMP3 on betaglycan shedding because of the positive effects of TGF-βs on TIMP3 and betaglycan expression. 93RS2 Sertoli cells were treated with different doses of rhTIMP3 for 48 h and the content of sBG was determined. The concentration of sBG was reduced in a dose-dependent and significant manner by up to ~60% with 20 nM rhTIMP3 (Fig. 1`

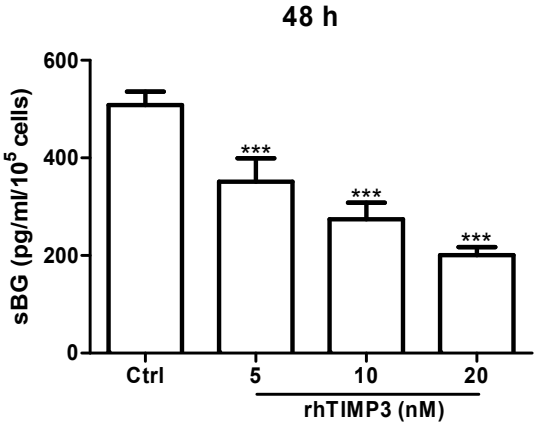


Figure 11. TIMP3 treatment reduces betaglycan shedding-7 × 10^{+} 93RS2 cells/well were incubated with rhTIMP3 for 48 h and supernatants analysed for sBG by ELISAs. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; ***P<0.001. **rhTIMP3**=recombinant TIMP3.

To investigate the impact of MMPs on betaglycan shedding experiments with the broad range MMP inhibitor GM6001 were performed. Treatment with of GM6001 (10 μM) reduced sBG levels by about ~50% after 24 h and 48 h (Fig. 12A, B).

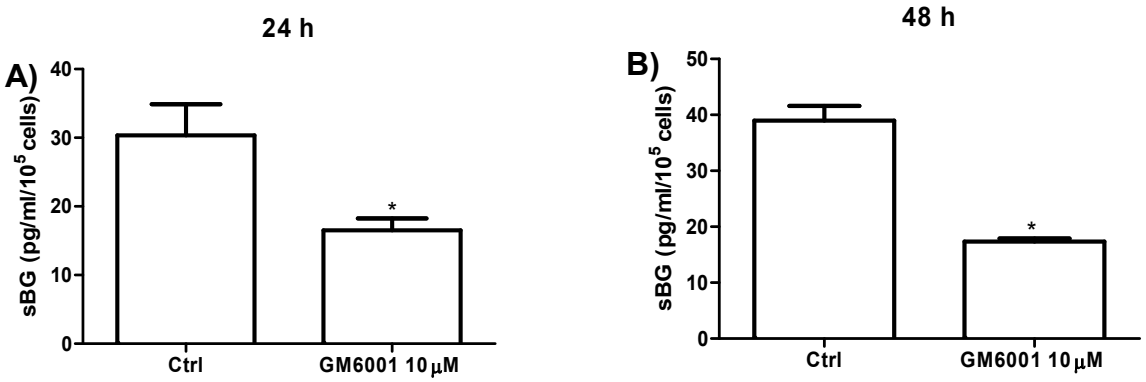


Figure 12. Inhibition of MMPs reduces shedding of betaglycan-7×10^4 93RS2 cells/well were incubated with 10 μM GM6001 for **(A)** 24 h and **(B)** 48 h and supernatant analysed for sBG by ELISAs. Each bar represents mean ± SEM of 3 independent experiments performed in duplicate. Student`s t-test was used for statistical analysis; *P≤ 0.05.

3.1.5 Effects of TIMP3 on TGF-β signalling

Because sBG is an antagonist of TGF-β signalling (Gatza et al., 2014), we hypothesized that TIMP3 might also modulate TGF-β signalling by inhibiting betaglycan shedding. Sertoli cells treated with rhTIMP3 increased Smad3 phosphorylation in a dose-dependent and significant manner (Fig. 13A). The specificity of signalling via TβRI was shown by incubation with the specific TβRI kinase inhibitor LY364964, which decreased the TIMP3-induced elevation of Smad3 phosphorylation dose-dependently (Fig. 13B). This suggests that TIMP3 modulates Smad3 phosphorylation indirectly via the TβRI.

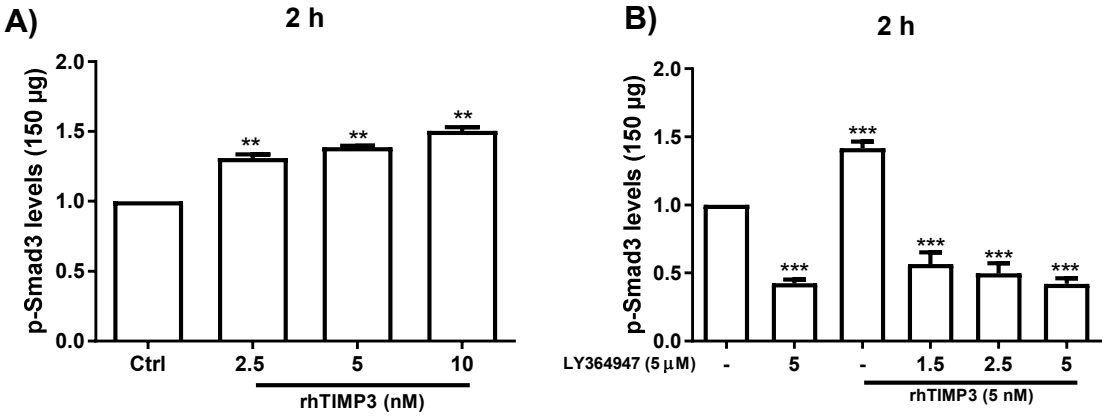


Figure 13. TIMP3 induces phosphorylation of Smad3 through TβRI-7×10^4 93RS2 cells/well were: **(A)** incubated with rhTIMP3 for 2 h and cell lysates analysed for p-Smad3 by ELISAs; and **(B)** pre-incubated with different concentration of LY364947 for 2 h and then treated with rhTIMP3 (5 nM) for 2 h and cell lysates analysed for p-Smad3 by ELISAs. Each bar represents mean ± SEM of 3 independent experiments performed in duplicate and p-Smad3 levels were standardized to 150 µg protein from lysates. Dunnett's test was used for statistical analysis; **P<0.01, ***P<0.001. **rhTIMP3**=recombinant TIMP3.

3.1.6 The indirect impact of betaglycan on TGF-β signalling

It has been reported that membrane-bound betaglycan inhibited TGF-β signalling by interfering with TβRI and TβRII (Eickelberg et al., 2002). Because TIMP3 treatment reduced betaglycan shedding, we wanted to elucidate the effects of increased membrane-bound betaglycan on TGF-β signalling. Thus, we pre-treated Sertoli cells with TIMP3 for 2 h and then stimulated with TGF-β1 or TGF-β2 for 2 h. The time point of 2 h for the p-Smad3 ELISA was optimized before the stimulation was done (data not shown). Of note, we observed that titrated TIMP3 reduced TGF-β1-dependent phosphorylated Smad3 levels at 2 h (Fig. 14A). Surprisingly, the converse occurred for TGF-β2. Titrated TIMP3 enhanced TGF-β2-dependent phosphorylation of Smad3 at 2 h (Fig. 14B). These results support the hypothesis that betaglycan is substantially required for TGF-β2 but not for TGF-β1-mediated signalling and highlight the potential of TIMP3 to modulate this difference.

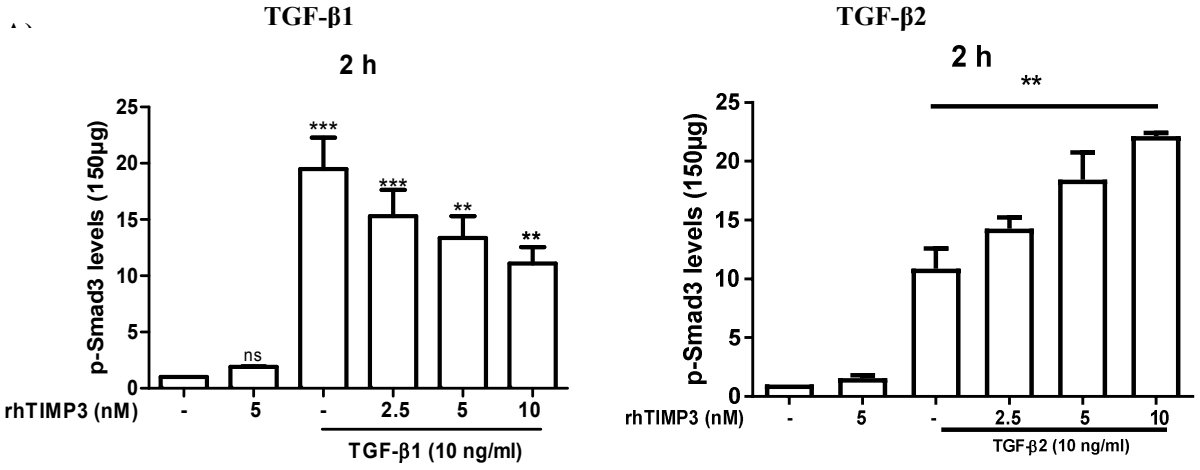


Figure 14. TIMP3 treatment increases Smad3 phosphorylation in TGF-β2 signalling-7×10^4 93RS2 cells/well were incubated with different doses of rhTIMP3 for 2 h and then treated with **(A)** TGF-β1 or **(B)** TGF-β2 (both 10 ng/mL) for 2 h. Cell lysates were analysed for p-Smad3 by ELISAs. Each bar represents mean ± SEM of 3 independent experiments performed in duplicate and p-Smad3 levels were standardized to 150 µg protein from lysates. Dunnett's test was used for statistical analysis; **P<0.01, ***P<0.001. **rhTIMP3**=recombinant TIMP3.

3.1.7 Betaglycan GAG chains influence the TGF-β1 and TGF-β2 signalling cascade

Betaglycan has heparan and chondroitin sulfate glycosaminoglycan chains attached to Ser535 and Ser546 (Lopez-Casillas et al., 1994; Zhang & Esko, 1994). The GAG chain is known to bind growth factors and thus, to modulate their biological activity. For instance, FGF2 requires the GAG chain for binding and activating signalling (Sterner et al., 2013). Furthermore, heparin and heparan sulfate chain (HS) interact with TGF-β1 to inhibit signalling (McCaffrey et al., 1989; Lyon et al., 1997; Eickelberg et al., 2002).

Reference to these molecular interactions and protein modulations, we aimed to investigate the role of GAG chains in TGF-β1 and TGF-β2 signal regulation in testis. Sertoli cells were incubated with GAG chain degrading enzymes (HI, HIII & C ABC) and then stimulated with TGF-β1 or TGF-β2 (Fig. 15). Results obtained from p-Smad3 ELISAs showed that degradation by chondroitase ABC (C ABC) reduced the TGF-β1-induced Smad3 phosphorylation by ~30%, whereas degradation by heparin I and III (HI, HIII) revealed no significant effects. Moreover, in TGF-β2 signalling, degradation of the GAG chain reduced p-Smad3 by 50% (Fig. 15). Collectively, these results indicated that GAG chains interact more with TGF-β2 signalling compared to TGF-β1 signalling. However, it has to keep in mind that betaglycan might not be the only carrier of GAG chains.

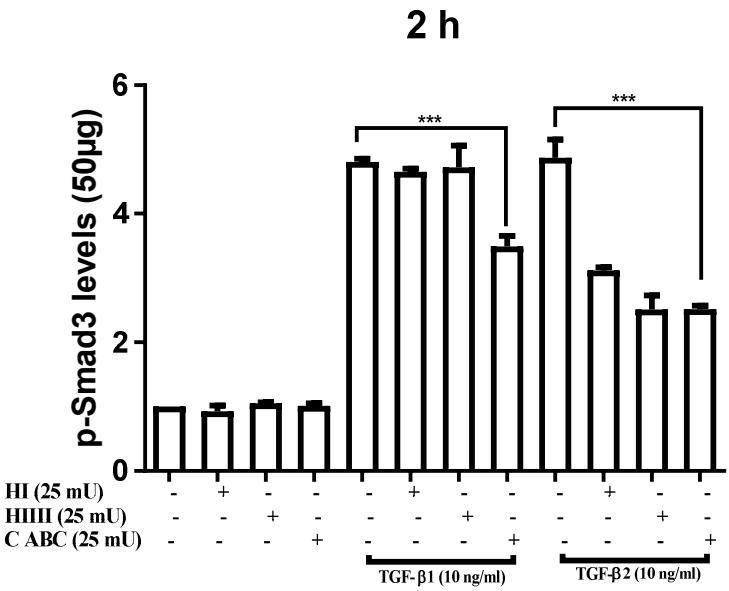


Figure 15. Degradation of GAG chain inhibits TGF-β2 signalling-7 × 10^4 93RS2 cells/well were incubated with Heparinase I &III, Chondroitinase ABC (each 25 mU) for 16 h and then treated with TGF-β1 or TGF-β2 (both 10 ng/mL) for 2 h. Cell lysates were analysed for p-Smad3 by ELISAs. Each bar represents mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; ***P<0.001. **HI-** Heparinase I; **HIII-** Heparinase III; **C ABC-** Chondroitinase ABC.

3.2 Role of activin A in regulating shedding of betaglycan in Sertoli cells and cross-talk with TGF-β signalling

3.2.1 Activin A-induced shedding of betaglycan

Inhibin, an extracellular activin A inhibitor, binds to betaglycan and thus prevents binding of activin A to the receptors ActRIIA or ActRIIB (Lewis et al., 2000; Chapman & Woodruff, 2001). TN4A5 Sertoli cells were used for these experiments as they respond to Activin A stimuli compare to 93RS2 Sertoli cells. To evaluate the potential effect of activin A on shedding of betaglycan, we collected the supernatants from TN4A5 Sertoli cells treated with activin A (different concentration) for 24 and 48 h and measured sBG by ELISAs (Fig. 16). We found that activin A in a dose-dependent significant manner induced cleavage of membrane-bound betaglycan into the soluble form compared to untreated controls; Activin A (~40-60% with 10 ng and 25 ng) induced shedding of betaglycan. These data suggest that activin A induces shedding of membrane-bound betaglycan into the soluble form, so it is no longer available for inhibin to bind and block the activin signalling pathway.

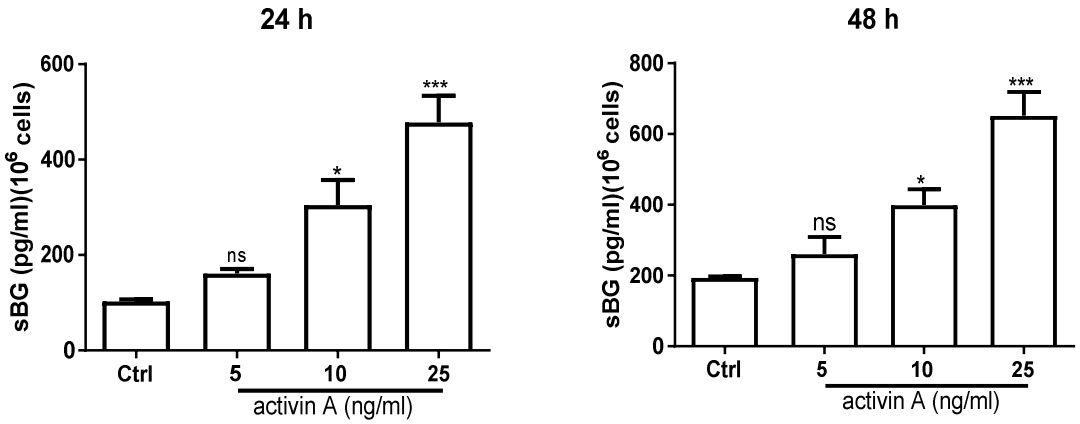


Figure 16. Activin A enhanced shedding of betaglycan-2×10^5 TN4A5 Sertoli cells were treated for 24 h and 48 h with activin A (5-25 ng/ml) and supernatants analysed by sBG ELISAs. Each bar represents mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *P ≤ 0.05, ***P < 0.001, sBG=soluble betaglycan; **ns**=not significant.

3.2.2 TIMP3 modulates activin A-induced phosphorylation of Smad3

Our earlier findings suggested the involvement of TIMP3 in blocking shedding of betaglycan in Sertoli cells (Fig. 11). Thus, the aim was to determine, whether TIMP3 could attenuate the effect of activin A on signalling by Smad3. We pre-incubated Sertoli cells with TIMP3 for 2 h and then treated with activin A for 1 h (Fig. 17). We found that TIMP3 reduced activin A-induced phosphorylation of Smad3 by ~50%.

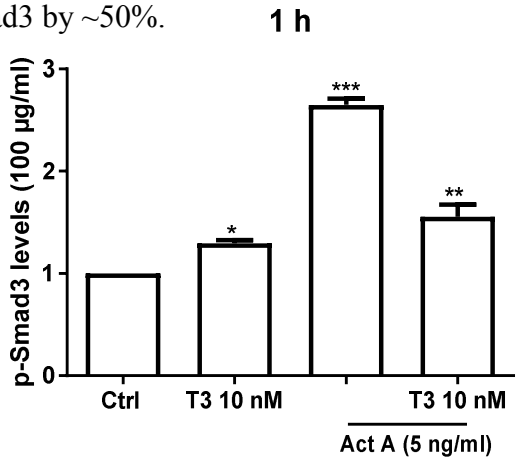


Figure 17. TIMP3 effect on activin A signalling-$1X10^5$ TN4A5 mouse Sertoli cells were treated 2 h with rhTIMP3 (10 nM) and then with activin A (5 ng/ml) for 1 h. Cell lysates were collected and p-Smad3 ELISAs were performed. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate and p-Smad3 levels were standardized to 100 µg proteins from lysates. Dunnett's test was used for statistical analysis; *P≤0.05, **P<0.01, ***P<0.001.**T3**-TIMP3; **Act A**-activin A.

We also performed qRT-PCR for TIMP3 and betaglycan expression and observed that activin A downregulated TIMP3 and betaglycan mRNA expression by ~50% ± 15% after 48h of treatment (Fig. 18A & B). These results suggest that TIMP3 is involved in blocking activin A signalling via Smad3.

