Involvement of Activin A in the Development of Chronic Testicular Inflammation and Fibrosis

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1. INTRODUCTION

1.1. The male reproductive tract

The male reproductive tract of both humans and rodents comprises the two testes, which are connected by the rete testis and the efferent ducts to the epididymis, where sperm mature and acquire their motility and capacity for fertilisation of the egg. Together with the epididymis, the testes are located in the scrotum surrounded by the tunica vaginalis. Secretions of accessory glands, including the seminal vesicles, prostate gland and bulbo-urethral glands, during ejaculation are needed for the completion of the semen, as sperm are transported from the epididymis via the vas deferens to the urethra where they exit the body through the penis (Hendry, 1983; Hedger and Hales, 2006).

1.1.1. Anatomy, histology and function of the testis

The testis as the male gonad has to fulfil two major functions: the generation of sperm and the production of androgens, especially testosterone. The testis is compartmentalised histologically and functionally, with spermatogenesis taking place in the seminiferous tubules and testosterone produced by the Leydig cells in the interstitial space, which is interspersed between the tubules **(Fig. 1)**. The highly coiled seminiferous tubules are surrounded by a lamina propria, which consists of a basement membrane, collagen and myoid peritubular cells (PTC). The PTC provide structural support and are able to transport the immotile spermatozoa by generating peristaltic waves through contraction and relaxation along the tubules, via the rete testis, into the epididymis (Fijak and Meinhardt, 2006; de Kretser *et al.*, 2016). In healthy men, several layers of PTC and extracellular matrix (ECM) proteins build the wall of the seminiferous tubules, while there is only one layer of PTC in mice (Mayerhofer, 2013).

The tubules are lined with a seminiferous epithelium, which consists of two types of cells: spermatogenic (or germ) cells and Sertoli cells (SC) **(Fig. 1)**. The main function of the columnar SC is to provide structural support, nutrients and growth factors to the developing germ cells. Together with the PTC, SC synthesise components of the basement membrane upon which the precursors of sperm cells, the mitotically dividing spermatogonia, reside. In adult vertebrates, the progressively maturing spermatogonia transition as spermatocytes through meiosis and develop into haploid spermatozoa. During this transition they progress from the tubule base towards the tubule lumen while they are continuously surrounded by cytoplasmic protrusions of the SC.

The interstitial space consists of Leydig cells, fibroblasts and different types of immune cells, including $CD4^+$ and $CD8^+$ T cells, macrophages and a few scattered mast cells, which contribute to the testicular defence against invading pathogens, and also help to maintain testicular functions under physiological conditions (Loveland *et al.*, 2017; Hedger, 1997; Heffner and Schust, 2010).

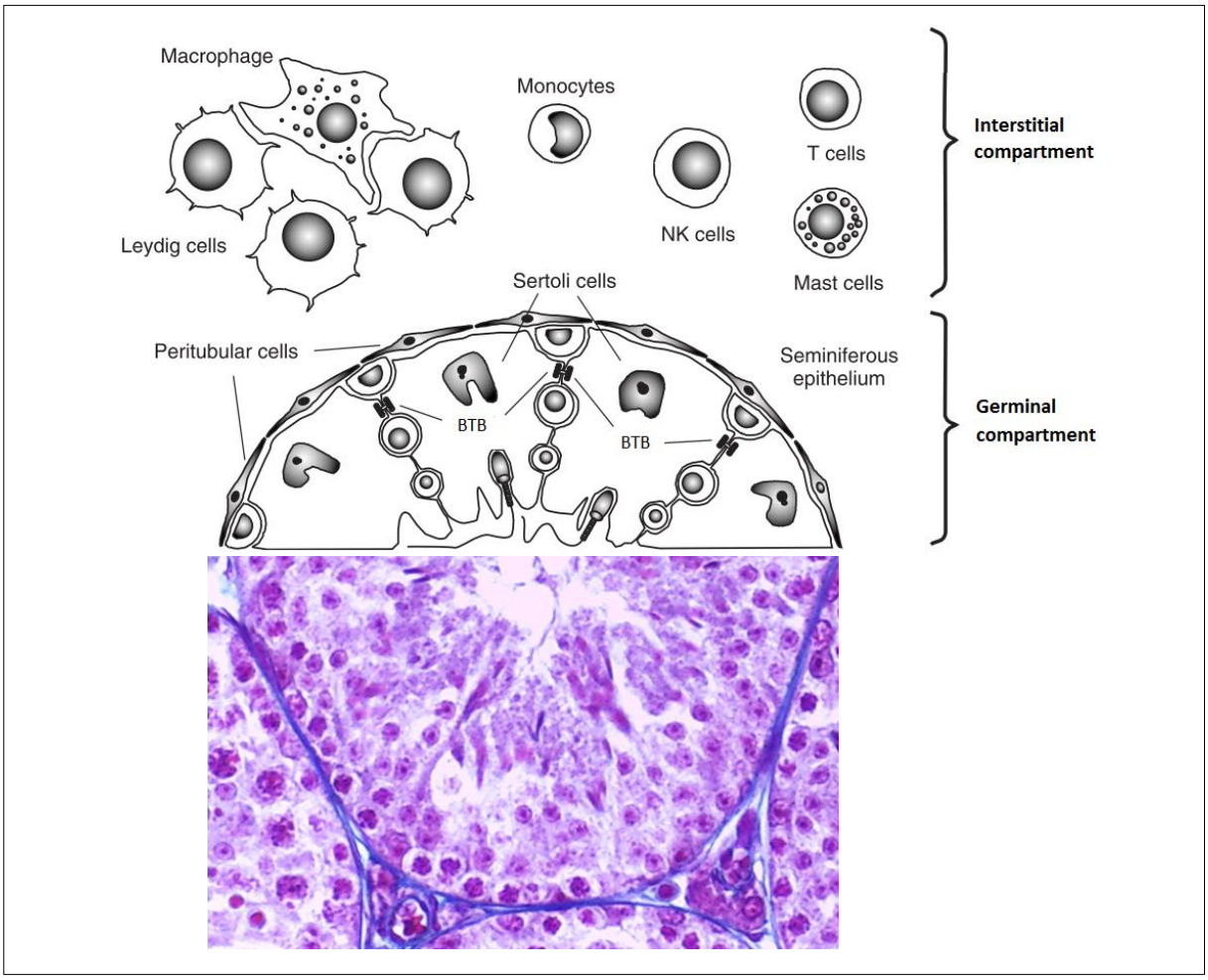

Figure 1: Schematic illustration and azan stained mouse testis section showing the interstitial space and seminiferous epithelium with spermatogenic progression and key cell types. BTB: blood-testis barrier. Illustration adapted from (Hedger and Hales, 2006).

1.1.2. Functions of the Peritubular cells (PTC)

The PTC are smooth muscle-like cells with a spindle-shaped morphology located at the periphery of the seminiferous tubules. In addition to co-operating with the SC to produce the ECM components of the basement membrane, the rhythmic contraction and relaxation of the PTC are required for the successful transport of the immotile spermatozoa along the tubule lumen. These are few of the presumably numerous functions of PTC, which make them crucially important for the maintenance of normal spermatogenesis and hence, male fertility (Welter *et al.*, 2013; Welsh *et al.*, 2009). Alpha-smooth muscle actin (αSMA) is one of the contractile markers expressed by the PTC (Schell *et al.*, 2010).