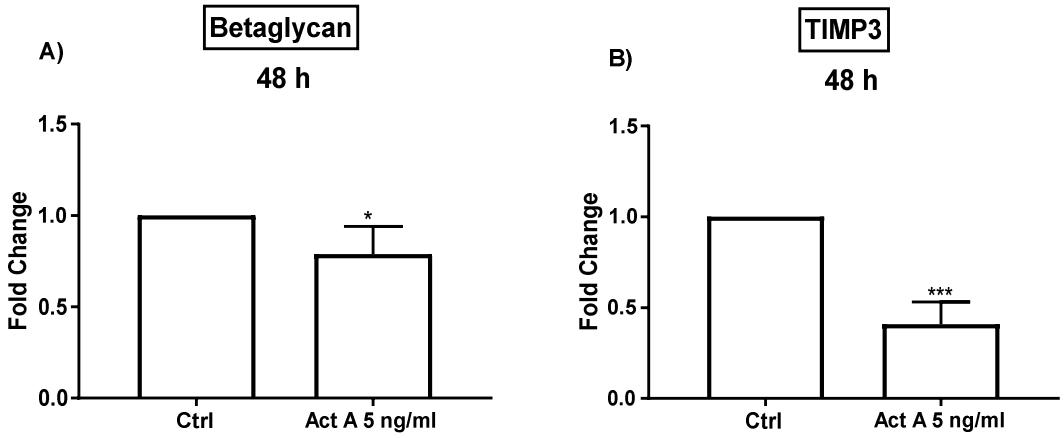


Figure 18. TIMP3 and betaglycan expression is reduced by activin A-1×10^5 TN4A5 cells/well were incubated with activin A (5 ng/ml each) for 48 h and analysed with qRT-PCR for **(A)** betaglycan and **(B)** TIMP3 gene expression. Data were normalized with the β-microglobulin housekeeping gene and are presented as the mean ± SD of three independent experiments. The t test was employed for statistical analysis. P-values ≤ 0.05 *, <0.001***. **Act A-**activin A; **Ctrl-**control.

3.2.3 GM6001 counteracts activin A-induced shedding of betaglycan in Sertoli cells

It is already known that MMPs are involved in shedding of betaglycan (Velasco-Loyden et al., 2004; Bilandzic & Stenvers, 2011) and TIMP3 blocks these MMPs actions (Gomez et al., 1997; Clark et al., 2008). Additionally, our previous results showed that GM6001 blocks the effects of MMPs on betaglycan shedding (Fig. 12). We further examined the involvement of MMPs in shedding of betaglycan in combination with activin A signalling in Sertoli cells.

We incubated the TN4A5 Sertoli cells for 2 h with GM6001 with different doses and then treated with activin A for 24 h and 48 h (Fig. 13). sBG ELISAs demonstrated that GM6001 abolished the effect of activin A-induced betaglycan shedding by ~50%. This indicates that MMPs might play a role in shedding of betaglycan. To further test the effects of MMPs on shedding the mRNA expression of MMPs in Sertoli cells was analysed.

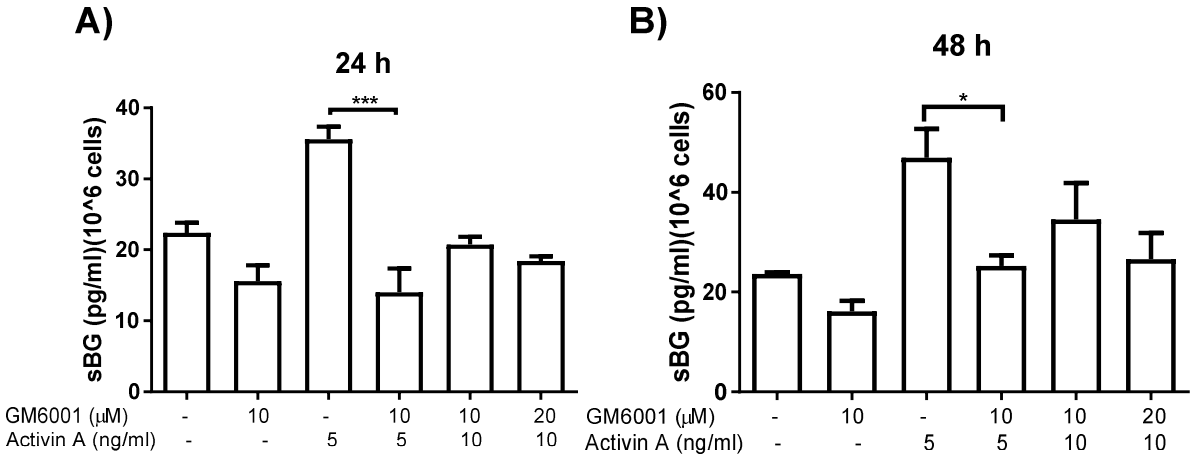


Figure 19. GM6001 blocks shedding of betaglycan-1 × 10^5 TN4A5 mouse Sertoli cells were incubated with the MMP inhibitor GM6001 for 2 h and then stimulated with activin A for 24 h **(A)** and 48 h **(B)** and supernatants analysed for sBG by ELISAs. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's t-test was used for statistical analysis; *P≤ 0.05, <0.001***.

3.2.4 Effects of TGF-β1/2 and activin A on MMPs abundance

Published data suggested that TGF-βs and activin A induce MMPs (Ogawa et al., 2000; Kim et al., 2004). To investigate whether TGF-βs and activin A modulate similar or different MMPs gene expression was analyzed.

Sertoli cells were treated with activin A, TGF-β1 or TGF-β2 for 24 h. As shown in Table 17 activin A increased expression of MMP-2, 9, 12 and 13, whereas TGF-β1 increased MMP-2 and MMP-11. In contrast, TGF-β2 reduced expression of MT1-3 (MMP-14-16). We further investigated whether MT1-MT3 might play a role in shedding of betaglycan as these MMPs were downregulated by TGF-β2 only.

Table 17. Gene expression of MMPs after stimulation with activin A, TGF-β1 or -β2

1 × 10^5 TN4A5 Sertoli cells were treated with activin A (5 ng/ml) and TGF-β1 or TGF-β2 (10 ng/ml) for 24 h. RNA isolation was done with the Qiagen RNA isolation kit. Each MMP product was generated after 35 PCR cycles. We used GAPDH and β-2-microtubulin as house-keeping genes. Results are presented as the means ± SD of three independent experiments.

Proteases	Ctrl	Act A (5 ng/ml)	TGF-β1 (10 ng/ml)	TGF-β2 (10 ng/ml)
MMP-2	+	+++	++++	+
MMP-3	++	-	+	++
MMP-7	+	-	-	+
MMP-8	++++	++++	++++	++++
MMP-9	+	+++	+++	+
MMP-10	++++	++++	++++	++++
MMP-11	++	++	+++	++
MMP-12	+	++	+	+
MMP-13	++	++++	+	++
MMP-14	++	+++	++	+
MMP-15	++	++	++	+
MMP-16	++	++++	++	+
MMP-19	+++	+++	+++	+++

The intensity of bands as determined by imageJ was as follows: **-** = No expression, **+** = Low expression, **++** = Moderate expression, **+++** = High expression, **++++** = Very high expression **Ctrl-**Control; **Act A**-activin A.

3.2.5 TGF-β2 downregulated protein expression of MT-MMP1-3 (MMP-14-16)

It is well documented that MT-MMP1-3 (MT1-MT3-MMPs) are involved in shedding of betaglycan (Velasco-Loyden et al., 2004; Zhao et al., 2004). To confirm the mRNA data by protein analysis, thus, the putative role of MT-MMPs on shedding of betaglycan in Sertoli cells was analysed. Furthermore, we investigated whether MT1-3 (MMP 14-16) levels are influenced by growth factors.

MMP-14-16 ELISAs were done with TN4A5 Sertoli cells stimulated with TGF-β1 or TGF-β2 (10 ng/ml), or activin A (5 ng/ml). As shown in Figure 20 (A & C) TGF-

β2 reduced MMP-14 & 16 abundance by ~50%, when compared to control, whereas MMP-15 downregulation by TGF-β2 was not significant (Fig. 20B). These results confirmed that TGF-β2 downregulated the abundance of MT-MMPs, possibly to reduce shedding of betaglycan.

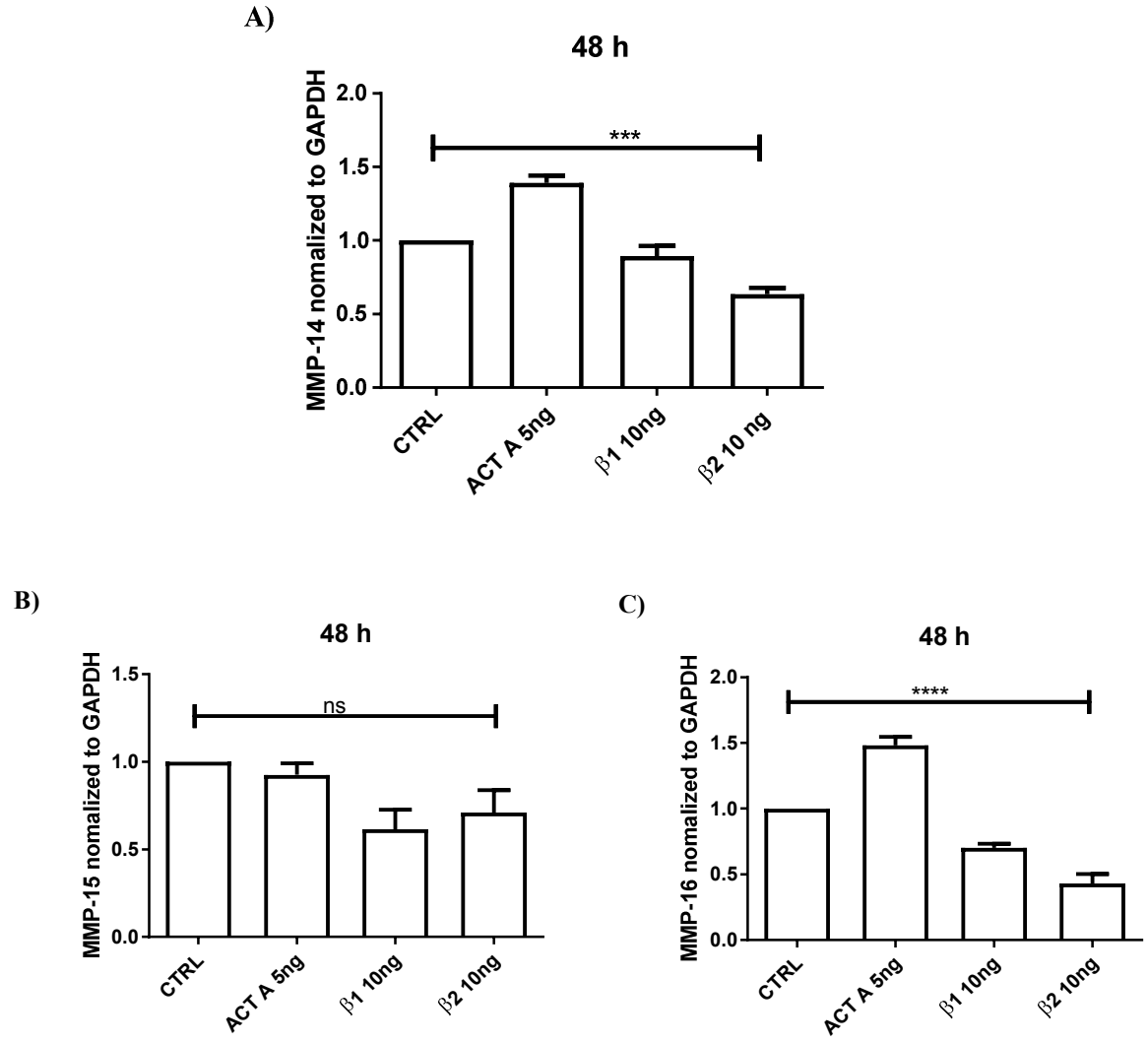


Figure 20. TGF-β2 reduces protein expression of MT-MMPs-1 × 10^5 TN4A5 mouse Sertoli cells were stimulated with activin A (5 ng/ml) or TGF-β1 and TGF-β2 (10 ng/ml) for 48 h. MMPs were measured by colorimetric Cell-Based ELISAs **(A)** MMP-14, **(B)** MMP-15 & **(C)** MMP-16. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Student`s t-test was used for statistical analysis because 3 different treatments were compared with the control; ***P<0.001, <0.0001****. **Act A**- activin A; **β1**-TGF-β1; **β2**-TGF-β2.

3.2.6 Activin A effects on proliferation of Sertoli cells

In recent studies it was shown that during rat testis development Sertoli cell proliferation is stimulated by activin A (Itman et al., 2006). Thus, further investigations of the effects of activin A on Sertoli cells were undertaken. We treated TN4A5 Sertoli cells with activin A in different concentration (Fig. 21A). The BrdU incorporation assay showed that activin A increased Sertoli cell proliferation 1.5-fold compared to controls and this effect was time and dose-dependent. In addition, when we added TGF-β1 or TGF-β2 together with activin A, the increased cell proliferation by activin A was attenuated by TGF-β1 as well as by TGF-β2 (Fig. 21B). To confirm these findings, we also performed a viability test by Tryphan blue exclusion and demonstrated 90-100% cell viability for all treatments (data not shown).

These results showed that TGF-β1 or TGF-β2 counteracted the proliferative effects of activin A signalling. To better understand this mechanism, we further investigated the cross-talk of activin A and TGF-β1 or TGF-β2 signalling cascades.

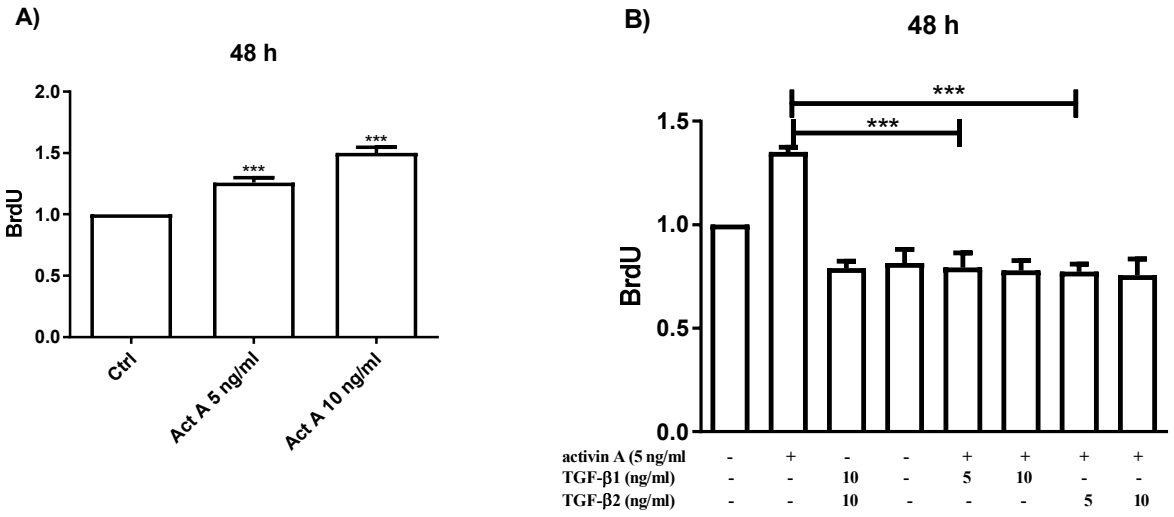


Figure 21. BrdU cell proliferation-Analysis of TN4A5 Sertoli cells treated with activin A (5 ng/ml) (**A)** and activin A (5 ng/ml) together with TGF-β1 or TGF-β2 (10 ng/ml) for 48 h **(B)**. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; ***P<0.001. **Act A**-activin A.

3.2.7 Activin A effects on secretion of TGF-β1 or TGF-β2

After confirmation of earlier results that immature Sertoli cell proliferation is induced by activin A (Fig. 21A) and that TGF-β1 or TGF-β2 had negative effects on proliferation (Fig. 21B), we investigated whether activin A influences secretion of TGF-βs.

Sertoli cells treated with 10 ng/ml of activin A reduced secretion of TGF-β1 by 70% compared to controls in both 24 h and 48 h (Fig 22 A & B). Similar effects were also observed for TGF-β2 which was reduced by activin A by 75% (Fig 16 C & D).

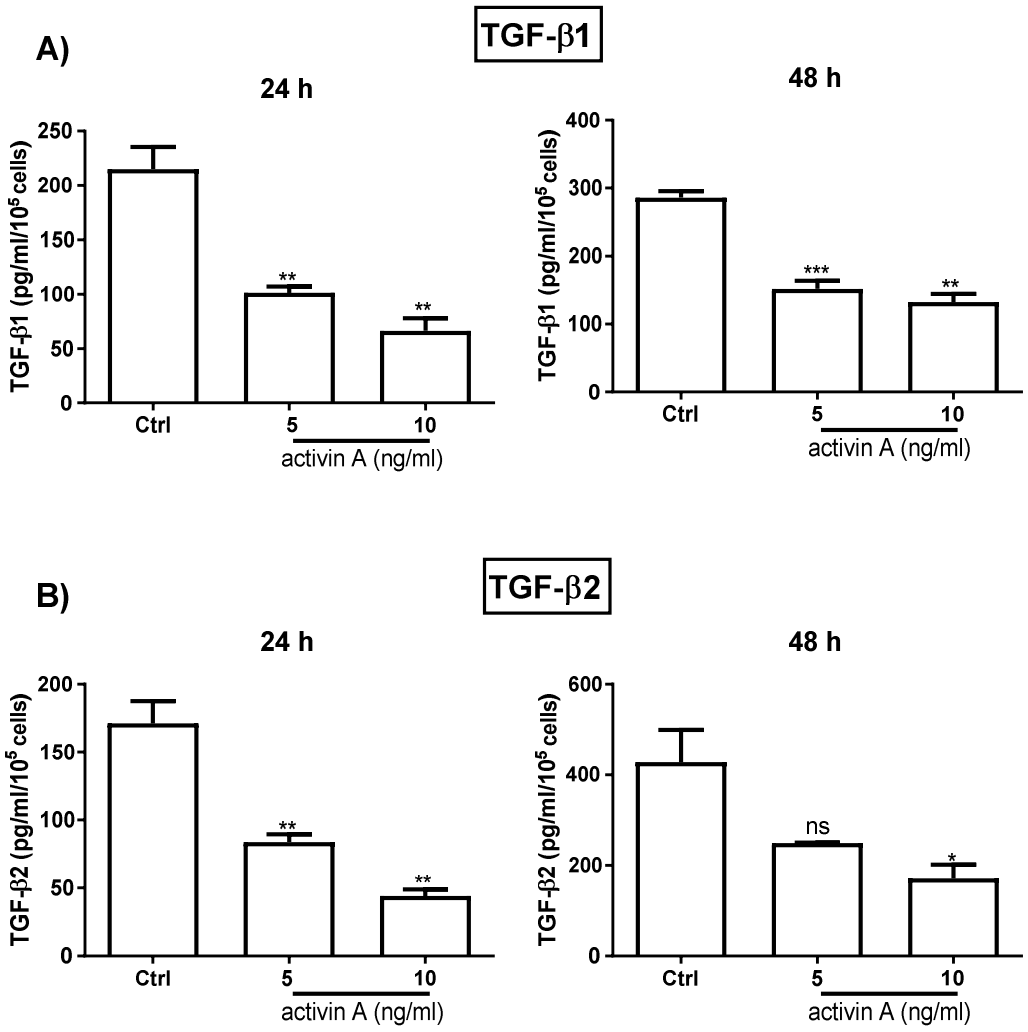


Figure 22. TGF-β1 and TGF-β2 secretion is reduced by activin A-1×10^5 TN4A5 cells/well were incubated with activin A with different doses for 24 & 48 h and supernatants analysed for **(A)** TGF-β1 and **(B)** TGF-β2 by ELISAs. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *P≤ 0.05. **P<0.01, ***P<0.001.

3.2.8 Cross-talk of activin A and TGF-βs with respect to Smad2 and Smad3 signalling

Our previous findings showed that the cross-talk between activin A and TGF-βs affected Sertoli cell proliferation (Fig. 21). Furthermore, we demonstrated cross-talks by targeting the downstream targets, namely phosphorylation of Smad2 and Smad3. It is well documented that both cytokines activate Smad2 and Smad3 (Shi & Massagué, 2003; Chen et al., 2006).

We treated the TN4A5 Sertoli cells with activin A and TGF-βs (β1 or β2) at different concentrations for 1 h. ELISAs for p-Smad3 (Fig. 23A) and p-Smad2 (Fig. 23B) showed that activin A reduced the TGF-β1-induced phosphorylation of Smad3 by 50%. In contrast, activin A together with TGF-β2 showed no effect. The dose-dependent activin A-reduced phosphorylation of Smad 2 & Smad3 was significant. These results suggest that activin A is competitive to TGF-β1 but not to TGF-β2 signalling.

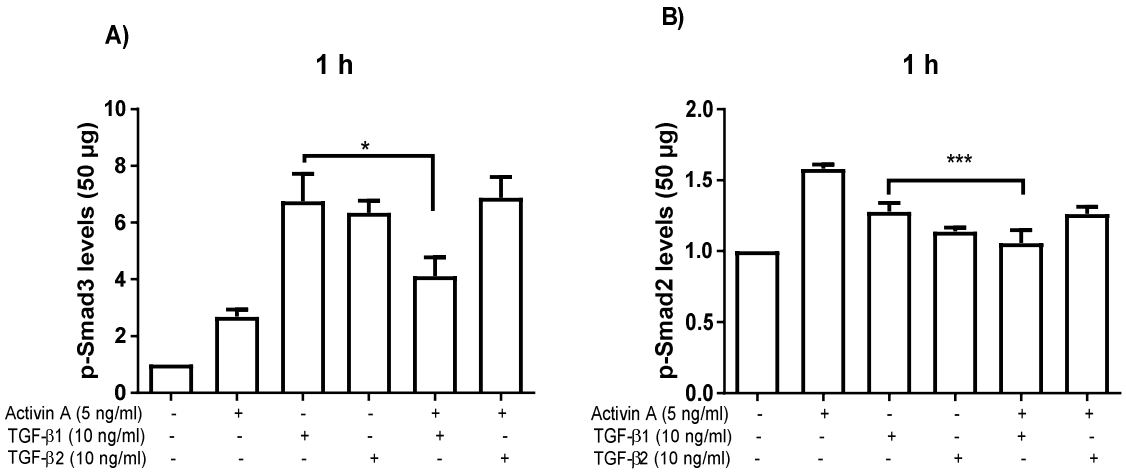


Figure 23. Activin A treatment reduced Smad2 & Smad3 phosphorylation in TGF-β1 signalling-7×10^4 TN4A5 cells/well were incubated together with activin A (5 ng/ml) and TGF-β1 or TGF-β2 (both 10 ng/mL) for 1 h. Cell lysates were analysed for **(A)** p-Smad3 & **(B)** p-Smad2 by ELISAs. Each bar represents mean ± SEM of 3 independent experiments performed in duplicate and p-Smad3 levels were standardized to 50 µg protein from lysates. Dunnett's test was used for statistical analysis; *P≤0.05, ***P<0.001.

3.2.9 TGF-β1-induced phosphorylation of Smad3 was reduced by activin A

The last experiments suggested that activin A has an influence on the TGF-β1 signalling cascade but not on TGF-β2 (Fig. 23). To examine the effect of TGF-β1 and activin A signalling on phosphorylated Smad3 in Sertoli cells, TN4A5 cells were treated together with TGF-βs and activin A for 1 h (Fig. 24). ELISA data of the dose- and time-dependent treatment of activin A and TGF-β1 showed that increased phosphorylation levels of Smad3 by TGF-β1 were significantly inhibited by activin A in a dose-dependent manner (Fig. 24A). Similar effects have also been observed in a time-dependent manner. After 60 min of incubation, a stronger effect was noted (Fig. 24B). In contrast to TGF-β2 signalling, activin A had no influence both in time- and dose-dependent treatments on TGF-β2 signalling (Fig. 24 A & B). Altogether these results suggest that activin A influenced TGF-β1-induced Smad3 signalling in a dose- and time-dependent manner.

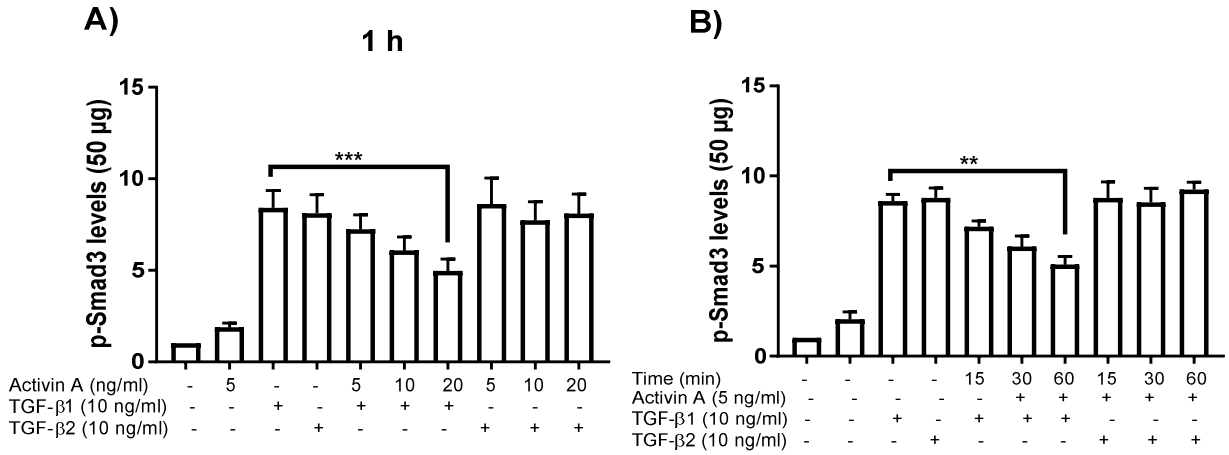
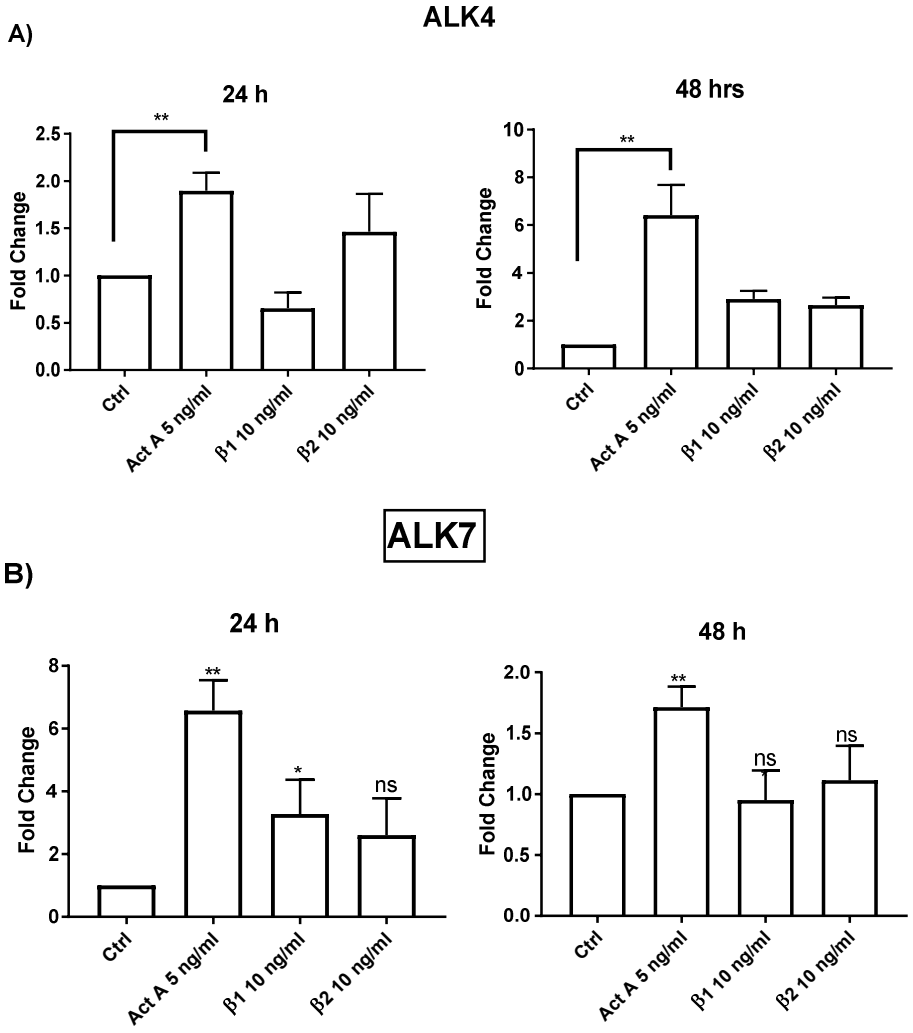


Figure 24. Activin A reduced TGF-β1-induced Smad3 phosphorylation in a dose- & time-dependent manner-7×10^4 TN4A5 cells/well were incubated together with activin A, TGF-β1 or TGF-β2 with different doses of activin A **(A)** and at different time points **(B)**. Cell lysates were analysed for p-Smad3 by ELISAs. Each bar represents the mean ± SEM of three independent experiments performed in duplicate and p-Smad3 levels were standardized to 50 µg protein from lysates. Dunnett's test was used for statistical analysis; **P<0.01, ***P<0.001.

3.2.10 mRNA expression of TGF-β family receptors in Sertoli cells

TGF-β type I receptors consist of activin-like-kinases (ALKs) and are differentially regulated during testis development (Itman et al., 2006). We used qRT-PCR to assess mRNA expression of AL4, ALK5, ALK7 and betaglycan in TN4A5 Sertoli cells. The observed gene expression is summarised as follows.

The type I receptors for activin A are ALK4 and ALK7. We found that ALK4 mRNA expression was 2-fold and 7-fold at 24 h and 48 h respectively, increased by activin A (Fig. 25A). Although ALK4 levels were approximately 2-fold elevated by TGF-β2 at 24 h, but after 48 h ALK4 levels after treatment with TGF-β1 or TGF-β2 were similar to 24 h. Whereas ALK7 mRNA expression was significantly upregulated only by activin A at 24 h and 48 h (Fig. 25B), TGF-β type-I receptor ALK5 was highly expressed in both treatments (24 h and 48 h) with TGF- β1 or TGF-β2 (Fig. 25C). Betaglycan expression was increased by ~6 fold only by TGF-β2 (Fig. 25D). This suggests again that betaglycan is required only by TGF-β2 for signalling.



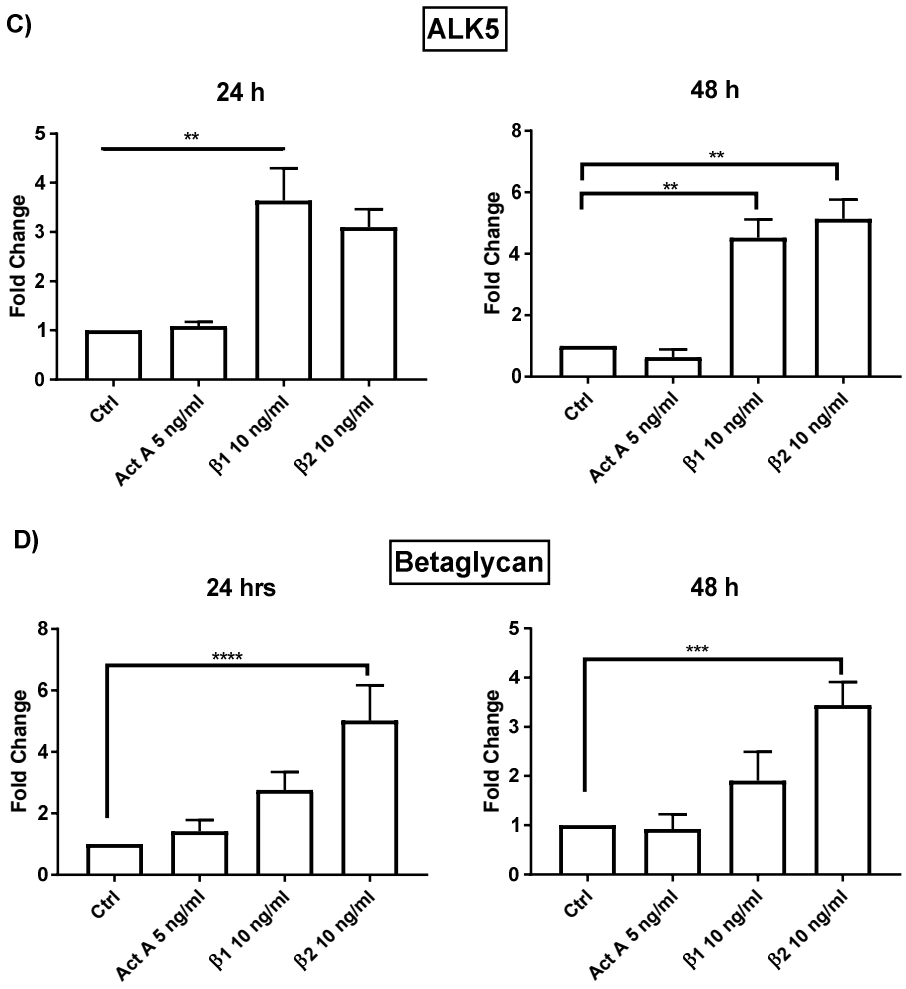


Figure 25. qRT-PCR analysis of ALK4, ALK5, ALK7, and betaglycan mRNA expression in Sertoli cells- 1×10^5 TN4A5 cells/well were incubated with activin A (5 ng/ml), TGF-β1 or TGF-β2 (10 ng/ml each) for 24 h and 48 h. **(A)** ALK4 **(B)** ALK7 **(C)** ALK5 and **(D)** betaglycan gene expression. Data were normalized with the β-microglobulin housekeeping gene and are presented as the mean ± SD of three independent experiments. Dunnett's test was employed for statistical analysis. P≤0.05*, <0.01**, <0.001***, <0.0001****. **Act A-** activin A; **β1**-TGF-β1; **β2**-TGF-β2.

3.3 Role of BMP in regulating shedding of betaglycan in Sertoli cells and cross-talk with activin A signalling

3.3.1 BMP signalling in Sertoli cells

The BMPs are members of the TGF-β superfamily and they influence event that help to establish male infertility (Puglisi et al., 2004). To elucidate BMP signalling in Sertoli cells, we first performed Western blot analysis with phospho-specific antibodies of Smad1/5 to

define the required dose to activate Smad1/5 signalling in Sertoli cells. Treatment of TN4A5 with BMP2, BMP7 or BMP8A resulted in phosphorylation of Smad1/5 in a dose-dependent manner (Fig. 20). In Western blot analysis Smad1/5 phosphorylation was detectable after treating with 10 ng/ml of BMPs, which persisted also at higher doses investigated (Fig. 26). Altogether, these results suggest that Smad1/5 phosphorylation is mediated by BMPs in Sertoli cells.

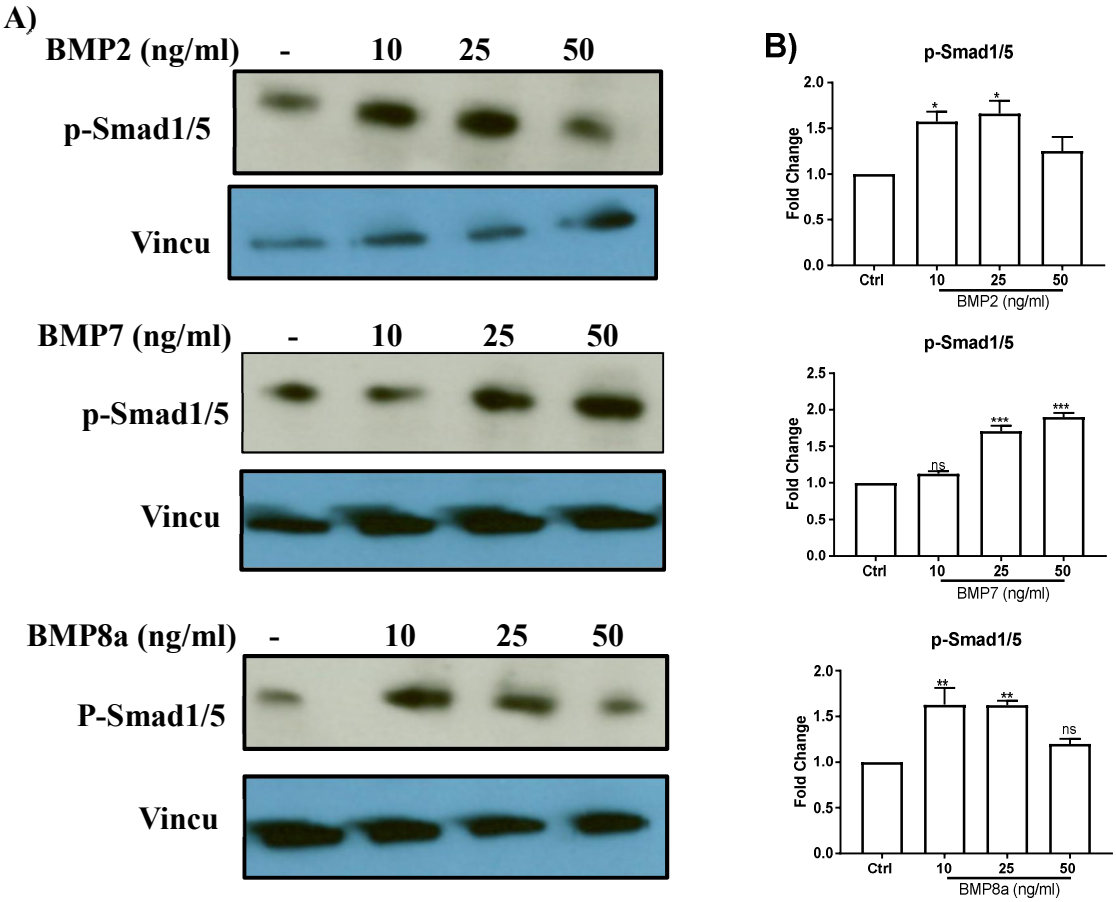


Figure 26. BMPs induced Smad1/5 phosphorylation in TN4A5-TN4A5 cells were stimulated with BMPs at different doses for 2 h. **(A)** Cells were lysed in SDS lysis buffer and equal amounts of proteins were resolved on 10% SDS-PAGE, blotted and probed with antibodies directed against phospho-Smad1/5. Membranes were reprobed with vinculin. N=3. **(B)** Phosphorylation levels were quantified by densitometric analysis of Western blots using Image J software. The graphs represent the data obtained from two independent experiments (mean ± SD).**Vincu**-Vinculin (124 kDa).