In patients with impaired spermatogenesis, such as Sertoli cell only syndrome (SCO) and mixed atrophy, infertility is commonly associated with increased deposition of ECM proteins, by this giving evidence for a fibrotic process in the wall of the seminiferous tubules and implying striking changes in the PTC layers. Investigations on PTC in infertility patients also revealed changes in nuclear morphology, size of the cells, reduced number of actin-positive cells as well as an increase in cells positive for vimentin associated with a progression of tubular sclerosis (Welter *et al.*, 2013).

1.1.3. Spermatogenesis

Mammalian spermatogenesis is a complex process which can be divided into three main phases: mitotic proliferation increases the number of cells; meiotic division produces genetic diversity; and spermiogenesis, the maturation of the germ cells involving extensive cellular remodelling for facilitation of the sperm transit and the penetration of the egg.

Until puberty, the initial diploid progenitor cells (spermatogonial stem cells) remain relatively dormant. After their activation at puberty, they on the one hand maintain their reservoir function through rounds of mitotic divisions, and on the other hand differentiate into spermatogonia, which themselves undergo a limited number of mitotic divisions before they form the primary spermatocytes. The first meiotic division results in cells which then are called secondary spermatocytes. After a second meiotic division the now haploid round spermatids are formed. The last step of spermatogenesis, or spermiogenesis, comprises the transformation of the early, round spermatid into a sperm cell that develops the microtubule-based tail and a head containing the highly compacted chromatin and anterior acrosome, a cap-like sac, which releases lytic enzymes during the fertilisation of the oocyte. This process also involves the removal of most of the spermatid cytoplasm, which forms the ‘residual body’ (Fijak and Meinhardt, 2006; de Kretser *et al.*, 1998; Heffner and Schust, 2010).

1.2. Regulation of steroidogenesis by the hypothalamic-pituitary-gonadal axis

Both functions of the testis, steroidogenesis and spermatogenesis, are regulated by the hypothalamus, the pituitary and various local factors comprising the hypothalamic-pituitary-gonadal axis **(Fig. 2)**. This axis consists of complex interactions of endocrine factors, i.e. the circulating gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are produced by the anterior pituitary after stimulation with gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus. The regulation of the axis also comprises paracrine factors produced in the testis, including androgens, growth factors, and cytokines such as activin and inhibin (Hedger and Hales, 2006; Weinbauer and Wessels, 1999). Androgens, especially testosterone, which is the most important steroid hormone produced by the testis, play a significant role in the development of the male phenotype and sexual behaviour, and regulate the somatic cells of the testis, in particular the SC and PTC (Wilson, 1999). In the male reproductive tract, LH regulates testosterone synthesis by the Leydig cells, whereas FSH initiates, and in combination with testosterone, maintains spermatogenesis via action on the SC. Hence, both LH/testosterone and FSH are needed to ensure quantitatively normal production of germ cells (Ramaswamy and Weinbauer, 2014; Dufau *et al.*, 1984).

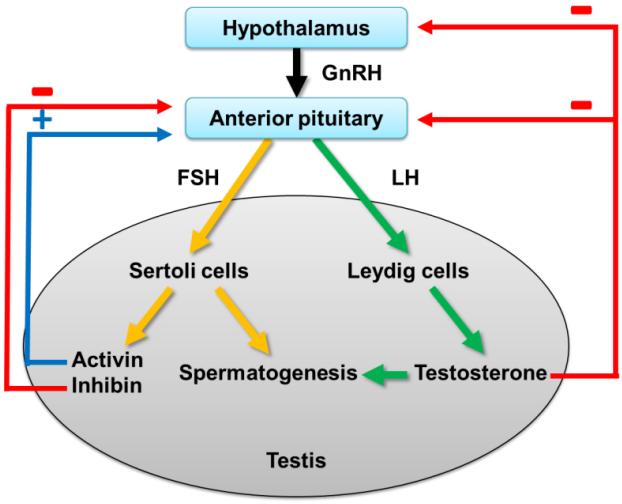

Figure 2: Schematic diagram of components and regulation of the hypothalamic-pituitary-testis axis. GnRH (gonadotropin releasing hormone) released from the hypothalamus stimulates the synthesis of the gonadotropins, FSH (follicle stimulating hormone) and LH (luteinizing hormone). LH stimulates testosterone synthesis by the Leydig cells. FSH, in combination with testosterone, regulates spermatogenesis via SC. Testosterone feeds back on the hypothalamus and the anterior pituitary thereby inhibiting FSH and LH production. Stimulation with activin leads to the production of FSH by the anterior pituitary, whereas inhibin inhibits its secretion. Picture adapted from http://slideplayer.com/slide/6994384/ on 03.04.2018.

Multiple feedback loops for FSH exists between the testis and the brain. One regulatory system comprises testosterone feeding back on both gonadotrophic hormones produced by the pituitary, and the other comprises inhibin, activin and follistatin. While inhibin suppresses FSH production by the pituitary and activin stimulates FSH release, follistatin antagonises actions of activin, which in turn suppresses FSH production. Receptors for FSH, LH and androgens are present in the testis, where they are confined to somatic cells (Gupta, 2006).

Gonadal failure results in increased LH levels, because of loss of the negative feedback of androgens. Decreased levels of sex steroids as well as the loss of inhibin, which is known to inhibit FSH production, following gonadal damage also result in increased FSH levels. Thus, primary gonadal failure from any cause results in decrease of sex steroid levels and elevation of serum gonadotropin levels (Klein, 2003).

1.3. The immune privilege of the testis

Together with the pregnant uterus, eye and brain, the mammalian testis is one of a small number of immune-privileged sites. Spermatogenesis, and hence emergence of large numbers of novel autoantigens, occurs first within the post pubertal period, long after establishment of immune self-tolerance during foetal and neonatal development. Hence, protection of the developing spermatogenic cells from the immune system throughout adult life is of critical importance, and so the testis has established several mechanisms to ensure this protection (Hedger, 2012).

An important mechanism for maintenance of the immune-privileged status is the formation of highly specialised tight junctions (zonula occludens) between adjacent SC at the onset of meiosis at puberty. This so called “blood-testis barrier” (BTB) **(Fig. 1)** divides the seminiferous epithelium into a basal and a luminal compartment, and protects post-meiotic germ cells from the immune cells that are present in the interstitial space, which otherwise would recognise these cells as foreign (Loveland *et al.*, 2017). Hence, the spermatogonia, leptotene, and zygotene spermatocytes are located in the basement compartment while the maturating germs cells, including meiotic pachytene and secondary spermatocytes, haploid spermatids and spermatozoa are protected in the luminal compartment. However, although cells present in the basal compartment also express novel antigens, they are still protected from the immune response, pointing out the existence of other mechanisms for maintaining immune privilege.

Besides this physical separation, immune privilege is also substantially controlled by the local immunosuppressive milieu and systemic immune tolerance (Chen *et al.*, 2016; Heffner and Schust, 2010).

Testicular cells produce a great number of anti-inflammatory cytokines, which generate a local immunosuppressive milieu. Several immunoregulatory factors are abundantly secreted by the SC, such as transforming growth factor-β (TGF-β) and activin A, which inhibit the expression of pro-inflammatory cytokines in dendritic cells and macrophages. Other SC-produced factors include galectin-1 and indolamine 2,3-dioxygenase (IDO). Furthermore, interleukin 10 (IL-10) is produced by testicular macrophages to prevent T cell activation (Chen *et al.*, 2016; Kaur *et al.*, 2014; Phillips *et al.*, 2009; Watanabe *et al.*, 2005). Testosterone produced by Leydig cells also was suggested to inhibit the systemic immune responses to autoantigens, and shown to act on SC to maintain immune privilege (Chen *et al.*, 2016; Meng *et al.*, 2011).