3.3.2 Effects of activin A on Smad1 phosphorylation

In recent publications, it has been shown that activin A induces also the non-canonical Smad1/5/8 signalling pathway via BMP type 1 receptors (Canali et al., 2016). To examine the cross-talks between activin A and BMP in Sertoli cells, we performed experiments by treating TN4A5 cells together with activin A and BMP2, BMP7 or BMP8a. We could show that phosphorylation of Smad1 was also initiated by activin A and the level of p-Smad1 was further increased 1.7-fold when activin A and BMPs were applied simultaneously (Fig. 27). These findings suggest that activin A activates also non-canonical Smad1 signalling in Sertoli cells.

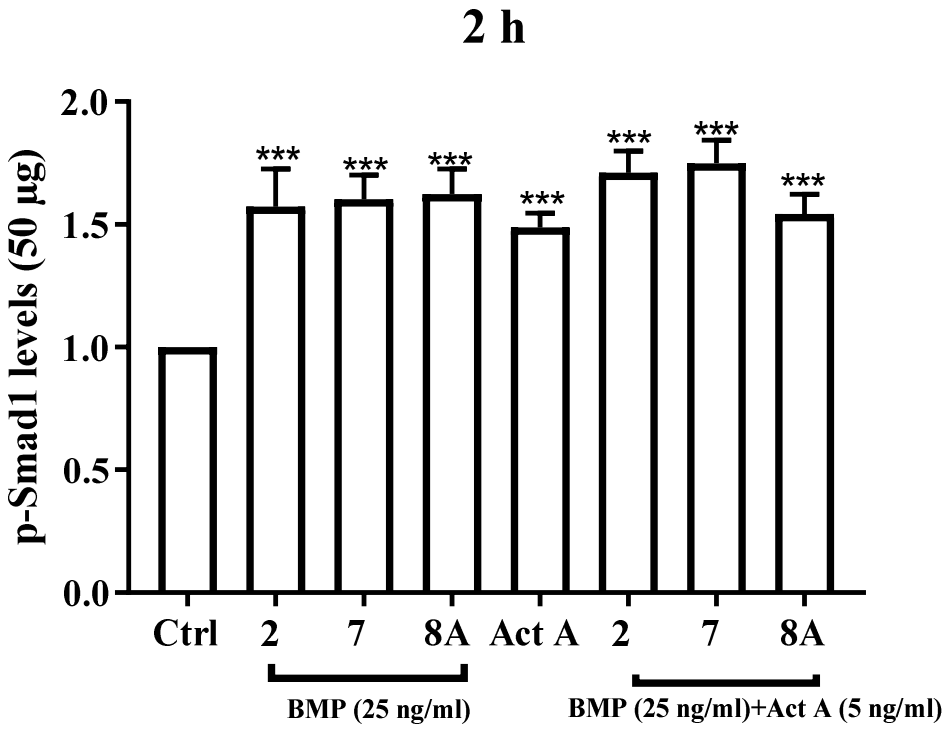


Figure 27. Activin A-induced BMP-specific Smad1 phosphorylation-7×10^4 TN4A5 cells/well were incubated together with activin A (5 ng/ml) and BMP2, BMP7 or BMP8A (25 ng/mL) for 2 h. Cell lysates were analysed for p-Smad1 by ELISA. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate and p-Smad1 levels were standardized to 50 µg proteins from the lysates. Dunnett's test was used for statistical analysis; ***P<0.001.

3.3.3 Effects of BMP2, BMP7 and BMP8a on shedding of betaglycan

The proportion of soluble to membrane-bound betaglycan has an influence on BMP signalling. Moreover the soluble form of betaglycan inhibits BMP-induced Smad1/5/8 phosphorylation (Gatza et al., 2014). Previous results showed the role of TGF-βs in modulating shedding of betaglycan in Sertoli cells (Fig. 7). To elucidate further the involvement of BMP in regulating betaglycan shedding, we performed experiments by treating Sertoli cells with BMP2, BMP7 or BMP8A for 24 h. As shown in Figure 28A & B BMP2 or BMP7 reduced shedding of betaglycan by 60-70% compared to the untreated control in a dose-dependent manner, whilst BMP8a induced shedding of betaglycan by ~100%. These findings showed that BMP8a is not dependent on betaglycan for signalling, in contrast to BMP2 and BMP7 which showed that they might require betaglycan for signalling.

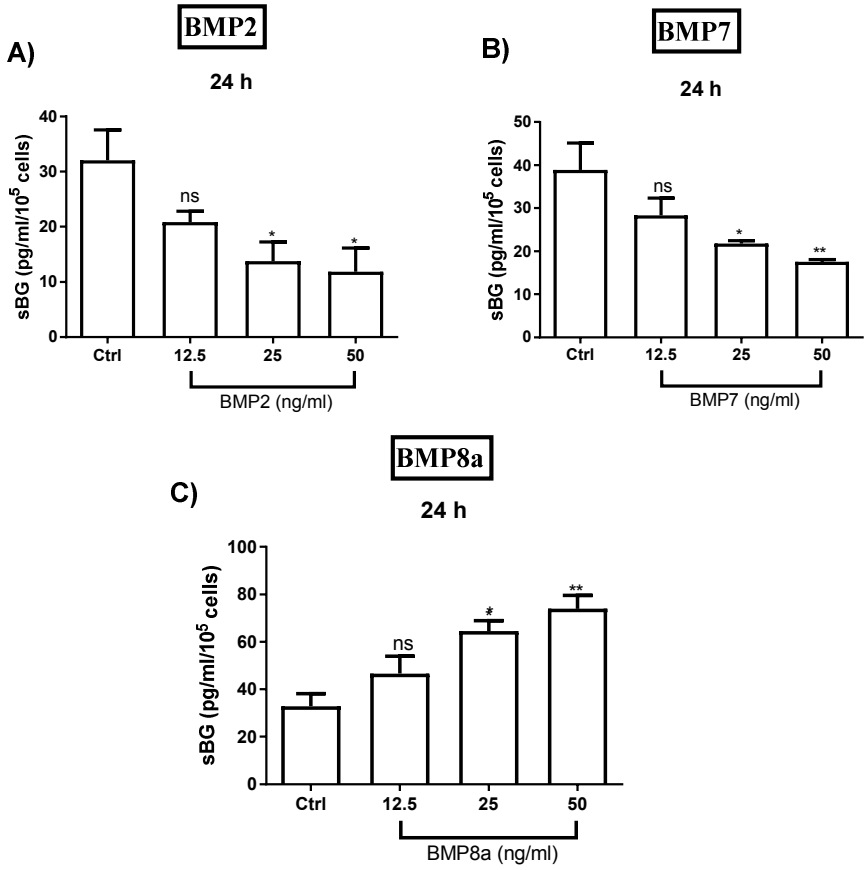


Figure 28. Effects of BMPs on shedding of betaglycan-1 × 10^5 TN4A5 Sertoli cells were treated with **(A)** BMP2, **(B)** BMP7 or **(C)** BMP8a (12.5-50 ng/ml) for 24 h and supernatants analysed by sBG ELISAs. Each bar represents mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *P ≤ 0.05, **<0.01. **sBG**=soluble betaglycan; **ns**=not significant.

3.3.4 Effects of BMP2 and BMP7 on MMP-14 & 16 abundance in Sertoli cells

We have previously demonstrated that TGF-β2 reduced abundance of MMP-14 and MMP-16, resulting in reduced shedding of betaglycan (Fig. 20). Further on, in the BMP signalling cascade, we showed that BMP2 and BMP7 reduced shedding of betaglycan (Fig 22). Therefore, we sought to examine the effects of BMPs on MMP-14 and MMP-16 protein expression.

TN4A5 Sertoli cells were treated with BMP2, BMP7 or BMP8a (each 25 ng/ml) on ELISA plates precoated with MMP-14 or MMP-16 specific antibodies (Fig. 23). ELISA data showed that MMP-14 abundance was reduced ~50 % by BMP2 and BMP7, whereas BMP8a increased MMP-14 abundance by 1.4-fold compared to the untreated control (Fig. 29A). A similar effect was observed also with MMP-16, where BMP2 and BMP7 reduced MMP-16 abundance by ~30 %. In contrast, BMP8a induced MMP-16 by 1.5-fold (Fig. 29B). Altogether these results suggest that MMP-14 and MMP-16 play a crucial role in regulating shedding of betaglycan in Sertoli cells.

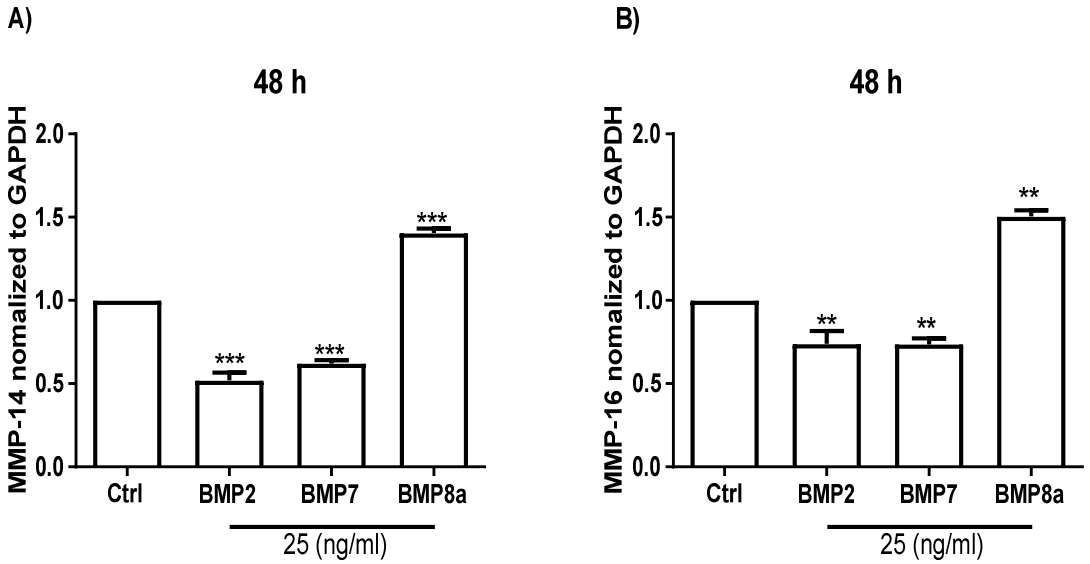


Figure 29. Effects of BMPs on the presence of MT-MMPs-1 × 10^5 TN4A5 mouse Sertoli cells were stimulated with BMP2, BMP7 or BMP8a (each with 25 ng/ml) on a 96-well plate pre-coated with MMP-14 or MMP-16 for 48 hr. MMP-14 (**A**) or MMP-16 (**B**) were measured by colorimetric Cell-Based ELISAs. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Student`s t-test was used for statistical analysis; **P <0.01; ***P <0.001.

3.3.5 BMPs effects germ cells migration

In immature 2-weeks old mouse testis BMP2, BMP7 and BMP8a wew highly expressed (Ciller et al., 2016). It is also known that BMP2 induces reorganisation of the actin cytoskeleton and cell migration (Gamell et al., 2008).

In order to elucidate the effects of BMPs on germ cell and Sertoli cell migration, GC-2 and TN4A5 Sertoli cells were treated with BMP2, BMP7 or BMP8a for 24 h. In Figure 30A BMP2 significantly induced migration of GC-2 by ~50% compared to control, whilst BMP7 and BMP8a had no effects. In contrast, Sertoli cell migration was reduced by BMP stimulation (Fig. 30B).

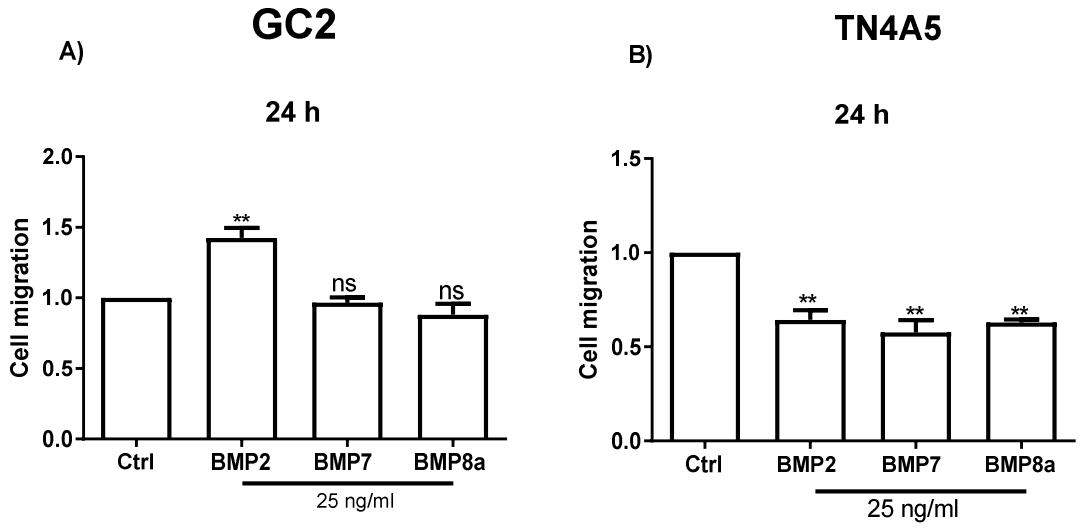


Figure 30. Effects of BMPs on germ cell and Sertoli cell migration-Experiments were carried out using the CytoSelect Cell Migration Assay Kit containing a polycarbonate membrane on inserts (8-µm pore size) in a 24-well plate. **(A)** 5×10^4 GC-2 germ cells or **(B)** $5x10^4$ TN4A5 Sertoli cells were seeded in 24-well inserts and then treated with BMP2, BMP7 or BMP8a for 24 h. The absorbance of each sample was determined at 450/550 nm using the M200 microplate reader. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *P ≤ 0.05, **P <0.01.

We then addressed the question whether migration of germ cells is also dose-dependent. In Figure 31 GC-2 cells treated with BMP2 showed increased cell migration in a dose-dependent manner; BMP2 at 50 ng/ml increased germ cell migration by ~50%.

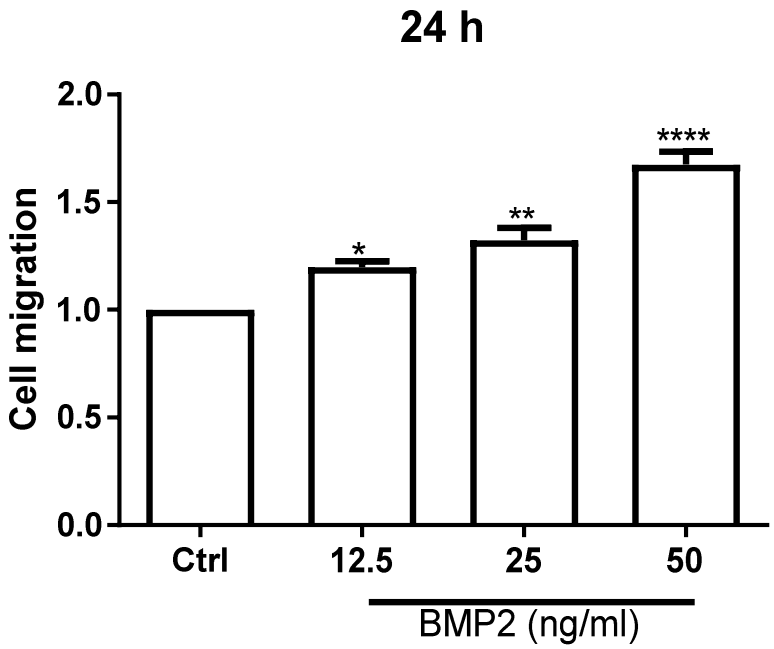


Figure 31. BMP2 enhances GC-2 cell migration in a dose-dependent manner-The experiment was carried out using the CytoSelect Cell Migration Assay Kit, containing polycarbonate membrane inserts (8-µm pore size) in a 24-well plate. $5x10^4$ GC-2 germ cells were seeded in 24-well inserts and then treated with BMP2 in dose dependent manner for 24 h. The absorbance of each sample was determined at 450/550 nm using the M200 microplate reader. Each bar represents mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *P ≤ 0.05, **P <0.01, ****P <0.0001.

3.3.6 Effects of BMPs on proliferation of germ cells

In the early stages of testis development BMP2 and BMP7 act as a proliferative signal upon Sertoli and germ cells (Puglisi et al., 2004; Ross et al., 2007).

To investigate whether BMP2, BMP7 and BMP8a affect proliferation of Sertoli cells and germ cells. TN4A5 Sertoli cells and GC-2 germ cells were cultured for 48 h in the presence of BMPs. In Figure 32A it is shown that BMP2, BMP7 and BMP8a increased germ cells proliferation compared to untreated controls in a dose-dependent manner. However, in Sertoli cells the higher dose of BMP8a reduced the BrdU incorporation (Fig. 32A). Interestingly, we observed that proliferation of Sertoli cell was not regulated by BMP2 and BMP7, except for BMP8a, which induced BrdU incorporation by 1.5-fold in Sertoli cells. These results suggest

that BMP8a induced germ and Sertoli cell proliferation, whereas BMP2 and BMP7 induced only germ cell proliferation.

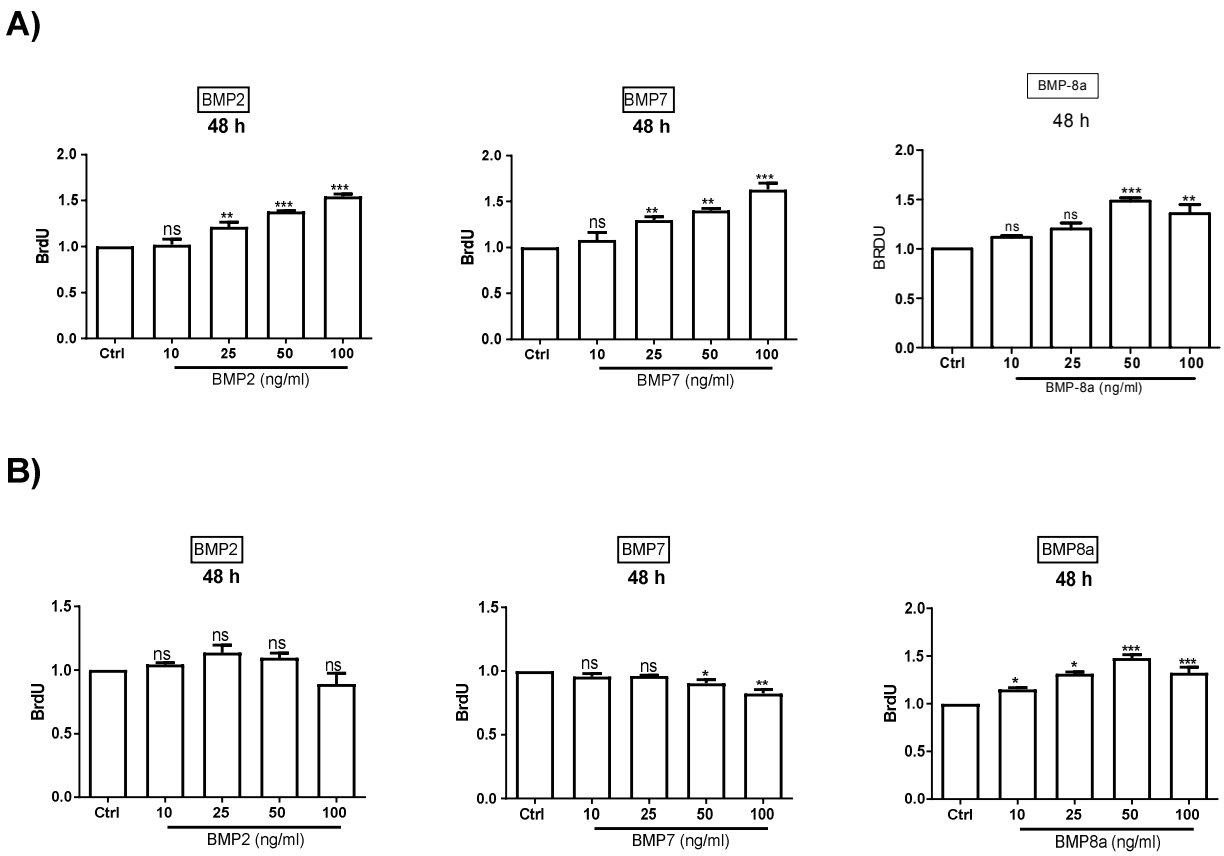


Figure 32. Effects of BMPs on proliferation of germ cells and Sertoli cells-Analysis of GC-2 germ cells and TN4A5 Sertoli cell line treated with BMP2, BMP7 or BMP8a **(A)** GC-2 **(B)** TN4A5 for 48 h. The absorbance of each sample was determined at 450/550 nm using the M200 microplate reader. Each bar represents mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; **P<0.01, ***P<0.001. **BrdU**-Bromodeoxyuridine.

3.3.7 Effects of BMP2 on Sertoli cell tight junctions

Sperm production is dependent on a testicular structure called the blood testis barrier (BTB) (Yan et al., 2008). This is formed by tight junctions between Sertoli cells (Mital et al., 2011). Previously published data showed that TGF-β2 and activin A downregulated Sertoli cell tight junctions (Yan et al., 2008; Nicholls et al., 2012). Retinoic acid (RA) stimulates BTB formation (Hasegawa & Saga, 2012).

To elucidate, whether BMP2 affects BTB formation 93RS2 Sertoli cells were used, as these cells are known to form barrier (Dietze et al., 2015). The cells were seeded on 24-well inserts to form a monolayer and then were treated with TGF-β1, TGF-β2, BMP2 or retinoic acid for 48 h. In tracer diffusion assays, the amount of FD4-FITC diffusing through the Sertoli cell monolayer treated with TGF-β1 showed impressive effects which were about 30% reduced compared to control; with BMP2 it was 35 % higher (highly significant, $p<0.001$) compared to the untreated monolayer (Fig. 33). Whereas TGF-β2 increased the diffusion of FD4-FITC by ~15% compared to control (Fig. 33) and we found no effect of the RA on the BTB (Fig. 33). These results showed that BMP2 have a negative influence on the blood testis barrier, but TGF-β1 had positive effects.

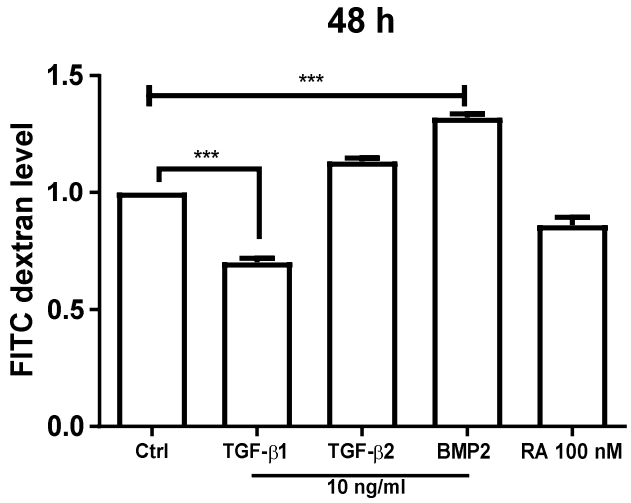


Figure 33. Effects of TGF-β1, TGF-β2, BMP2 or retinoic acid (RA) on the BTB-5×10^4 93RS2 cells were seeded on 24-well insert and grown to form a monolayer after 48 h. Treatment with TGF-β1, TGF-β2, BMP2 (each 10 ng/ml) or RA (100 nM) was done for 48 h. Tracer diffusion assays were done and the amount of FD4-FITC dextran passed through the membrane was measured. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; ***P<0.001; **RA-** Retinoic acid.

3.3.8 Modulation of the BMP signalling cascade by GAG chains

Previous data showed that GAG chains influenced the TGF-β signalling cascade in 93RS2 Sertoli cells (Fig. 15). It has also been reported that heparin sulfate chain influences the activity of BMP (Irie et al., 2003; Fisher et al., 2006). Specifically, BMP2 and BMP7 interact with the GAG chain (heparin and chondroitin sulfate chain; Takada et al., 2003).

To analyze the effect of GAG chain interaction with BMP signalling in 93RS2 Sertoli cells, we performed experiments by treating 93RS2 Sertoli cells with GAG chain degrading enzymes for 16 h prior to stimulation with BMP2, BMP7 or BMP8a for 2 h (Fig. 28). P-Smad1 ELISA analysis showed that p-Smad1 was increased 1.5-fold by BMP2, when the heparin III chain was degraded (Fig 34A). However, when all other GAG chains were digested, BMP2 signalling was abrogated to control levels (Fig 34A). Similarly, BMP7 and BMP8a-induced p-Smad1 were downregulated, when the GAG chains were degraded by enzymes (Fig 34A & B). These results indicate that BMP binds to the GAG chain to activate canonical Smad signalling.

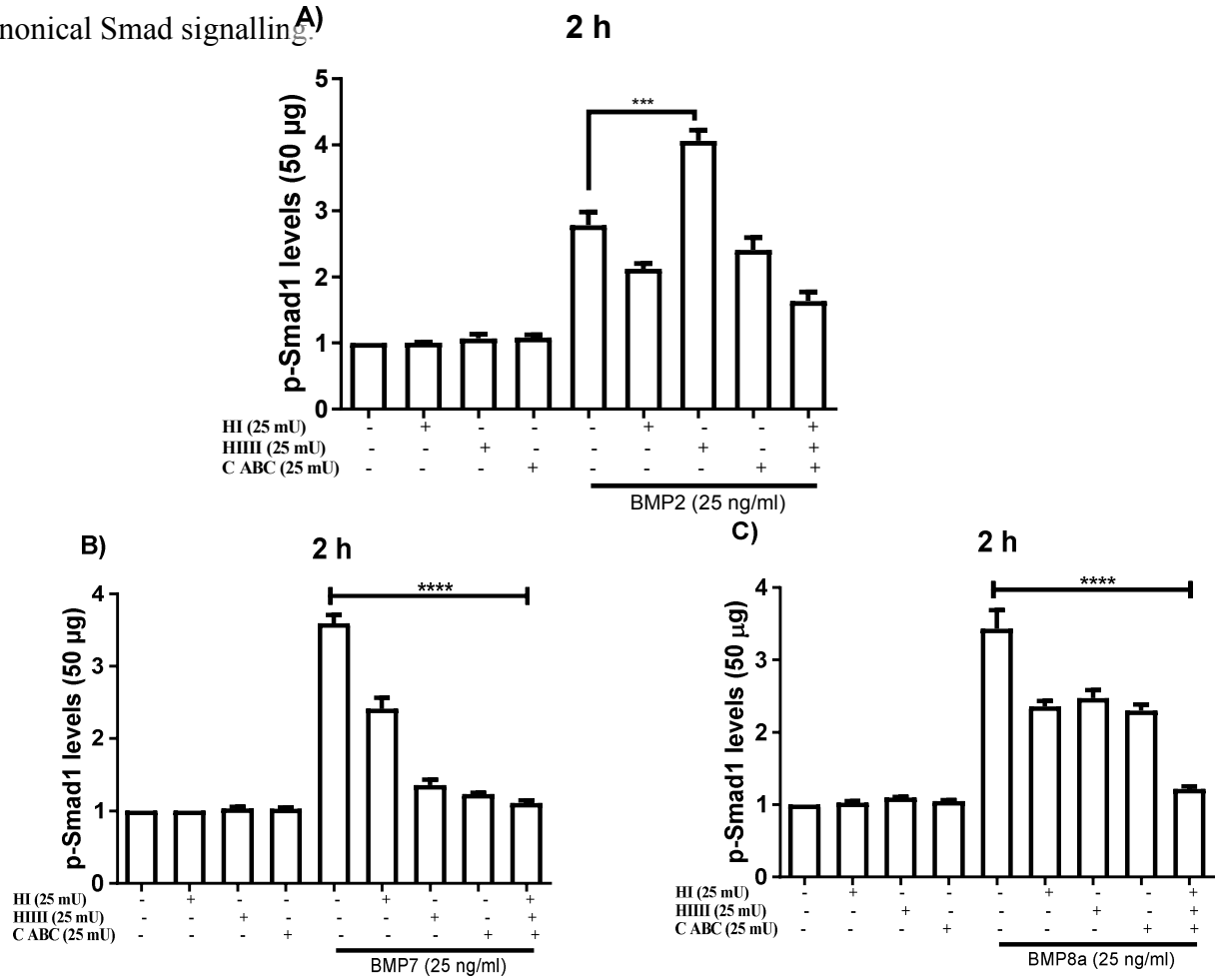


Figure 34. BMP signalling is modulated by GAG chains-7×10^4 93RS2 cells/well were incubated with Heparinase I & III, Chondroitinase ABC (each 25 mU) for 16 h and then treated with BMP2, BMP7 or BMP8a (each 25 ng/mL) for 2 h. Cell lysates were analysed for p-Smad1 by ELISAs. Each bar represents mean ± SEM of 3 independent experiments performed in duplicate and p-Smad1 levels were standardized to 50 µg protein from lysates. Dunnett's test was used for statistical analysis; ***P<0.001, ****P<0.0001. **HI**-Heparinase I; **HIII**- Heparinase III; **C ABC**-Chondroitinase ABC.

3.4 Influence of FSH on Sertoli cells

3.4.1 Effects of FSH on proliferation of Sertoli cells

Follicle-stimulating hormone (FSH) plays a major role in testis development. In rat gonads after 14.5 days of gestation low levels of FSH receptor activity are detected, but at about 19.5 to 21.5 fetal days the FSH binding activity of the receptors increased (Warren et al., 1984). It is also well documented that FSH induces Sertoli cell proliferation (Allan et al., 2004).

To confirm these finding in our study, we treated TN4A5 Sertoli cells, which express the FSH receptor, with FSH at different dosages and incubated for 24 h and 48 h (Fig. 35 A & B). FSH stimulated Sertoli cell proliferation by 1.5-fold after 24 h (Fig. 35A) and up to 1.7-fold at 48 h (Fig. 35B).

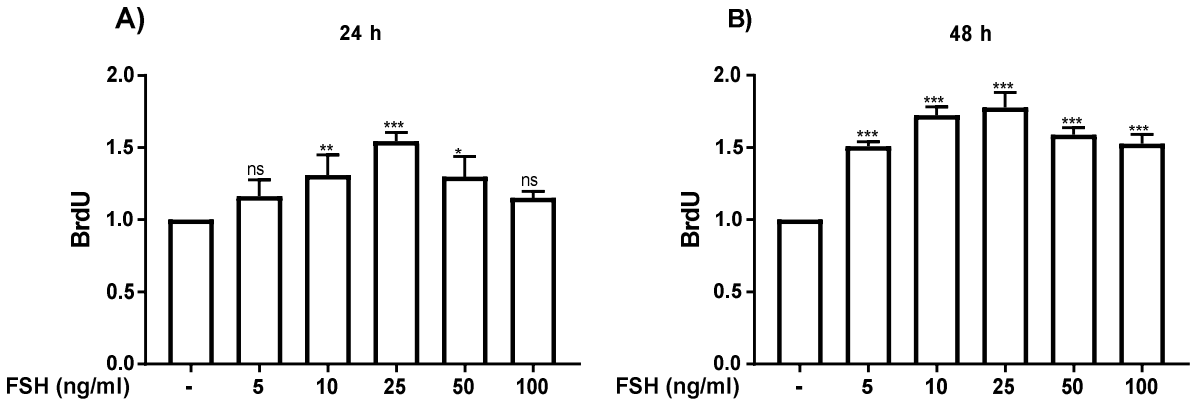


Figure 35. FSH-induced Sertoli cell proliferation-BrdU analysis of TN4A5 Sertoli cell line treated with FSH (different dosages in ng/ml) for **(A)** 24 h and **(B)** 48 h. The absorbance of each sample was determined at 450/550 nm using the M200 microplate reader. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; **P<0.01, ***P<0.001.

Then, we looked further for secretion of TGF-β1 & TGF-β2, as we already know that FSH reduced secretion of total TGF-β2 in Sertoli cells (Konrad et al., 2000). ELISA data analysis of total TGF-β1 and TGF-β2 showed that secretion of TGF-β1 was reduced by FSH treatment, which was significantly higher with 50 & 100 ng/ml FSH (Fig. 36 A & B). A similar effect was also observed with TGF-β2 (Fig. 36 C & D); FSH significantly reduced TGF-β2 in a dose-dependent manner in Sertoli cells. These data suggests that FSH induces Sertoli cell proliferation and at the same time reduced secretion of TGF-βs.

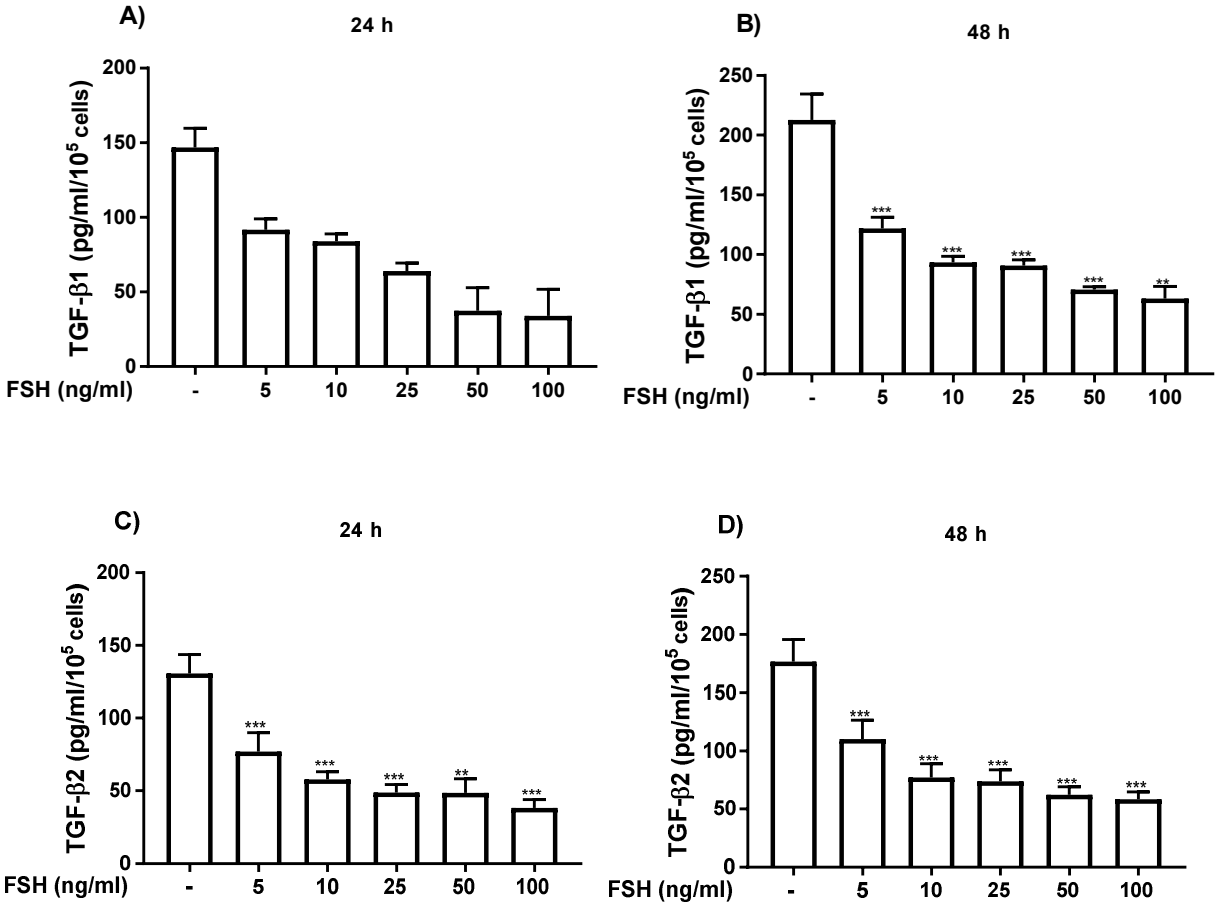


Figure 36. FSH reduced TGF-β1 and TGF-β2 secretion-1 × 10^5 TN4A5 cells/well were incubated with FSH at different dosage for 24 h & 48 h. Supernatants were analysed for TGF-β1 **(A)** 24 h and **(B)** 48 h and TGF-β2 **(C)** 24 h and **(D)** 48 h by ELISA. Each bar represents the means ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; **P<0.01, ***P<0.001.