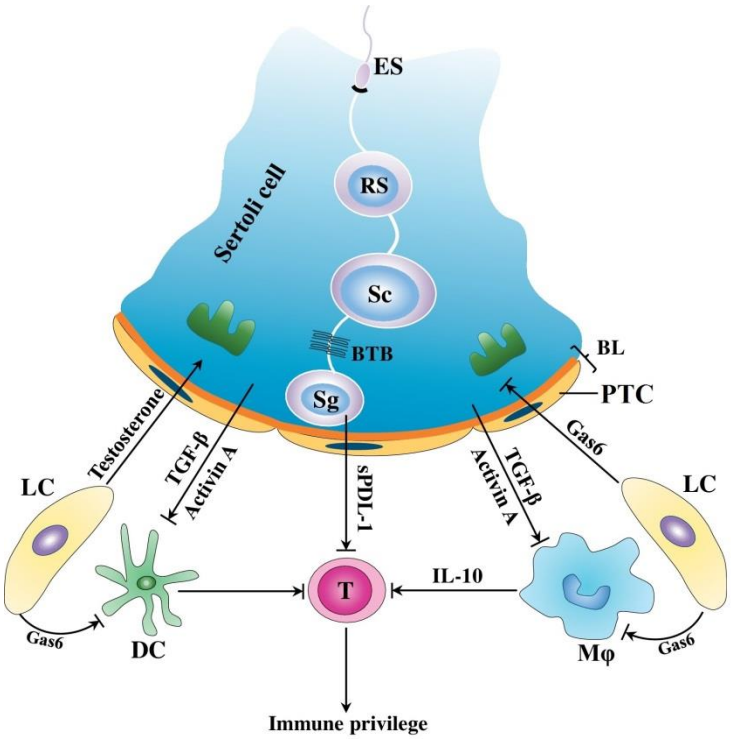

Figure 3: Schematic summary of local mechanisms underlying immune privilege of the testis. Two adjacent Sertoli cells form the blood–testis barrier (BTB) near the basal lamina (BL). The BTB sequesters the autoantigens of the late stage of germ cells (elongated spermatid [ES], round spermatid [RS], spermatocyte [Sc]) within the adluminal compartment from immune cells. Dendritic cells (DC) exhibit immature phenotype within the testis and suppress the activation of T lymphocytes (T). Macrophages (Mφ) produce IL-10 to inhibit activation of T lymphocytes. TGF-β and activin A, secreted by SC, inhibit the expression of inflammatory cytokines in Mφ and DC. Leydig cells (LC) produce testosterone, which regulates immune privilege by acting on Sertoli cells. Growth arrest-specific gene 6 (Gas6), also produced by LC, inhibits innate immune responses in DC, SC and Mφ. Soluble programmed death ligand-1 (sPDL-1) is released by spermatogonia (Sg) and is able to induce T lymphocyte apoptosis. PTC: peritubular cell. Picture adapted from (Chen *et al.*, 2016).

However, various other mechanisms were shown to contribute to the immune privilege of the testis. Tolerance of peripheral T cells is one of these mechanisms and different pathways have been revealed by which immune privilege can lead to T-cell tolerance: clonal deletion, clonal anergy, immune deviation, and T-cell suppression (Streilein *et al.*, 1997). It was demonstrated that some meiotic germ cell antigens are discarded by spermatids during spermiation and egress from seminiferous tubules with an intact BTB into the interstitial space. These antigens are able to induce systemic tolerance by maintaining antigen-specific Treg cells in peripheral lymphoid organs (Tung *et al.*, 2017).

A summary of some local mechanisms underlying the testicular immune privilege is shown in **Fig. 3**.

1.4. The immune system

The immune system has the ability to destroy a broad range of pathogens and to clear a variety of toxins. Its main elements comprise leukocytes, antibodies, the complement system and the lymphatic system, which consists of lymphatic organs such as tonsils, thymus, spleen, bone marrow and lymph nodes. Broadly defined, the immune system includes the innate and the adaptive (acquired) immune responses.

1.4.1. The components of the immune system

The cells of the immune system are called leukocytes. They originate from haematopoietic stem cells in the bone marrow. Initially, these stem cells give rise to stem cells of more limited potential, the progenitors of the two main categories of leukocytes: myeloid cells and lymphoid cells. The myeloid progenitor is the precursor of the cells of the innate immune system, such as granulocytes, macrophages, dendritic cells and mast cells. Dendritic cells are specialised to take up antigens, process them, and display them for recognition by T lymphocytes (T cells) in a process called antigen presentation. Macrophages, the mature form of monocytes, are one of three main phagocytic cell types of the immune system. They are widely distributed in all body tissues, and are also able to present antigens to the T lymphocytes (Janeway *et al.*, 2005).

The common lymphoid progenitor gives rise to the lymphocytes. There are three major types of lymphocytes: (i) B lymphocytes (B cells), which differentiate after activation into antibody secreting plasma cells; (ii) T lymphocytes, which can differentiate on activation into different classes; and (iii) the natural killer cells. $CD8^+$ T cells, also termed cytotoxic T cells, principally kill cells infected with viruses.

$CD4^+$ T cells are commonly further divided into regulatory T (Treg) cells and T helper cells. T helper cells ($CD4^+$) activate other immune cells, such as B cells and macrophages, while Treg cells regulate and suppress other cells in the immune system in response to self and foreign antigens, and help prevent autoimmune disease (Janeway *et al.*, 2005).

1.4.2. The principles of innate and adaptive immunity

The components of the innate immune system include physical barriers, cells, soluble proteins and bioactive small molecules, which are either constitutively present in biological fluids (such as the complement proteins) or are released from cells as they are activated (including cytokines, chemokines, reactive free radical species), as well as membrane bound receptors and cytoplasmic proteins that bind common molecular patterns, expressed on the surfaces of invading pathogens. The macrophages and neutrophils of the innate immune system provide a first line of defence against a wide range of infectious organisms as they are immediately available without requiring prior exposure to pathogens (Chaplin, 2010).

In contrast, the T and B lymphocytes, as part of the adaptive immune system, have evolved to provide a more versatile defence through targeting a particular pathogen antigen. They are involved in the later stages of inflammatory responses since they require the activation by an antigen presenting cell carrying a specific antigen, which has drained from the site of infection via the afferent lymphatics to the lymph nodes, where the activation of T and B lymphocytes takes place (Janeway *et al.*, 2005).

As macrophages and dendritic cells, which have phagocytosed pathogens and become activated, are required for the antigen presentation and the production of cytokines for the activation of T lymphocytes, cells of the innate immune system play a crucial role in the initiation and direction of adaptive immune responses (Chaplin, 2010).

The adaptive immune response is primarily based on antigen-specific receptors expressed on the surfaces of lymphocytes. These receptors, unlike the germ-line-encoded recognition molecules of the innate immune response, are encoded by genes that are assembled by somatic rearrangement of germ-line gene elements to form intact T cell receptor and immunoglobulin genes. This process permits the formation of a vast number of different antigen receptors, each with a unique specificity for a particular target antigen. Once activated, a single lymphoid progenitor cell gives rise to a large number of identical effector cells by a process called clonal expansion (Chaplin, 2010).