4 DISCUSSION

TGF-βs are one of the growth factors which are important for the development of the testis. Furthermore, the members of the TGF-β family and their receptors are expressed during development as well as maturation of somatic and germ cells in testis (Itman et al., 2006).

In the present study, we investigated the influence of members of the TGF-β superfamily on Sertoli and germ cells. We also analysed the crosstalk between the family members and their impact on Sertoli cell functions. Later on, we explored the effects of betaglycan shedding on signalling, and furthermore, the effects of hormones on Sertoli cells. Here for the first time, we show that the activin A and TGF-βs signaling pathways intertwine and influence Sertoli cell functions.

4.1 The role of betaglycan and interaction with TGF-βs

The TGF-β co-receptor betaglycan binds all three TGF-β ligands with high affinity (Hinck & O'Connor-McCourt, 2011). However, in contrast to TGF-β1 and TGF-β3 showing a high affinity for TβRII and not requiring the co-receptor to signal, TGF-β2 has only a weak affinity for TβRII and thus requires betaglycan for signalling (Bilandzic & Stenvers, 2011). It was recently shown that betaglycan binds TGF-β2 with a 1:1 stoichiometry and concentrates TGF-β2 on the cell surface for binding to TβRII (Villarreal et al., 2016). Thereafter, betaglycan is replaced by TβRI in the receptor complex for signalling. Of note, membrane-anchored betaglycan can be cleaved into a soluble form by MMPs (Velasco-Loyden et al., 2004) and the resulting soluble form acts as an antagonist (López-Casillas et al., 1994; Vilchis-Landeros et al., 2001; Villarreal et al., 2016). However, the role and regulation of betaglycan shedding in Sertoli cells have not been previously analyzed.

During the early stages of testis development, an increase in betaglycan expression occurs in Sertoli and other testicular cells (Sarraj et al., 2007), as well as TIMPs are similarly up-regulated (Guyot et al., 2003). Based on these parallels studies, we hypothesized that TIMPs might be involved in the modulation of betaglycan shedding and consequently in TGF-β signalling.

4.1.1 Influence of TGF-βs on shedding of betaglycan

Betaglycan is expressed within the interstitial compartment of the fetal testis, predominantly by Leydig cells, but its expression shifts inside the seminiferous (epithelium) cords at birth (Sarraj et al., 2007). Betaglycan knockout mice show fetal testis dysgenesis and compromised

Leydig cell function (Sarraj et al., 2007; 2010). Up to date, it is not known whether TGF-βs modulate shedding of betaglycan in Sertoli cells.

In the first set of experiments, we analyzed whether TGF-βs influenced betaglycan shedding and found not only that ligand binding inhibited betaglycan shedding as also shown by others (Philip et al., 1999), but also that both TGF-β1 and TGF-β2 reduced it. This reduction of betaglycan shedding suggests to result in the non-availability of the receptor for TGF-β signalling. Our results agree with an earlier report (Sarraj et al., 2013), which showed that the betaglycan-null fetal testis has a compromised cord development and a disrupted TGF-β2 function, as both cord maintenance and somatic cell development are highly sensitive to the levels of TGF-β2. Since MMPs and especially MT1-3MMPs were described to be involved in betaglycan shedding (Velasco-Loyden et al., 2004), we also analyzed the influence of TIMPs.

4.1.2 TGF-β2 induces secretion of TIMP3

Since the membrane and soluble forms of betaglycan have opposite effects regulating the availability of TGF-β, it is key to further characterize shedding of betaglycan. Earlier studies showed that MT-MMPs are involved in shedding of betaglycan (Velasco-Loyden et al., 2004) and are inhibited by TIMPs (Liano et al., 1999; English et al., 2000).

In undifferentiated gonads (11.5 dpc) TIMP1-3 were detected (Nuttal et al., 2004; LeMagueresse-Battistoni, 2008). Specifically, TGF-βs induce TIMP3 expression via the Smad3 canonical pathway, but also non-canonically via ERK (Leivonen et al., 2013). We suspected that TGF-β2 induces TIMP3 secretion in Sertoli cells. After treating Sertoli cells with TGF-βs, we found that only TGF-β2 induced secretion of TIMP3 in Sertoli cells, whereas TIMP1 and TIMP2 were not found to be present (regardless of treatment with TGF-βs). These results showed that betaglycan might be required for TGF-β2 signalling and suggested that TGF-β2 might induce TIMP3 secretion to suppress shedding of betaglycan by inhibiting activation of MMPs. To test these hypotheses we investigated the role of TIMP3 in shedding of betaglycan.

4.1.3 Role of TIMP3 in reducing shedding via suppressing MMPs

The balance between the activities of MMPs and TIMPs is very important in tissue remodeling. *In vivo* TIMP3 is the main MMP inhibitor because it is present in nearly all tissues and can inhibit all MMPs (Leco et al., 1994; Baker et al., 2002; Shen et al., 2010). Our

data showed that TGF-β2 induced secretion of TIMP3; after treating Sertoli cells with recombinant TIMP3, we found a reduction in shedding of betaglycan in a time-dependent manner. This suggests that TGF-β2 induced TIMP3 secretion to reduce shedding of betaglycan in Sertoli cells.

The involvement of MMPs was determined by using GM6001, a well-known inhibitor of human MMPs (Xu et al., 2018). We found that after inhibiting MMPs, shedding of betaglycan was reduced in a time-dependent manner. These results indicated that TIMP3 blocks the activation of MMPs, thus rendering MMPs unavailable to cleave the bound form of betaglycan, resulting in the soluble form and antagonising TGF-β2 signalling in Sertoli cells. Nonetheless, it remained unclear whether these effects may have a positive or negative influence on TGF-β signalling.

4.1.4 Involvement of TIMP3 in regulating TGF-β signalling

As clearly shown in our study TIMP3 increased phosphorylation of Smad3, one of the main transducers of TGF-β signalling (Moustakas et al., 2001). In order to analyze, whether this effect was direct or indirect, we investigated the influence of TIMP3 in parallel with LY364947, a highly effective inhibitor of TβRI (Sawyer et al., 2003). We unequivocally demonstrated that LY364947 completely abolished the effect of TIMP3 on Smad3 phosphorylation. Thus, we propose that TIMP3-mediated phosphorylation of Smad3 is indirect and occurs via TβRI.

It is reported that membrane-bound betaglycan can inhibit Smad-dependent TGF-β signalling by interfering with TβRI and TβRII after stimulation with TGF-β1 (Eickelberg et al., 2002; Tazat et al., 2015). Because we have shown that TIMP3-reduced shedding of betaglycan resulted in an increased Smad3 phosphorylation, we wanted to analyse whether the presence of either TGF-β1 or TGF-β2 together with TIMP3 might also inhibit TGF-β signalling. Thus, Sertoli cells were pre-treated with recombinant TIMP3 and then stimulated with TGF-β1 or TGF-β2. First, we observed that TIMP3 together with TGF-β1 strongly reduced p-Smad3 levels consistent with published results (Eickelberg et al., 2002; Tazat et al., 2015). Remarkably, however, Smad3 phosphorylation was increased when Sertoli cells were treated with TIMP3 together with TGF-β2. These results suggest that betaglycan is required for TGF-β2 but not for TGF-β1 signalling.

Taken together, we found that both TGF-β1 and TGF-β2 reduced betaglycan shedding but that only TGF-β2 but not TGF-β1 induced secretion of TIMP3. Since TIMP3 treatment reduced secretion of both TGF-β1 and TGF-β2 and importantly reduced betaglycan shedding, it is highly likely that TIMP3 is an important regulator of betaglycan shedding (Fig. 37). This was underscored by the finding that TIMP3 increased phosphorylation of Smad3, albeit mediated indirectly by TβRI. We also found that ligand binding together with reduced shedding of betaglycan strongly attenuated TGF-β1-dependent Smad3 phosphorylation. However, ligand binding and reduced betaglycan shedding increased TGF-β2-dependent Smad3 phosphorylation. Thus, we have clearly demonstrated the importance of TIMP3 in betaglycan shedding and the substantial need for betaglycan in TGF-β2 signalling.

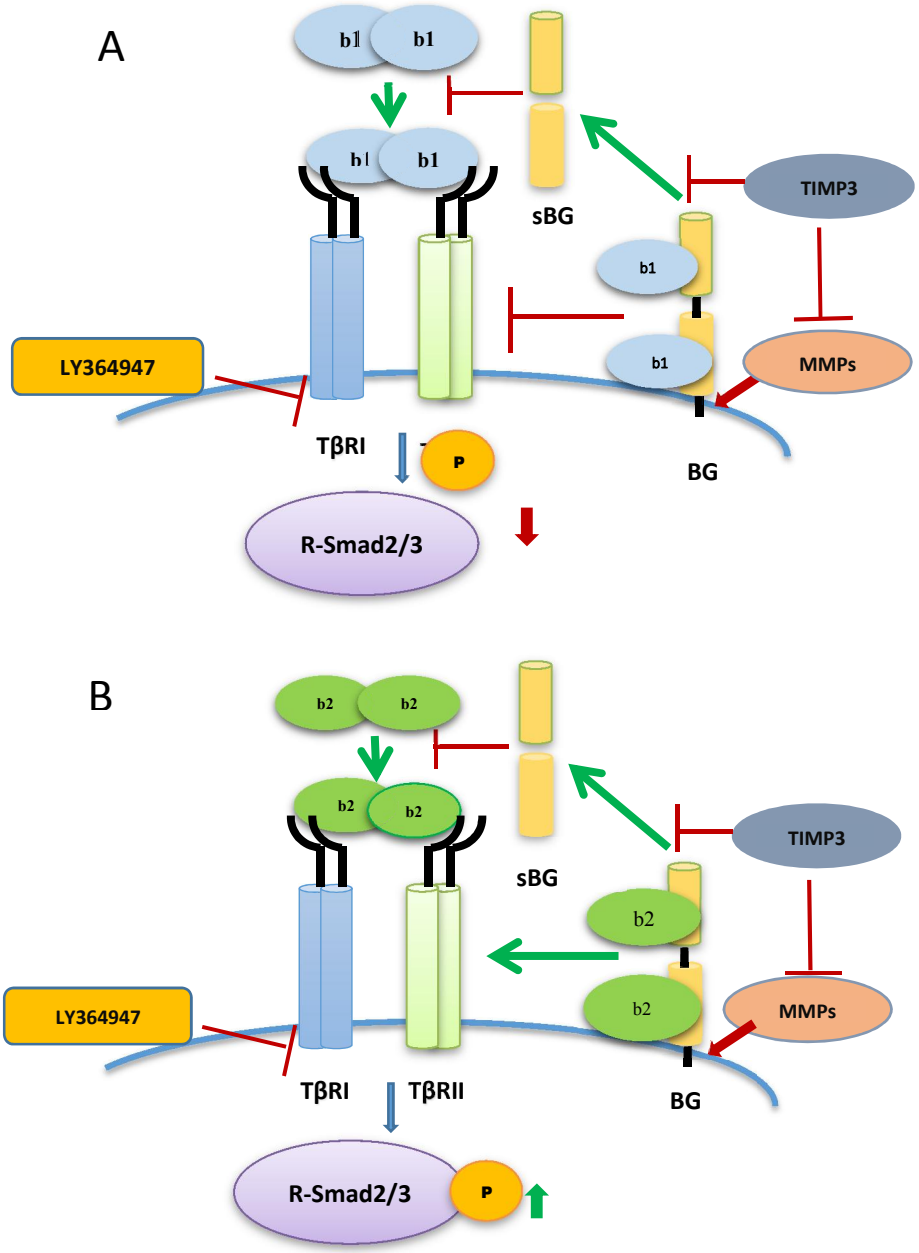


Figure 37. Scheme for the different modes of signalling by TGF-β1 and TGF-β2 under the influence of betaglycan and TIMP3 **(A)** normally, binding of TGF-β1 (b1) to the TGF-β receptor complex results in phosphorylation of Smad3. However, TIMP3 counteracts MMP mediated betaglycan (BG) shedding, thus resulting in interference of membrane-bound and ligand-activated betaglycan with the TGF-β receptor complex. This interaction attenuates TGF-β1-dependent Smad3 phosphorylation (red arrow) via TβRI as shown by the inhibitor LY364947. Soluble betaglycan (sBG) reduces binding of TGF-β1 to the TGF-β receptor complex and thus counteracts TGF-β1 signalling. **(B)** In contrast, binding of TGF-β2 (b2) to betaglycan enhances phosphorylation of TGF-β2-dependent Smad3 via TβRI as shown by the inhibitor LY364947. TGF-β2-dependent Smad3 phosphorylation is further increased (green arrow) when TIMP3 inhibits MMP-dependent betaglycan shedding. Soluble betaglycan (sBG) reduces binding of TGF-β2 to the TGF-β receptor complex and thus counteracts TGF-β2 signalling.

4.1.5 Effects of GAG chains on TGF- β signalling

Earlier studies showed that the GAG chain is attached to Ser 535 and Ser 546 sites on betaglycan (Lopez-Casillas et al., 1994). These residues are located in Ser-Gly sequences surrounded by acidic residues, which is a consensus site for GAG chain attachment (Bourdon et al., 1987). A mutation within any serine residue prevents GAG chain attachment completely (Lopez-Casillas et al., 1994).

In Sertoli cells, betaglycan contains both heparan sulfate and chondroitin sulfate chains (Skinner et al., 1985). Variable modifications of heparan sulfate proteoglycans have been shown to influence the biological activity of FGF-2 (Fiore, 2001). The GAG chains influence TGF-β1 binding to type-1-receptors and block downstream signalling (Eickelberg et al., 2002; Koehler et al., 2017). The GAG chain can also play a dual role in modulation of signalling pathways, for example; the sulfated GAG chains on betaglycan regulate canonical Wnt3a signalling. Wnt interacts with the heparan sulfate chain, which results in blocking of Wnt signalling, whereas the chondroitin sulfate chain promotes Wnt3a signalling (Jenkins et al., 2016). In some studies they also have shown that alteration of GAG chain has no influence on the ability of betaglycan to bind TGF-β and thus, to activate signalling (Lopez-Casillas et al., 1994), suggesting that the type and amount of GAG chains may trigger different outcomes in different cells.

By investigating the effects of GAG chains on TGF-β signalling in Sertoli cells, we found that after degrading the GAG chains with enzymes p-Smad3 signalling was partially reduced for TGF-β1. However, a stronger effect was observed for TGF-β2 signalling. Phosphorylation of Smad3 was strongly reduced in the absence of GAG chains. We hypothesize that TGF-β2 might bind to GAG chains and then to betaglycan for signalling. Further experiments are required to understand the structural basis for the GAG chain effect and the possibility that GAG chain modifications represent a mechanism, whereby other growth factors might influence TGF-β signalling. Our data suggest significant implications for the understanding of betaglycan GAG chain function in the regulation of TGF-β signalling.

4.2 Role of activin A on shedding of betaglycan and cross-talks with TGF-β

Activin A plays an important role in regulating male fertility like gonadal function, hormonal homeostasis, growth and differentiation of musculoskeletal tissue (Wijayarathna & de Kretser, 2016). Particularly, activin A effects the proliferation of Sertoli cells and development of the testis (Mendis et al., 2011). Shortly after sex determination, from E12.5 to birth (0 dpp), the activin A subunit transcript (Inhba) level rises in testis (Mendis et al., 2011).

Inhibins, heterodimeric proteins composed of α and β subunits linked together by disulphide bonds, act as activin A antagonists (Chapman & Woodruff, 2001). Activin A stimulates FSH secretion, whereas inhibin inhibits secretion of FSH from the pituitary (Ling et al., 1987; de Kretser et al., 2002). Inhba-/- mice, which lack activin A at the day of birth showed a significant reduction in testis weight compared with wild-type littermates (Mendis et al., 2011). Interestingly, their testis had a significantly lower number of Sertoli cells, but twice the number of gonocytes, indicating that activin A is essential in maintaining a normal germ cell-to-Sertoli cell ratio. In fetal mouse testis abrogation of activin A signalling resulted in the failure of testicular cord elongation, testicular dysgenesis, and reduced Sertoli cell numbers (Archambeault & Yao, 2010). Activin A disturbs the blood-testis-barrier in Sertoli cells (Nicholls et al., 2012) and thus, might be playing a role in spermatogenesis. In the current study, we investigated the influence of activin A on Sertoli cell proliferation and the cross-talks with TGF-βs.

4.2.1 Activin A induces shedding of betaglycan

Activins and inhibins are members of the TGF-β superfamily (Massagué, 1998) and are mutually antagonistic regulators of reproductive and other functions (Buzzard et al., 2004). Activins bind specifically to type II receptor serine kinases (ActRII or IIB) to activate and transphosphorylate the type I receptor serine kinases, ALK4 or ALK7 (Bernard et al., 2006), which then regulate gene expression by activating Smad2/3 proteins (Lu et al., 2017). Inhibins also bind to type II activin receptors but do not recruit ALK4, providing a competitive model for the antagonism of activin by inhibin (Lebrun & Vale, 1997).
The betaglycan receptor can function as an inhibin co-receptor together with ActRII. Betaglycan binds inhibin with high affinity, enhances binding in cells co-expressing ActRII, and blocks the binding of activin A to its receptor (Lewis et al., 2000).