1.4.3. Autoimmunity

Immune responses to self-antigens are called autoimmunity. They can lead to autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus or multiple sclerosis, which are characterised by the formation of autoreactive T helper cells, autoantibodies and tissue damage. Under physiological conditions, a variety of mechanisms generates a state of self-tolerance in which an individual's immune system does not attack the normal tissues of the body (Janeway *et al.*, 2005).

Interestingly, antigens sequestered in immunologically privileged sites are often the targets of autoimmune attacks (Janeway *et al.*, 2005). In a mouse model for multiple sclerosis, called experimental autoimmune encephalomyelitis (EAE), immunisation with myelin basic protein causes substantial infiltration of the brain with antigen-specific T helper cells inducing a local inflammatory response (Constantinescu *et al.*, 2011). This shows that at least some antigens expressed in immunologically privileged sites induce neither immune tolerance nor activation of lymphocytes under normal conditions, but if autoreactive lymphocytes are activated elsewhere, these autoantigens become possible targets for an autoimmune attack. As immune privileged sites can harbour infection, and therefore, the access of effector cells at these sites during infection is necessary, it is not surprising that effector T cells are able to enter such sites. However, accumulation of T cells is only seen when an antigen is recognised in the site, triggering the production of cytokines that alter protective tissue barriers (Janeway *et al.*, 2005).

1.4.4. Inflammation

The word “inflammation” is derived from the Latin *inflammare* and means “to set on fire”. Inflammation is part of the non-specific immune response and hence considered as a mechanism of the innate immunity, in contrast to adaptive immunity, which is pathogen-specific. It is the protective response of the body to any type of harmful stimuli, such as invasive pathogens, chemicals or trauma, and aims to eliminate the initial cause of the injury, remove damaged tissue and finally initiate the repair process. Inflammation in the absence of microorganisms is termed ‘sterile inflammation’. The initial protective response of the body is named acute inflammation. It induces a cascade of signals that leads to the recruitment of inflammatory cells, particularly neutrophils and macrophages. These cells, in turn, phagocytose cell debris and infectious agents and produce pro-inflammatory cytokines and chemokines, including TNF and interleukin-1 (IL-1), which activate lymphocytes and adaptive immune responses (Chen and Nuñez, 2010; Rivas, 2010).

Although inflammation is absolutely important for prevention of tissue destruction and eradication of invasive pathogens, and is a self-limiting process under normal conditions, in some cases the body is unable to remove the offending agent, resulting in prolonged, chronic inflammation. Chronic inflammation commonly includes a persistent activation of macrophages and other local inflammatory cells, as well as the adaptive immune response. This ultimately leads to a progressive shift in the cell types present at the site of inflammation, resulting in tissue destruction, fibroblast proliferation and fibrosis. Chronic inflammation has been implicated in several diseases, such as atherosclerosis, rheumatoid arthritis, and cancer (Ferrero-Miliani *et al.*, 2007; Chen and Nuñez, 2010; Ross, 1999; Coussens and Werb, 2002; Wong and Lord, 2004; Wynn, 2008).

In the human testis, sterile inflammation is often accompanied by impaired spermatogenesis. It is also characterised by increased numbers of mast cells and macrophages that accumulate in the wall of seminiferous tubules, which frequently shows fibrotic remodelling. Testicular mast cells and macrophages secrete vast amounts of TNF, which can target PTC and stimulate their secretion of IL-6 and macrophage chemoattractant protein 1 (MCP-1). This process may promote inflammation and infertility and point to an important role of PTC (Mayer *et al.*, 2016; Fijak *et al.*, 2018).

1.4.5. Resident immune cells of the testis

Resident macrophages constitute the second most numerous cell type, after the Leydig cells, in the interstitial tissue of the normal testis of most species, including human, monkey, mouse and rat. A majority of these macrophages has an anti-inflammatory (or M2) phenotype that displays an immunosuppressive profile, specialised to help maintaining the immune privilege of the testis and protecting the developing germ cells (Niemi *et al.*, 1986; Fijak and Meinhardt, 2006; Suescun *et al.*, 2003). M2 macrophages are usually identified by the co-expression of the markers F4/80 and CD206 (Jaiswal *et al.*, 2014).

Lymphocytes comprise the second largest population of immune cells in the testis and also participate in immunological surveillance. In normal rat and human testes, T lymphocytes but not B lymphocytes can be found. Notably, a minor population of Treg cells resides in the testis, which is believed to contribute to the immune privilege of the testis by inhibiting effector T cell activation (Chen *et al.*, 2016; Fijak *et al.*, 2018). Dendritic cells and granulocytes (mast cells and eosinophils) also reside in the testis under physiological conditions. However, the distribution of granulocytes appears to the strongly species-specific.

While they are absent from the testicular parenchyma and mostly found associated with blood vessels of the testicular capsule in the testes of rat, mouse, bull, cat and dog, they are present throughout the interstitial compartment in human and equine testes.

Testicular dendritic cells are generally immature and hence favour the immune privilege of the testis (Chen *et al.*, 2016; Hedger and Hales, 2006; Anton *et al.*, 1998; Rival *et al.*, 2006a; Rival *et al.*, 2007).

1.4.6. Cytokines, chemokines and modulators in the testis

1.4.6.1. Tumour necrosis factor (TNF)

TNF is a multifunctional cytokine, which modulates inflammation, immunoregulatory responses and apoptosis (Fiers, 1991; Beutler, 1995; Baker and Reddy, 1998). Secreted TNF forms biologically active homotrimers; interestingly, trimerisation of TNF may also occur with other TNF protein family members forming biologically-active membrane-anchored heterotrimers (Lysiak, 2004). TNF is produced by a range of cell types. In mouse testis, TNF mRNA is expressed in pachytene spermatocytes, round and elongated spermatids, and activated macrophages, while TNF production was mainly found in monocytes, macrophages and round spermatids (Xiong and Hales, 1993; De *et al.*, 1993; Moore and Hutson, 1994).

TNF secretion by testicular T lymphocytes and macrophages has been linked to progression and severity of experimental autoimmune orchitis (EAO) (Yule and Tung, 1993; Suescun *et al.*, 2003) and recent observations confirmed an increase of TNF mRNA levels in murine EAO (Nicolas *et al.*, 2017a).

Two families of TNF receptors (TNFR) have been described. The TNFR type 1 family members have the ability to induce either cell death or cell survival, which is determined by adaptor proteins. The TNFR type 2 family comprises TNFR2, CD30, CD40, lymphotoxin receptor, RANK, and BAFF, and is generally unable to elicit apoptosis. However, in the murine and human testis, TNFR1 is the main receptor and expressed by SC, Leydig cells, macrophages, lymphocytes, and germ cells (Lysiak, 2004; Yao *et al.*, 2007).

1.4.6.2. Interleukin-1 (IL-1)

IL-1 is an important regulator of the inflammatory process through mediating chemotactic and immunoregulatory actions. It is involved in spermatogenesis and Leydig cell steroidogenesis under normal conditions and can occur in two isoforms, IL-1α and IL-1β. Both isoforms bind to the same receptor and exert almost identical functions (Guazzone *et al.*, 2009). Generally, activated monocytes and macrophages are a major source of IL-1.

Its expression was also found in many testicular cells, including SC, germ cells, Leydig cells and macrophages whereas SC were shown to be the predominant producers (Guazzone *et al.*, 2009; Lysiak, 2004; Rozwadowska *et al.*, 2005). Treatment of rat SC with IL-1 was shown to stimulate the βA-subunit expression and the production of activin A (Kazutaka *et al.*, 2011).

Recent research showed the regulatory role of IL-1α, together with TGF-β3 and TNF, on SC tight junction dynamics. While TGF-β3 and TNF facilitate the opening of the tight junction by lowering occluding levels, IL-1α affects the opening by modulating the actin cytoskeleton (Guazzone *et al.*, 2009; Yan *et al.*, 2008; Sarkar *et al.*, 2008). *In vitro*, an inflammatory stimulus was shown to increase the production of IL-1α by SC (Guazzone *et al.*, 2009).

1.4.6.3. Interleukin-6 (IL-6)

IL-6 is a potent cytokine that promotes inflammatory processes through expansion and activation of T cells, differentiation of B cells as well as induction of the acute phase response (Kamimura *et al.*, 2003). In the testis, it is involved in the regulation of spermatogenesis and steroidogenesis (Hedger and Meinhardt, 2003). In the rat testis, IL-6 is produced by most cells including interstitial macrophages, Leydig cells, SC, PTC and germ cells under physiological conditions. Its production was shown to be stimulated by FSH, testosterone, IL-1, TNF and bacterial lipopolysaccharide (LPS) in rat SC (Rival *et al.*, 2006b).

IL-6 was shown to be involved in the development of autoimmune diseases. During the development of EAO in rat, PTC and activated macrophages increase their production of IL-6, while SC downregulate IL-6 expression during the chronic stage of the disease. Results of this study also suggest that IL-6 may be involved in the pathogenesis of EAO by promoting testicular inflammation and germ cell apoptosis (Rival *et al.*, 2006b; Fijak *et al.*, 2011). In contrast, the exogenous administration of IL-6 was shown to prevent development of disease in murine EAO (Li *et al.*, 2002). However, in mouse EAO, no change of testicular IL-6 levels was detected (Nicolas *et al.*, 2017a).

1.4.6.4. Monocyte chemoattractant protein 1 (MCP-1; CCL2)

MCP-1 (also referred to as chemokine C-C motif ligand 2; CCL2) is a potent chemokine involved in recruiting immune cells, including both monocytes and lymphocytes, to the site of inflammation. It is present in the testis at low but physiologically relevant levels (Guazzone *et al.*, 2009; Gerdprasert *et al.*, 2002).

In vitro, it is produced by human and rat PTC and Leydig cells, and its transcripts were also detected by quantitative real-time PCR (qRT-PCR) in SC, while it was not detectable in germ cells (Aubry *et al.*, 2000; Guazzone *et al.*, 2009).

MCP-1 has an important role in recruiting immune cells to the testis in rats undergoing autoimmune orchitis. Its expression was found to be upregulated in mononuclear, endothelial, Leydig and peritubular cells in rat EAO and it was also expressed in SC of rats with severe orchitis (Guazzone *et al.*, 2003; Fijak *et al.*, 2011; Iosub *et al.*, 2006).

Its mRNA levels were also found elevated in murine EAO suggesting an important function in disease development (Nicolas *et al.*, 2017a). MCP-1 expression was shown to be increased by inflammatory cytokines, including IL-1α, IL-1β, TNF, interferon-gamma (IFN-γ), and by LPS in cultured rat PTC, while it was markedly upregulated in SC in response to Toll-like receptor (TLR) agonists (Aubry *et al.*, 2000; Schell *et al.*, 2008; Starace *et al.*, 2008; Riccioli *et al.*, 2006). TLRs are pattern-recognition receptors that recognise structurally conserved molecules derived from pathogens such as LPS. The specific MCP-1 cell-surface receptor, CC-chemokine receptor-2 (CCR2) belongs to the G-protein-coupled seven-transmembrane receptor superfamily and is known to mediate inflammatory diseases, such as asthma, obesity, multiple sclerosis, and chronic obstructive pulmonary disease (Xia and Sui, 2009). Moreover, MCP-1 and CCR2 play a crucial role in the pathogenesis of EAE (Mahad and Ransohoff, 2003).

1.5. Activins

1.5.1. Formation and structure

The activins are highly conserved members of the TGF-β superfamily of growth and differentiation cytokines (Ling *et al.*, 1986). They are a homologous family of homo- and heterodimers. Activin A, which is the best studied family member, is a homodimer of the βA subunit of the gonadal hormone inhibin A, which itself is formed by the dimerisation of α and β subunits **(Fig. 4)**.

Activin B is a homodimer of the inhibin βB subunit and activin AB is formed by the hetero-dimerisation of the two β subunits, βA and βB . Several other activin subunits were identified, including βC and βE, which are mostly found within the liver (Fang *et al.*, 1997; O'Bryan *et al.*, 2000; Hedger and de Kretser, 2013; Hotten *et al.*, 1995). In mammals, the subunits of activin A and B are encoded by two separate genes, inhibin βA (*INHBA*) and inhibin βB (*INHBB*), while the βC, and βE subunits are products of *INHBC*, and *INHBE* genes, respectively.

Activins are synthesised as larger precursors, consisting of an N-terminal signal peptide, a poorly conserved pro-domain of 250 – 350 residues, and the smaller mature domain. After clevage of the pro-domain and dimerisation, disulphide-linked activin A and B dimers have a mass of approximately 25 kDa. They are highly conserved across species, displaying 100% protein sequence conservation between human and mouse (Loveland and Hedger, 2015; Wang *et al.*, 2016; Hardy *et al.*, 2015; Hedger and de Kretser, 2013).