This ability of betaglycan to facilitate inhibin antagonism against activin A provides a variation on the emerging roles of betaglycan in testis function. Consequently, we hypothesised that activin A induces shedding of betaglycan, thus rendering betaglycan incapable of binding inhibin. We showed for the first time activin A-increased shedding of betaglycan in Sertoli cells in a time-dependent and dose-dependent manner, thus suggesting that activin A induces cleavage of membrane-bound betaglycan into the soluble form.

It has been reported that alteration of the binding site of betaglycan reduced the inhibitory effect of inhibin on activin A (Makanji et al., 2007). Here, we demonstrated that TIMP3, which reduced shedding of betaglycan, when administered together with activin A in Sertoli cells, attenuated activin A-induced shedding of betaglycan compared to activin A alone. These findings were confirmed by mRNA expression of betaglycan and TIMP3, which were reduced in Sertoli cells by activin A. These results showed that activin A reduced betaglycan expression at the mRNA and protein level. This provides evidence for the involvement of MMPs (as TIMPs reduce the activity of MMPs) and TIMPs expression to be downregulated by activin A in Sertoli cells, which indirectly indicated that MMPs might play a role in activin A-induced shedding of betaglycan. While activin A activates Smad2/3 as a canonical downstream target, interestingly, we found that activin A-induced Smad3 phosphorylation was also reduced after TIMP3 stimulation. To conclude, betaglycan can readily influence activin A downstream targets.

Furthermore, as TIMP3 is a potent inhibitor of the MMPs (Arpino et al., 2015), activin A increased expression of MMP2, 3, 7 and 9 in endometrial epithelial cell and inhibin A counteracted expression of MMPs (Jones et al., 2006). MMPs involvement in activin A-induced betaglycan shedding was tested by treating Sertoli cells with GM6001. After pre-incubation with GM6001, activin A-induced shedding of betaglycan was partially reduced after blocking MMPs as compared to controls. Altogether, these results suggested that activin A induces shedding of betaglycan by increasing expression of MMPs, which cleave the membrane-bound betaglycan into its soluble form; consequently, the membrane-bound form is not available for inhibin to bind, and thus to antagonise activin A signalling. The differential expression of MMPs was the subject of the following section.

4.2.2 TGF-β2 downregulates MMPs responsible for shedding of betaglycan

Cells secrete TGF-βs as homodimeric proproteins in an inactive form. Hence, TGF-βs needs to be activated: to be released from the ECM and from the bound proteins that inhibit its activity. MMPs play a crucial role in the activation of TGF-βs. Members of the MMP superfamily, specifically MMP14, MMP13 (collagenase 3), MMP9 and MMP2 mediate the activation of latent TGF-β complexes (Jenkins, 2008; Wipff & Hinz, 2008). TGF-β1 induces expression of MMP2 and MMP9 in breast epithelial cells (Kim et al., 2004). It is also known that MT1-3 MMPs are candidates for shedding of betaglycan (Velasco-Loyden et al., 2004).

Our data showed interesting results which were not demonstrated before in as much Sertoli cells that TGF-β1, TGF-β2 or activin A, differentially regulate the expression of MMPs. As shown in Table 2, activin A increased expression of MMP2, 9, 12 and 13, whereas TGF-β1 increased MMP2 and MMP11. In contrast, TGF-β2 reduced expressions of MT1-3 (MMP14-16) were increased by activin A and TGF-β1 in Sertoli cells. In agreement with the observations that these MT-MMPs are involved in shedding of betaglycan (Velasco-Loyden et al., 2004), we looked for the expression of MT1-3 (MMP14-16) by qPCR or semi-quantitative PCR in Sertoli cells, and found that the levels of MT1-3 were reduced by TGF-β2, whereas TGF-β1 and activin A increased expression of MT1-3. Similar results were also obtained with ELISAs, in which MT1-3 levels were reduced as well; TGF-β2 reduced MT3-MMPs levels compared to TGF-β1 and activin A.

Remarkably, MMPs are the key regulator of shedding of betaglycan in both activin A and TGF-β signalling cascades. Interfering with either one of them affects the outcome of the

signalling pathway. Although at this moment the mechanism is unknown, all these data allow us to conclude that MT-MMPs are involved in shedding of betaglycan in Sertoli cells.

4.2.3 Effect of activin A on Sertoli cell proliferation and cross-talk with TGF-βs

Cell numbers in tissues are usually determined by the rate of proliferation or cell death (Sommer & Rao, 2002). It was shown that activin A influences Sertoli cell proliferation by modulating secretion of FSH (Fragale et al., 2001). Deletion of the activin A receptor II (AcrRII) in male mice showed a delay in reaching puberty and decreased the overall cell number (Matzuk et al., 1995). Considering all these data, we investigated the effects of activin A on Sertoli cell numbers. Activin A increased the overall Sertoli cell numbers, a similar observation to those of Fragale et al. (2001) and Mendis et al. (2011) who showed that activin A increased Sertoli cell proliferation.

Both TGF-β1 and TGF-β2 have been shown to cause apoptosis of rat gonocytes at 14 dpc (Olaso et al., 1998). Furthermore, in this study we showed the negative effects of TGF-β1 or TGF-β2 on cell numbers along with activin A, similar to Meresman et al. (2003) and Sui. (2012), who showed that treatment with TGF-βs reduced the cell numbers. However, our data are in contrast to Sun et al. (2008), who showed that TGF-βs did not change proliferation of Sertoli cells.

Up to date, the connection between the TGF-βs and activin A pathways still remains unclear. Since activin A increases Sertoli cell proliferation and TGF-βs has no such effect, we suspect there might be a competition between TGF-βs and activins when both growth factors are present at the same time in the extracellular milieu. After treating Sertoli cells with activin A together with TGF-β1 or TGF-β2, we surprisingly found that the increase in Sertoli cell proliferation by activin A was attenuated by TGF-βs (discussed in section 4.2.5). Of note, TGF-β1/β2 did not cause cell death, based on the evaluation of live/dead Sertoli cell numbers after counting tryphan blue-stained cells (data not shown). We also demonstrated that activin A completely reduced secretion of both TGF-β1 as well as TGF-β2 in Sertoli cells.

Collectively, this suggests a possible cross-talk between the TGF-βs and activin A pathways, as both pathways utilize Smad 2/3 as their signal transducers from the cell surface to the nucleus. Cross-talk between growth factors takes place at the receptors level or at the downstream Smads, for example they may phosphorylate Smads at different sites (Abdollah et al., 1997; Liu et al., 2003). The cross-talk between the two pathways is described in the following section.

4.2.4 Cross-talks of the TGF-β and activin A pathway

TGF-βs and activin A both bind to specific type II receptors, phosphorylates Smad proteins namely Smad2 or Smad 3 (Massagué, 1998; Chen et al., 2006). Activated Smads bind Smad4 in the cytoplasm and are transported into the nucleus to facilitate signalling; in fact, both ligands activate the same Smads (Pangas & Woodruff, 2000; Massagué & Xi, 2012). Thus, it might be crucial to determine whether or not a cross-talk exists between the two pathways in Sertoli cells in order to deepen our understanding of the roles of the two pathways in the testis.

We observed that TGF-β1-induced Smad2 and Smad3 phosphorylation was reduced by activin A when co-stimulated, but this effect of activin A was not observed when cells were treated with TGF-β2. This might be due to the presence and active role of betaglycan in TGF-β2 signalling, as TGF-β2 upregulates betaglycan expression, and betaglycan itself can block activin A signalling (Lewis et al., 2000).

Moreover, we showed for the first time the suppressive effects of activin A on TGF-β1 signalling, which was dose- and time-dependent, as evidenced by Smad2/3 regulation by activin A in cross-talks with TGF-β1.

In conclusion, activin A has a negative effect on TGF-β1 but not on TGF-β2 signalling. The exact mechanism is still unknown and further investigation is needed to understand the role of their cross-talks in testis. To further elucidate cross-talks between the TGF-βs and activin A pathways, we measured the receptor mRNA expression in Sertoli cells.

4.2.5 Effects of TGF-βs and activin A on receptors

TGF-βs and activin A type I receptors are differentially regulated during testis development (Itman et al., 2006). In immature rat testis TGF-β type-I receptor ALK5 is highly expressed (Itman et al., 2011; Miles et al., 2013). Inhibition of ALK4/5/7 or ALK5 at E11.5 prevent testis cord formation (Miles et al., 2013). These studies showed that ALK4/5/7 are important regulators for proper testis formation and development. Betaglycan which is the co-receptor of the TGF-β superfamily is expressed in all somatic cells in the testis and expression increased at birth (Sarraj et al., 2007). In fetal testis, the loss of betaglycan expression results in compromised Leydig cell function and changes in testis cord development at the time of sex determination (Sarraj et al., 2010). Up to date, the regulation of TGF-βs and activin A

receptors by growth factors in testis remain unclear, therefore we investigated the mRNA expression pattern of the receptors after growth factor stimulation.

Our results suggest that activin A increased ALK4 mRNA expression (Fig. 19A), whereas ALK7 mRNA expression was significantly upregulated only by activin A but not by the TGFβs, these findings are similar to Miles et al. (2013), who showed that ALK4/7 are essential for testis development and these are the preferred receptors of activin A signalling (de Kretser et al., 2002). TGF-β1 or TGF-β2 increased ALK5 expression in Sertoli cells, which implies that ALK5 is essential for TGFβ signalling. The expression of the co-receptor betaglycan was increased only by TGF-β2, whereas activin A reduced betaglycan mRNA expression. This downregulation might countereact inhibin binding to betaglycan which blocks activin signalling (Lewis et al., 2000; Namwanje & Brown, 2016). These data suggest again that betaglycan is required only by TGF-β2 for signaling, but might be also important for activin A to counteract inhibin.

4.3 Role of BMPs in shedding of betaglycan and the cross-talks with activin A

BMPs and their receptors (BMPR-1A, -1B and -2) are members of the TGF-β superfamily, and they influence events that affects male fertility by effecting germ and somatic cells behaviour throughout fetal and postnatal life (Itman & Loveland, 2008). An *in vivo* study showed that in the fetus BMP8a and BMP8b regulate primordial germ cells (PGC). Furthermore both BMPs are also highly expressed during adult spermatogenesis (Zhao et al., 1998, Ying & Zhao, 2001, Hu et al., 2004). Whereas *in vitro* juvenile spermatogonal proliferation is enhanced by BMP2 and BMP7 (Puglisi et al., 2004), immature Sertoli cells also respond to BMP2 stimulation and activate signalling (Itman & Loveland, 2008). Mutations in BMP4, BMP7 and BMP8a resulted in either compromised fertility or complete infertility in male mice (Zhao et al., 1998, 2001; Hu et al., 2004). BMPs initiate its signalling cascade by activating specific Smads (Smad1, Smad5 & Smad8) and then Smads activate specific genes responsible for proper development of the testis. Conditional triple knockouts of Smad1, Smad5 & Smad8 resulted in infertile mice and development of metastatic granulosa cell tumours (Pangas et al., 2008). We investigated the role of BMPs in Sertoli cell and germ cell proliferation, migration, and cross-talks with other pathways. We also looked for betaglycan effects on the BMP signalling cascade.

4.3.1 BMPs activated the canonical pathway and cross-talk with activin A in Sertoli cells

Previously, it has been shown that the BMP receptors - BMPRIa, BMPRIb, and BMPRII, as well as BMP2, BMP7 and BMP8a are expressed in mouse testis at all stages (Ciller et al., 2016). Conditional deletion of Smad1 and Smad5 in somatic cells of male leads to metastatic tumour development in mice (Pangas et al., 2008). Despite many studies on Smad proteins, there is so far no direct evidence implicating Smad1/5/8 as an intermediate protein in BMP signalling pathway in Sertoli cells (Itman & Loveland, 2008).

In our experiments, we showed that BMP2, BMP7, and BMP8a activated Smad1/5 in Sertoli cells, and phosphorylation is further increased with dosage. Altogether, these results suggested that BMPs activate the canonical pathway by activating Smad1/5 in Sertoli cells.

Activin A was reported to bind to ALK4 or ALK7 (type 1 receptors) and form a complex with ACVR2A or ACVR2B, which activates Smad2 or Smad3 (Walton et al., 2012). At the same time, activins bind and signal via bone morphogenetic protein receptor type II (BMPR2) and ACVR2 (Rejon et al., 2013). Activin B induces non-canonical Smad1/5/8 signalling via BMP Type I receptors in hepatocytes (Canali et al., 2016).

We investigated whether or not activin A activates Smad1. For the first time, we demonstrated that in Sertoli cells phosphorylation of Smad1 was also initiated by activin A and the level of p-Smad1 was further increased when BMPs were applied simultaneously with activin A. This observation leads us to conclude that activin A activates the non-canonical pathway in Sertoli cells, but the consequence of this cross-talk still remains unclear.

4.3.2 Effect of BMPs on betaglycan shedding

Many signalling pathways are modulated by co-receptors (Kirkbride et al., 2005). Modulating the betaglycan reduces the affinity of BMPs to bind to their receptors (Kirkbride et al., 2008). In breast cancer studies an increase in shedding of betaglycan significantly reduced BMP-mediated Smad1/5/8 phosphorylation, migration, and invasion (Gatza et al., 2014). Betaglycan is also able to inhibit BMP signalling by promoting the binding of inhibin to its cognate receptor BMP type II receptor, as inhibin opposes the actions of BMPs (Wrana et al., 1994; Lewis et al., 2000). Thus, we hypothesized that betaglycan is required also for BMP signalling.

We showed that BMP2 and BMP7 reduced shedding of betaglycan, whereas BMP8a induced shedding of betaglycan in Sertoli cells. Altogether, this shows that betaglycan may serve as a cell surface receptor for BMP2 and BMP7, whereas for BMP8a signalling betaglycan might play an inhibitory role in Sertoli cells. Furthermore, we investigated regulation of MT1-3 MMPs by BMPs in Sertoli cells, because from our previous results we know that MMP14 and MMP16 are important in shedding of betaglycan. After treatment with BMPs in Sertoli cells, we found that BMP2 and BMP7 reduced the abundance of MMP14 and MMP16, whereas BMP8a increased the presence of MMPs. These results confirmed our previous results that BMP2 and BMP7 require betaglycan in contrast to BMP8a.

Further, we investigated the implication of the GAG chains, heparan sulphate and chondroitin sulfate proteoglycan, of betaglycan (Segariul & Seyedin, 1988). These GAG chains modulate different signalling cascade, for example, fibroblast growth factor binding to TβRIII is mediated by glycosaminoglycan modifications (Andres et al., 1992). We found that GAG chains influence TGF-β2 signalling and furthermore, this is comparable to observations showing that BMP has a strong affinity for heparan sulfate chain on betaglycan (Irie et al., 2003). We then hypothesised that modifications of GAG chains might affect the BMP signalling pathway as well. We showed that GAG chain degradation in Sertoli cells influenced the BMP-induced Smad1 signalling. BMP2 increased Smad1 phosphorylation when the heparin III chain was degraded. Even more, when all other GAG chains were digested, BMP2 signalling was abrogated to control levels (Fig 28A). Similarly, BMP7 and BMP8a-induced p-Smad1 levels were decreased, when the GAG chains were degraded by enzymes. These results indicate that BMP binds to the GAG chain to indirectly activate canonical Smad signalling via betaglycan and BMP type-II receptor complex.

Combining all the data indicates that MMP14 and MMP16 are key regulators of shedding of betaglycan and thus can modulate BMP signalling. Further studies are needed to investigate the effect of betaglycan modulation of BMP signalling in testis.

4.3.3 Effects of BMPs on migration and proliferation in testis

BMP-2 induces a rapid rearrangement of actin filaments and cell migration (Gamell et al., 2008). During germ cell migration BMPs and their receptors are known to be expressed (Dudley et al., 2007). In testis development BMPs play a major role and are highly expressed during spermatogenesis (Itman & Loveland, 2008). Specifically, blocking of BMP8a resulted in a 50% reduction in germ cell numbers, showing the important role of BMP8a in the

maintenance of spermatogenesis (Zhao et al., 1998). This finding is especially interesting as it suggests that BMP2 might induce migration of germ cells, also through the blood-testis barrier to undergo spermatogenesis (Itman & Loveland, 2008).

In our study we could show that after treatment with BMP2 only germ cells migrated, but not Sertoli cells. Whereas BMP7 and BMP8a had no effect on germ cell migration, in contrast, they reduced Sertoli cell migration to control levels. This behaviour might be associated with the BTB formed by Sertoli cells (Mruk & Cheng, 2015) to differentiate the luman compartment from the interstitial compartment, where germ cells migrate through the BTB to undergo further differentiation in the luman (Smith & Braun, 2012). Sperm production is dependent on an intact BTB structure (Yan & Cheng, 2005), which is formed primarily by tight junctions between Sertoli cells (Mital et al., 2011). Previously published data showed that TGF-β2 and activin A downregulated Sertoli cell tight junctions (Yan et al., 2008; Nicholls et al., 2012).

We investigated the BTB integrity by tracer diffusion and measured FD4 diffusion through a monolayer of Sertoli cells. Surprisingly we found that BMP2 induces more FD4 diffusion through the Sertoli cell monolayer. These results suggest that BMP2 might induce migration of germ cells and at the same time it also impairs the integrity of the barrier. TGF-β isoforms showed different impacts on the BTB, as published earlier, they downregulate the BTB. Especifically TGF-β2/β3 open the BTB (Lui et al., 2003; Yan et al., 2008), but we observed a different behavior with TGF-β1, which reduced FD4 diffusion through the BTB. This shows that TGF-β1 can be a possible candidate to study further the BTB barrier integrity. We also investigated claudin-11 expression (data not shown). Claudin-11 is a tight junction protein characterised by a high expression in the Sertoli cell tight junctions (Dietze et al., 2015). After induction with BMP2 claudin-11 expression was also downregulated. This result confirms the findings that BMP2 might play an important role in regulating the BTB by disturbing the Sertoli tight junctions, which allows the germ cells to migrate through it.

Furthermore our results agree with previously published data by Puglisi et al. (2004) and Ross et al. (2007), who showed that in immature mouse testis spermatogonia and Sertoli cell proliferation is mediated by BMP2 and BMP8a. Moreover, BMP2 & 8a expression were higher in spermatogenesis and placental development (Zhao et al., 1998). Similar results were observed after treating Sertoli and germ cells with BMP2 and BMP8a, We found a dose-dependent increased Sertoli and germ cell proliferation, whereas BMP7 induced only germ

cell proliferation, but had no effect on Sertoli cell proliferation. These data agree with Ross et al. (2007) who stated that germ cell proliferation is mediated by BMP7. These data indicated that BMPs induce germ cell proliferation.

Future work will elucidate the coordinate roles of BMPs and other molecular signals in the regulation of germ cell development in testis.

4.4 FSH induced Sertoli cell proliferation

The testicular size, germ cell numbers and spermatozoa output per testis is determined by the number of Sertoli cells (Orth et al., 1988). In the testis, the factors required for germ cell maturation into spermatozoa are regulated mainly by Sertoli cells by transducing signals from FSH (Walker & Cheng, 2005). FSH interacts with many pathways to maintain the proper balance of the cell numbers in testis (Walker & Cheng, 2005). FSH acts indirectly to increase androgen production by the Leydig cells and directly on the Sertoli cells to increase germ cell numbers. The concentration of FSH varies throughout testis development (Allan et al., 2004; Atanassova et al., 2005; O'Shaughnessy et al., 2010). FSH receptors knockout (FORKO) mice showed a decline in spermatogenesis both qualitatively and quantitatively (Krishnamurthy et al., 2000). In mice strong activation of FSHR triggers spermatogenesis which was completely independent of testosterone (Oduwole et al., 2018). Our results are in agreement with the previous findings, FSH induced Sertoli cell proliferation after 24 h and the effect of the increase in number was further potentiated at 48 h (Allan et al., 2004).

Furthermore, we investigated secretion of TGF-β1 and TGF-β2. Earlier data suggested that TGF-β isoforms are expressed in Sertoli cells in rat testis (Mullaney & Skinner, 1993). Specifically, Sertoli cells secrete bioactive TGF-β1 in pig (Avallet et al., 1987). During puberty and spermatogenesis induction, FSH reduces TGF-β2 expression (Teerds & Dorrington, 1993). We found that TGF-β1 and TGF-β2 secretion was downregulated in a dose-dependent manner after FSH induction of Sertoli cells, similar to Konrad et al. (2000), who showed that FSH reduced TGF-β2 secretion in Sertoli cells. These data allow us to conclude that secretion of TGF-β1 and TGF-β2 by Sertoli cells might affect this proliferation, ensuring a proper development of the testis.

4.5 Schematic diagram of betaglycan interactions with different pathways

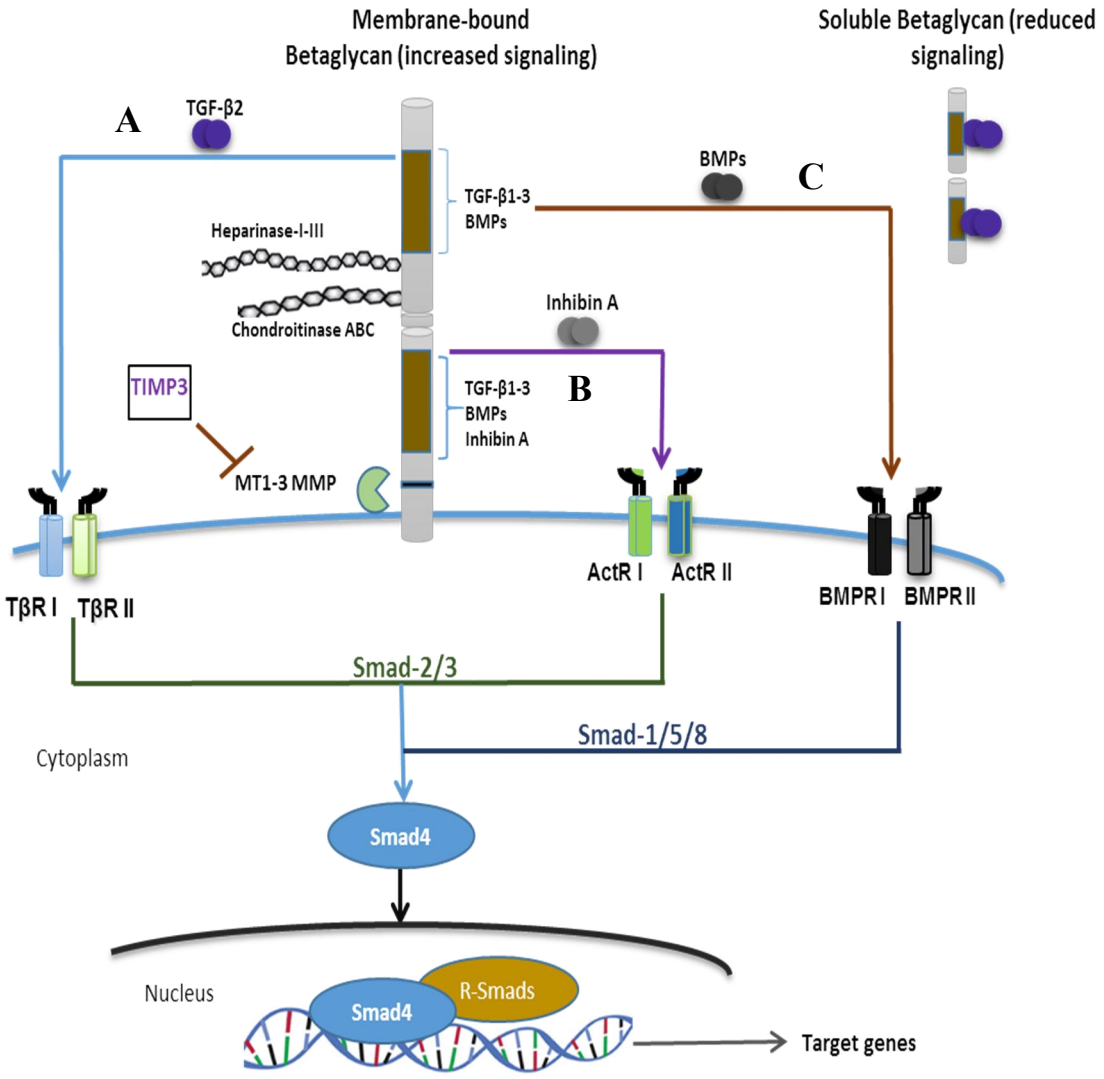


Figure 38. Scheme for the different modes of signalling by TGF-βs, activin A and BMPs with the contribution by betaglycan. Normally TGF-βs, activin A and BMPs binding to specific type-I receptor complex results in phosphorylation of Smad2/3 in case of TGF-βs and activin A and of Smad1/5/8 in BMPs. However, betaglycan can modulate this signalling. **(A)** Betaglycan binds TGF-β2 and presents the ligand to type-II receptors, and thus activates Smad2/3 signalling. In the absence of betaglycan, TGF-β2 has a lower affinity to bind to its receptors. **(B)** Betaglycan also binds inhibin and enhances inhibin binding to activin A type-II receptors resulting in blocking activin A signalling, which makes it an antagonist of activin A. **(C)** Betaglycan also binds BMPs and activates the downstream targets Smad1/5/8. A specific GAG chain (Heparin I&III, Chondroitin ABC) also has an influence in TGF-βs and BMP signalling. Shedding of betaglycan plays a key role in the testis, as evidenced by the

presence of TIMP3 - an inhibitor of MMPs and regulates betaglycan shedding. The interplay between BMP, inhibin, and TGF signalling suggests a broader role for betaglycan in orchestrating TGF-superfamily signalling.

5 SUMMARY

The transforming growth factor-beta (TGF-β) superfamily ligands regulate growth, differentiation, adhesion and apoptosis in various cell types and play a major role in testis development and spermatogenesis. Betaglycan (type III TGF-β receptor) is an important co-receptor and modulator of TGF-β superfamily ligands that acts through binding of distinct BMPs, inhibin, and TGF-β1-3. It is highly expressed in testis and has a considerable impact on Leydig cell and seminiferous cord development. The regulation of TGF-βs signalling by betaglycan is both positive and negative. Although the membrane-bound form of betaglycan increases binding of TGF-βs to their receptors, the soluble form reduces actions of TGF-βs. BMPs, inhibin and TGF-β2 share betaglycan as a co-receptor and TGF-β2 isoform is particularly dependent on betaglycan for signalling. However, little is known about interactions between these factors under physiological conditions. The betaglycan GAG chains can also influence TGF-βs, activin A and BMPs ability to bind to their receptors. TGF-βs, activins and BMPs are expressed significantly during testis development and thus might be involved in regulating functions of somatic and germ cells. The aim of this study was to investigate the effects of TGF-β family signalling and possible cross-talks with other TGF-β family pathways and their outcomes. Our study aims to elucidate the role of betaglycan conversion into its cleaved soluble form for TGF-β superfamily signalling in a key testis target cell type, the Sertoli cell.

We identified several levels in the regulation of signalling of activin A, TGF-βs and BMPs. The first level starts at the receptors; the level of Smad phosphorylation was strongest with activin A, modest with TGF-βs and lowest with BMPs, when applied simultaneously. Of note, TGF-β2 signalling was not influenced by activin A, because we revealed a pivotal role of betaglycan for TGF-β2. In line with these results, we found that TGF-β1/β2, BMP2 and BMP7 reduced shedding of betaglycan, which in contrast was increased by activin A.

The second level of regulation was identified while analyzing regulation of betaglycan shedding. Betaglycan shedding was attenuated by TIMP3 which was positively regulated by TGF-β2. Of note, TIMP3-reduced betaglycan shedding and increased phosphorylation of Smad3 indirectly via the type-I receptor (ALK5) and thus again highlighting the importance of betaglycan for the TGF-βs, especially TGF-β2. We also found the involvement of MMPs14-16 in shedding of betaglycan and found a decreased MMPs14-16 expression after treatment of Sertoli cells with TGF-β2, BMP2 or BMP7. In contrast to an increase with

activin A. This results in increased betaglycan shedding and consequently reduced inhibin and thus increased activin A signalling. A third level of regulation was revealed by the analysis of GAG chain, which influenced the binding ability of BMPs and TGF-βs, especially of TGF-β2 to betaglycan; in the absence of GAG chains Smads signalling was reduced.

From these results, we propose that betaglycan shedding is differentially regulated among the TGF-β family and leads to different physiological outcomes. Furthermore, we demonstrated for the first time the involvement of GAG chains in binding the ligands and activating the signalling cascade in Sertoli cells. In addition, we showed the importance of FSH in regulating the proliferation of Sertoli cells and germ cells. These findings might provide new insights into the roles of TGF-β family members in testis function. However, more studies are needed in TGF-β signalling pathways in testicular cells to elucidate the role of the cross-talks on the function of somatic and germ cells. Our study paves the way to further understand the involvement of betaglycan in TGF-β superfamily signalling in Sertoli and germ cells. We hypothesise that the observed betaglycan shedding plays a major role in testicular TGF-β2 signalling, and that further research to understand its physiological implications is warranted.

6 ZUSAMMENFASSUNG

Die transforming growth factor-beta (TGF-β) Superfamilie Liganden regulieren Wachstum, Differenzierung, Adhäsion und Apoptose in verschiedenen Zelltypen und spielen eine wichtige Rolle bei der Entwicklung des Hodens und der Spermatogenese. Betaglycan (Typ III TGF-β-Rezeptor) ist ein wichtiger Co-Rezeptor und Modulator der Liganden der TGF-β Superfamilie, der durch die Bindung spezifischer BMPs, Inhibin und TGF-β1- β3 agiert. Betaglycan wird im Hoden stark exprimiert und hat einen erheblichen Einfluss auf die Entwicklung der Leydig Zellen und der Tubuli seminiferi. Die Regulierung der TGF-β Signale durch Betaglycan ist sowohl positiv als auch negativ. Obwohl die membrangebundene Form von Betaglycan die Bindung der TGF-βs an ihre Rezeptoren verstärkt, reduziert die lösliche Form die Aktivität der TGF-βs. Die Konversion von membrangebundenem zu löslichem Betaglycan wird auch als Shedding bezeichnet. In jüngerer Zeit wurde gezeigt, dass Betaglycan zusätzlich als Co-Rezeptor für Inhibin fungiert. Obwohl BMPs, Inhibin und TGF-β2 Betaglycan als Co-Rezeptor teilen, ist über potentielle Interaktionen zwischen diesen Faktoren unter physiologischen Bedingungen wenig bekannt. Die Betaglycan GAG Ketten können ebenfalls die Fähigkeit der TGF-βs, Aktivin A und BMPs beeinflussen, ihre Rezeptoren zu binden. Die TGF-β2 Isoform ist besonders abhängig von Betaglycan für die Signalvermittlung. TGF-βs, Aktivine und BMPs werden während der Hoden Entwicklung besonders stark exprimiert. Damit könnte die TGF-β Superfamilie an der Regulierung der Funktionen von somatischen Zellen und Keimzellen beteiligt sein. Ziel dieser Studie war es, die Auswirkungen der durch TGF-βs ausgelösten Signale und potentielle cross-talks mit anderen Signalkaskaden und deren Ergebnissen zu untersuchen. Unsere Studie zielt darauf ab, die Rolle der Betaglycan Konvertierung in die abgespaltene lösliche Form für die Vermittlung der Signale der TGF-β Superfamilien in Sertoli-Zellen als wichtigen Hoden Zelltyp, aufzuklären.

Wir haben mehrere Ebenen in der Regulation der Signale von Aktivin A, TGF-βs und BMPs identifiziert. Die erste Ebene beginnt bei den Rezeptoren; unter vergleichbaren Bedingungen war der Smad-Phosphorylierungsgrad bei Aktivin A am stärksten, bei den TGF-βs mäßig und bei den BMPs am niedrigsten. Bemerkenswert ist, dass die TGF-β2-Signale nicht durch Aktivin A beeinflusst wurden, da eine entscheidende Rolle von Betaglycan für TGF-β2 nachgewiesen werden konnte. In Übereinstimmung mit diesen Ergebnissen fanden wir, dass TGF-β1 / β2, BMP2 und BMP7 das Shedding von Betaglycan verminderten, während Aktivin A es erhöhte.

Die zweite Ebene der Regulierung wurde durch die Regulation des Betaglycan-shedding identifiziert. Das Betaglycan-shedding wurde durch TIMP3 abgeschwächt, das wiederum durch TGF-β2 positiv reguliert wurde. Bemerkenswert ist, dass TIMP3 das Shedding von Betaglycan reduziert und die Phosphorylierung von Smad3 indirekt über den Typ-I-Rezeptor (ALK5) erhöht und damit erneut die Bedeutung von Betaglycan für die TGF-βs, insbesondere TGF-β2, unterstreicht. Wir fanden auch die Beteiligung der MMP-14-16 an der Freisetzung von Betaglycan und fanden nach Behandlung von Sertoli-Zellen mit TGF-β2, BMP2 oder BMP7 eine verminderte MMP-14-16 Expression. Im Gegensatz dazu führt Aktivin A zu vermehrter MMP-14-16 Expression. Dies führt zu einem verstärkten Betaglycan-shedding, gefolgt von verringerten Inhibin-Signalen und damit verstärkten Aktivin A-Signalen. Eine dritte Regulationsebene wurde durch die Analyse der GAG-Kette gezeigt, die die Bindungsfähigkeit von BMPs und TGF-βs, insbesondere von TGF-β2, an Betaglycan beeinflusste. In Abwesenheit von GAG-Ketten wurde die Phosphorylierung der Smad-Proteine reduziert.

Anhand dieser Ergebnisse schlagen wir vor, dass Betaglycan-shedding die TGF-β Familie differenziert reguliert und zu unterschiedlichen physiologischen Ergebnissen führen kann. Darüber hinaus haben wir zum ersten Mal die Beteiligung von GAG-Ketten an der Bindung der Liganden und der Aktivierung der TGF-β Signalkaskade in Sertoli-Zellen demonstriert. Weiterhin haben wir gezeigt, wie wichtig Hormone bei der Regulierung der Proliferation von Sertoli-Zellen und Keimzellen sind. Diese Erkenntnisse könnten neue Einblicke in die Rolle der TGF-β Familienmitglieder in die Hoden Funktion geben. Weitere Studien zu TGF-β Signalwegen in Hoden-Zellen sind nötig, um die Rolle der identifizierten cross-talks bei der Funktion von somatischen Zellen und Keimzellen aufzuklären. Unsere Studie ebnet den Weg, um die Beteiligung von Betaglycan an der Regulation der Signale der TGF-β Superfamilie in Sertoli- und Keimzellen weiter zu verstehen. Wir vermuten, dass das beobachtete Betaglycan-shedding eine wichtige Rolle bei den TGF-β2 Signalen im Hoden spielt und dass weitere Forschungen, um die physiologischen Implikationen zu verstehen, nötig sind.

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9 OWN PUBLICATIONS

- ***"TIMP3 influences betaglycan (TβRIII) shedding and TGF-β signalling in rat 93RS2 Sertoli cells"***,Pradeep Kumar Kudipudi ,Sebastian Galuska, Raimund Dietze, Kate Loveland, Lutz Konrad (Manuscript finished).
- ***'Signalling of TGF-betas in tubule cultures of adult rat testis'*** Kai-Hui Chan[1], Sebastian Galuska[2], Pradeep Kumar Kudipudi[1], Mohammad Assad Riaz[1], Kate Loveland[3], Lutz Konrad[1]*(American Journal of Translational Research).
- ***'Signalling via the interleukin-10/signal transducer and activator of transcription 3 pathway establishes the immunosuppressed phenotype of testicular macrophage*** Pradeep Kudipudi[1], Ming Wang[1], Nour Nicolas[1], Vera Michel[1], Lutz Konrad[2], Monika Fijak[1], Andreas Meinhardt[1], and Sudhanshu Bhushan[1*](Manuscript finished) in Reproduction.
- ***'Neoplasia in human testis: functional polarization of macrophage and dendritic cells'*** Pueschl D, Kudipudi PK, Bhushan S, Hedger MP, Bergmann M, Schuppe H C, Loveland KL[*](Manuscript finished)**.**

Conference Abstracts

- 10th Society for the study of reproduction, Washington DC, USA. (13-16 July. 2017) [Poster Presentation]
- The 9th Annual GGL annual meeting; Giessen, Germany. (21-22 Sep. 2017) [Poster presentation Presentation]
- 19th European Testis Workshop Saint Malo, France. (11-15 Jun. 2016) [Poster presentation Presentation]
- IRTG Milestone meeting, Melbourne, Australia (23-28 Nov. 2015). [Oral Presentation]
- Abstract submitted to EMDS (European macrophage dendritic cell society) annual conference 2014 held in Vienna on topic "role of IL-10 in dampening the inflammatory response of testicular macrophage ".
- 7th International Congress on Male reproduction; Hyderabad, India (9-13 Nov. 2013). [Oral Presentation]

10 EHRENWÖRTLICHE ERKLÄRUNG

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Giessen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Giessen, den _____ Pradeep Kumar Kudipudi