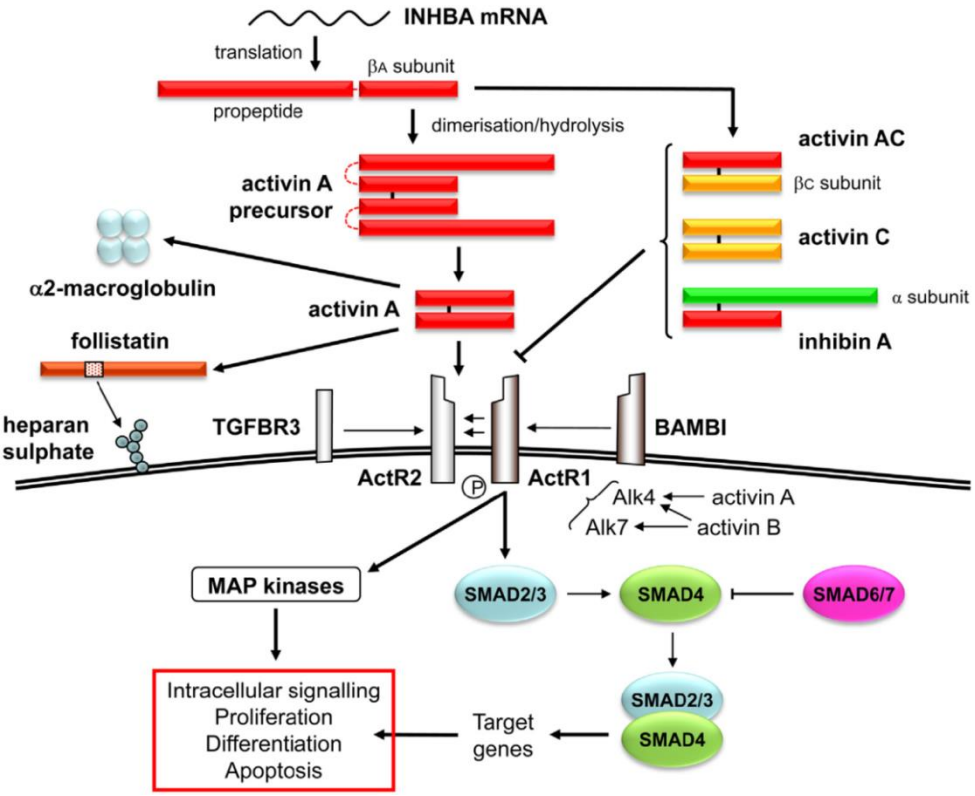

Figure 4: Activin A production, structure and signalling. Activin A is dimer of two inhibin βA subunits which are encoded by the *INHBA* gene. Binding of activin A to one of two activin type 2 (ActR2) receptors, ACVR2A and ACVR2B, on the surface of target cells leads to their association with type 1 (ActR1) receptors (Alk4 in the case of activin A). This in turn initiates signalling via SMAD2/3 and MAP kinases, and induces the expression of target genes responsible for proliferation, differentiation or apoptosis of cells. Activin A activity is regulated by the formation of inhibin A which is a heterodimer consisting of inhibin βA and α subunits and the formation of activin AC and activin C, which compete for the activin receptor but are not capable of transmitting a signal. Further regulation of activin A occurs by proteins, such as follistatin, and on level of the activin receptors by membrane-bound co-receptors (TGFBR3 and BAMBI [bone morphogenetic protein and activin membrane-bound inhibitor]). Image adapted from (Hedger and de Kretser, 2013).

1.5.2. Activin signalling

Members of the TGF-β superfamily exert their biological effects in target cells by binding complexes of type I and type II receptor serine/threonine kinases **(Fig. 4)** (Mathews and

Vale, 1991). Activins bind to one of two activin specific type II receptors, ActR2a and ActR2b, which then dimerises with an activin-specific type I receptor serine/threonine kinase (activin receptor-like kinase, ALK) in a juxtamembrane domain (GS box). ALK4 (also known as ActR1b) is considered as the only activin A-specific type I receptor. It was shown that ALK2, another type I receptor, binds activin A, but does not transduce the ligand's intracellular signals (Tsuchida *et al.*, 2004; Tsuchida *et al.*, 2009; Vale *et al.*, 2004; ten Dijke *et al.*, 1994; Allendorph *et al.*, 2006).

Upon activation of the type I receptor, the transcription factors SMAD2 and SMAD3 become phosphorylated. This allows the formation of a complex with SMAD4 and translocation into the nucleus, where the complex accumulates and regulates the expression of target genes involved in proliferation, differentiation and survival of cells (Heldin *et al.*, 1997; Itman *et al.*, 2009). Studies examining the effects of SMAD2 or SMAD3 knock-down revealed that mice lacking SMAD2 exhibit embryonic lethality, while mice lacking SMAD3 develop to adulthood, indicating that SMAD2 and SMAD3 are not functionally redundant (Waldrip *et al.*, 1998; Zhu *et al.*, 1998).

In addition, activins can also act in several alternative inflammation and stress-mediated signalling pathways via tumour necrosis factor receptor associated factor 6 (TRAF6) and downstream activation of the mitogen-activated protein kinases (MAPK), p38 MAPK, c-Jun N-terminal protein kinases (JNK) and extracellular signal-regulated kinases ERK1/2 (Huang *et al.*, 2006; Heldin *et al.*, 2009; Hedger and de Kretser, 2013).

1.5.3. Inhibition

Several endogenous inhibitors are known to regulate the bioactivity of activins. Inhibins, which are almost exclusively produced by the testis or ovary, have the ability to inhibit FSH production due to the blockade of endogenous activin activity within the anterior pituitary itself (Bilezikjian *et al.*, 2004). Inhibins are also able to regulate the amount of activin produced, since their formation (α-β heterodimers) reduces the availability of the β subunit (Wijayarathna and de Kretser, 2016). In the testis, inhibin B is the main form produced, and the SC are the principal site of production (Anawalt *et al.*, 1996; Ramaswamy *et al.*, 1999; Sharpe *et al.*, 1999). Activin C is another activin A antagonist. It competitively binds to βA and βB subunits and this way decreases the levels of activin A (Wijayarathna and de Kretser, 2016).

Further regulation of activins occurs at the receptor level through expression of the pseudoreceptor BAMBI, which is related to the type I receptors of the TGF-β family but lacks an intracellular kinase domain, and therefore is not capable of transducing a signal (Deli *et al.*, 2008).

1.5.3.1. Follistatin

Follistatin is a monomeric glycosylated polypeptide, which endogenously binds activin with high affinity, thereby obstructing the activin receptor binding site, and targets the complex to cell surface proteoglycans and lysosomal degradation (Anderson *et al.*, 1998; Nakamura *et al.*, 1990). By splicing of the follistatin gene, *FST*, two forms of follistatin are produced; they are named FST288 and FST315 based on the length of the polypeptide chains (288 and 315 amino acids, respectively). FST315 is the main circulating form in the blood, while FST288, which has a constitutively exposed positively charged site that binds to negatively charged heparin sulphate proteoglycans on cell surfaces, is predominantly tissue-bound (Lerch *et al.*, 2007; Sugino *et al.*, 1993).

As is the case for activin, follistatin is produced by many tissues. In the rat testis, follistatin mRNA is expressed in several germ cell stages, SC and endothelial cells. Moreover, the protein has been localised to Leydig cells, SC, spermatogonia, primary spermatocytes (except zygotene stage), spermatids and endothelial cells (Meinhardt *et al.*, 1998; Anderson *et al.*, 1998; Wada *et al.*, 1996; Nakamura *et al.*, 1990; Majdic *et al.*, 1997). In the human testis, follistatin was expressed in SC and Leydig cells (Anderson *et al.*, 1998; Wada *et al.*, 1996). Due to its ability to prevent activin A actions, follistatin is a potential therapeutic target for several inflammatory and fibrotic diseases (Mather *et al.*, 1990; Mather *et al.*, 1993; Hardy *et al.*, 2013; Hardy *et al.*, 2015; Boitani *et al.*, 1995).

1.5.4. Production and function of activins

Activins are expressed in most tissues and organs, with βA and βB subunit genes being highly expressed in male and female reproductive tracts under physiological conditions. High levels of the βA subunit mRNA were also found in the bone marrow, central nervous system, placenta, liver, fat, heart as well as adrenal glands (Schneider *et al.*, 2000; Hedger and de Kretser, 2013).

Activins were discovered and named by their ability to stimulate the secretion of FSH from the anterior pituitary (Ling *et al.*, 1986). However, activins are now known to exert various effects on a range of systems, including haematopoiesis and immune cell development (Hedger *et al.*, 2011; Broxmeyer *et al.*, 1988; Locci *et al.*, 2016).

They also play crucial roles in inflammation, immunity and fibrosis where they were found to be increased in the epithelial, endothelial, myeloid, lymphoid and stromal cells of affected tissues as well as in the blood (Werner and Alzheimer, 2006; Hedger and de Kretser, 2013). Activin A especially is known to be a crucial regulator of inflammation and fibrosis in a range of organs, including lung, kidneys, heart and liver (Karagiannidis *et al.*, 2006; Gregory *et al.*, 2010; de Kretser *et al.*, 2012; Patella *et al.*, 2006; Yamashita *et al.*, 2004; Hu *et al.*, 2016).

Activin A was also shown to be involved in epithelial invasiveness and tumorigenesis (Le Bras *et al.*, 2014). In contrast to activin A, little is known about the roles of activin B in inflammation and fibrosis, and its function in the testis.

1.5.5. Activin A function in fibrosis

Several studies implicate activin A as a crucial regulator and an important intermediary of fibrosis. Activin A has a stimulatory effect upon fibroblast proliferation and differentiation into myofibroblasts (Ota *et al.*, 2003; Ohga *et al.*, 1996; Hu *et al.*, 2016). It also has the ability to induce the expression of ECM proteins like collagen type I, and to stimulate the production of regulators and intermediates of fibrosis, including αSMA and connective tissue growth factor, in a range of cell types (Yamashita *et al.*, 2004; Ohga *et al.*, 1996; Ota *et al.*, 2003; Hu *et al.*, 2016). Moreover, activin A upregulates the production of TGF-β, which is known to be a crucial regulator of the fibrotic response in renal and lung fibroblasts as well as pancreatic stellate cells (Karagiannidis *et al.*, 2006; Ohnishi *et al.*, 2003).

1.5.6. Activin A function in inflammation and immunity

Evidence from several studies implicates activin A as a critical mediator of inflammation and immunity. Activin A, particularly during inflammatory responses, is produced by a range of activated cells of the myeloid lineage, such as monocytes, macrophages, neutrophils, mast cells and dendritic cells, as well as bone marrow stromal cells and some lymphoid cells, including activated murine splenic $CD4^+$ T cells and thymocytes. Furthermore, activin A levels are systemically elevated in many acute and chronic inflammatory conditions (Leto *et al.*, 2006; Zhang *et al.*, 2009; Yang *et al.*, 2015; Palacios *et al.*, 2015; Phillips *et al.*, 2009).

Activin A modulates several aspects of the inflammatory response, including nitric oxide production, immune cell activity and release of a variety of key pro-inflammatory cytokines, such as IL-1β, TNF and IL-6, thereby promoting the onset of the inflammatory response (inflammatory phase) (Hedger *et al.*, 2011). However, in the subsequent immune response (immunity phase), activin A inhibits crucial immune functions of activated myeloid and lymphoid cells, indicating an immunoregulatory role of activin (Hedger *et al.*, 2011).

These data point out the important dual functions of activin A to either promote or suppress immune responses, depending on its environment and activation status (Phillips *et al.*, 2009).

A schematic representation of some of the effects of activin A on immune cells is depicted in **Fig. 5**.

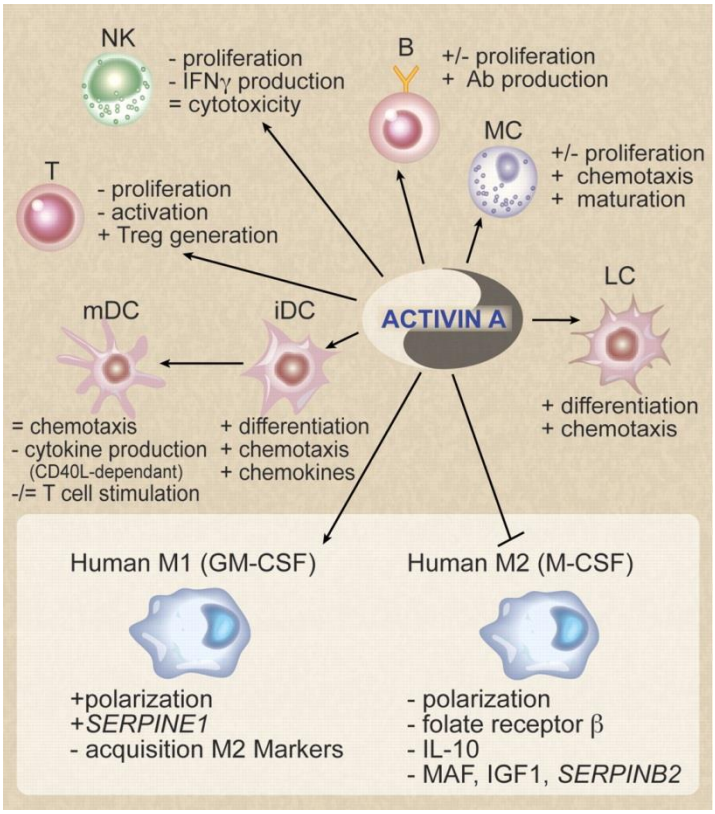

Figure 5: Activin A has stimulating and inhibitory effects on immune cells. Activin A acts inflammatory by promoting the differentiation and migration of Langerhans cells, mast cells and immature dendritic cells as well as production of antibodies by B cells. It is also able to polarize macrophages towards M1 pro-inflammatory phenotype. In contrast to these pro-inflammatory effects, Activin A is able to act anti-inflammatory by controlling and inhibiting Th1 and Th2 responses through the induction of antigen-specific regulatory T cells. Finally, Activin A was reported to suppress cytokine production by activated NK cells, with no effect on their cytotoxic activity. LC: Langerhans cells; iDC: immature dendritic cells; mDC: mature dendritic cells; MC: mast cells; NK; natural killer cells; +: stimulation; −: inhibition; =: no effect. Picture taken from (Sozzani and Musso, 2011).

1.5.7. Activin production and function in the testis

Numerous scientific studies and clinical observations revealed the importance of activins in the control of reproductive functions (de Kretser *et al.*, 2002; Welt *et al.*, 2002). In the male reproductive tract, activins and their receptors act as crucial autocrine and paracrine modulators of germ cell development and SC proliferation.

The changing spatial and temporal distributions of activins indicate that their modulated production and action are important during the onset of murine spermatogenesis (Barakat *et al.*, 2008).

The βA subunit mRNA and activin A protein have been localised to SC, PTC, macrophages, mast cells, Leydig cells and endothelial cells, as well as spermatogonia, spermatocytes and round spermatids in the testes of developing and adult rats, mice and humans.

However, data implicate the SC as the predominant source of activin A, with potentially significant additional contributions from the PTC (Barakat *et al.*, 2008; Okuma *et al.*, 2006; de Winter *et al.*, 1994; Buzzard *et al.*, 2004; Fragale *et al.*, 2001; Kaipia *et al.*, 1992; Mendis *et al.*, 2011). Both immature and post-mitotic SC express activin receptors. Additionally, ActR2a and ActR2b are expressed by interstitial cells and gonocytes during foetal development. In the adult testis, activin A synthesis by SC is stimulated by phagocytosis of spermatogenic cells and residual cytoplasm, to regulate the proliferation and development of spermatogonia and spermatocytes, and in this way regulate spermatogenesis (Hedger and Winnall, 2012; de Winter *et al.*, 1992; Barakat *et al.*, 2008).

Moreover, under physiological conditions activins inhibit pro-inflammatory cytokine production, thereby acting as an anti-inflammatory cytokine and potentially maintaining the immune privilege of the testis (Loveland and Hedger, 2015).

In contrast, activin overexpression in the testis has been shown to result in ablation of the formation of tight junctions in rat SC, as well as disruption of spermatogenesis and BTB function, resulting in infertility in adult mice (Nicholls *et al.*, 2012; Tanimoto *et al.*, 1999). A strong increase of activin A production by SC and by infiltrating immune cells in the interstitium was reported within the chronically inflamed mouse testes from studies on EAO. Moreover, the increase in activin A correlated with the severity of EAO (Nicolas et al., 2017).

Other studies showed the stimulation of activin A production in all testicular cell types except the spermatogenic cells, but particularly in PTC and SC, by incubation with inflammatory stimuli such as LPS, IL-1 or TNF. Pro-inflammatory factors also strongly induced the activin A production in cell types outside the testis (Kazutaka *et al.*, 2011; Jones *et al.*, 2007; Scutera *et al.*, 2008; Okuma *et al.*, 2005b; Winnall *et al.*, 2009; Okuma *et al.*, 2005a; Takahashi *et al.*, 1992; Shao *et al.*, 1992).

Investigations in rat SC and other cell types demonstrated that regulation of the βA subunit involves activation of TRAF, as well as the p38 MAPK and JNK pathways, via the adapter protein MyD88 by ligands of the Toll-like receptors (TLR) and IL-1 **(Fig. 6)**. TNF stimulates activin A production by directly activating the MAPK without the intervention of MyD88 (Bradley, 2008; Hedger and Winnall, 2012).

In addition, other cytokines, including several regulators of fibrosis such as IL-13, TGF-β, endothelin, angiotensin and thrombin, that can activate these pathways, were shown to stimulate production of activin A in various cell types and tissues; for example TGF-β, known as a crucial driver of fibrotic processes, induces the expression of activin A by activation of MAPK via signalling through TRAF6 (Hedger and de Kretser, 2013; Heldin *et al.*, 2009; Yamashita *et al.*, 2004; Wada *et al.*, 2004).

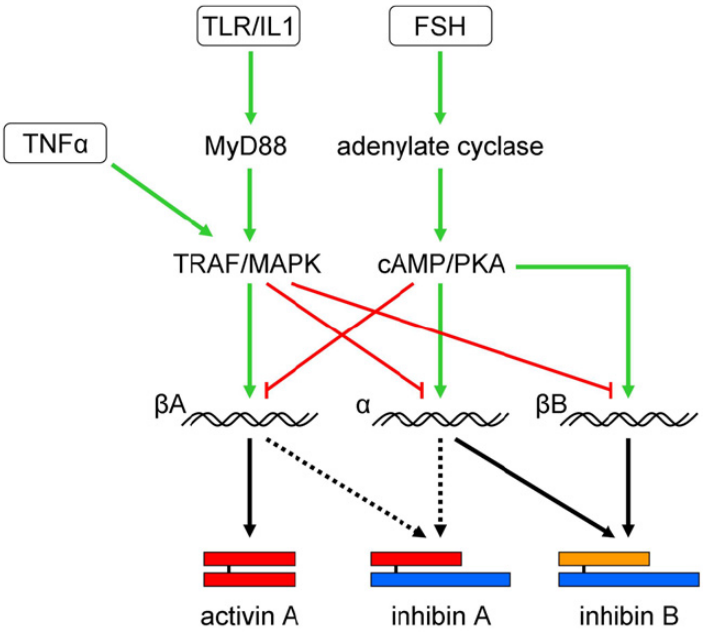

Figure 6: Regulation of activin production in SC. Production of the βA subunit involves direct activation of TRAF and MAPK by TNF as well as ligands of the TLR and IL1 via MyD88. Negative regulation of the βA subunit occurs through FSH via production of cAMP and subsequent activation of protein kinase A (PKA). Scheme adapted from (Hedger and Winnall, 2012).

A range of other immunoregulators, such as IFN-γ, CD40L, colony-stimulating factor 2, and reactive oxygen species, stimulates the expression of the βA subunit highlighting the importance of activin A secretion during inflammatory reactions.

However, not all inflammatory cytokines stimulate activin A production; for example studies on rat SC showed that IL-6, which acts primarily via the JAK/STAT signalling pathway, had no effect on activin A production. In rat SC, the expression of the βA subunit and hence activin A production are negatively regulated via a signalling pathway activated by FSH (Loveland and Hedger, 2015; Scutera *et al.*, 2008; Hedger and Winnall, 2012; Okuma *et al.*, 2005a).

1.6. Male Infertility

1.6.1. Infertility in men and testicular inflammation

Infertility is a significant medical problem worldwide. Studies showed that approximately 15% of couples in Europe (Jungwirth *et al.*, 2012; Rouchou, 2013) experienced infertility, which is defined by the WHO as the “inability of a sexually active, non-contracepting couple to achieve pregnancy in one year” and involves male factors in approximately 50% of all infertile couples (Dohle *et al.*, 2005; Rowe *et al.*, 2000).

Other recent publications estimate that one in eight women (12.5%) and one in ten men (10.1%) in UK had experienced infertility (Barratt *et al.*, 2017).

Although many causes of human male infertility are reported in the literature, 60 – 75% of male infertility cases are categorised as idiopathic disturbance of spermatogenesis (Filipponi and Feil, 2009; Naito *et al.*, 2012b). However, previous studies of this condition have reported that lymphocytic infiltration and immune deposits were present in approximately 10% of idiopathic male infertility cases (Hofmann and Kuwert, 1979; Jahnukainen *et al.*, 1995; Jorgensen *et al.*, 2014; Pollanen *et al.*, 1985; Suominen and Soderstrom, 1982). This indicates that inflammatory or immunological factors can contribute to the development of tissue damage in the testis. In a systematic re-examination of testicular biopsies from asymptomatic men with impaired fertility, lymphocytic infiltrates were even recorded in over 50% of the cases (Schuppe *et al.*, 2008).

The degree of lymphocytic infiltration, predominantly consisting of $CD4^+$ and $CD8^+$ T cells, ranged from widely scattered, single lymphocytes to focal or multifocal dense infiltrates, and correlated with the strength of characteristic tubular pathologies, including loss of germinal epithelium, thickening of the lamina propria and tubular fibrosis. However, numbers of non-resident $CD68^+$ macrophages and mast cells have also been found increased in inflamed testis and have been associated with tissue remodelling and fibrosis (Schuppe *et al.*, 2008; Frungieri *et al.*, 2002; Meineke *et al.*, 2000).

Mast cells are known to promote fibrosis by inducing fibroblast proliferation and production of collagen. Hence, increased numbers of these cells are frequently found in fibrotic testes of infertile men (Chen *et al.*, 2016).

Spermatogenic failure, such as SCO, maturation arrest at different levels of early round spermatids, primary spermatocytes, or spermatogonia, and hypospermatogenesis is often observed in idiopathic sub- and infertility patients.

While in most oligozoo- or azoospermic patients, testicular biopsy reveals a “mixed atrophy”, which describes the simultaneous occurrence of different spermatogenic defects in adjacent seminiferous tubules including SCO tubules or even only lamina propria (tubular shadows), patients with complete SCO are always azoospermic. The lamina propria of SCO tubules is often thickened due to increased deposition of collagen type IV and increased thickness of the basal lamina, which is thought to be related to spermatogenic dysfunction (Sigg, 1979; Behre *et al.*, 2000).

Known causes of inflammatory or immunological damage are bacterial (*E. coli*) or viral (mumps) infections, trauma and sterile autoimmune orchitis (Schuppe *et al.*, 2008; Schuppe and Bergmann, 2013). Autoimmune orchitis can occur spontaneously in several species, including dogs and mink, and can be induced experimentally in vasectomised guinea pigs and rabbits, and in mice that have been thymectomised within 4 days of birth (Fritz *et al.*, 1976; Tung *et al.*, 1981; Bigazzi *et al.*, 1976; Tung, 1978).

Pathologic characteristics, similar to autoimmune orchitis, have been described in several human idiopathic diseases associated with male sub- or infertility, and inflammation of the testis in the presence of specific anti-sperm antibodies (Yule and Tung, 1993). However, as chronic inflammatory conditions in testis are often asymptomatic, and disease symptoms can only be identified on the basis of histological evaluation of testicular biopsies, they tend to be neglected in clinical diagnosis (Schuppe *et al.*, 2008; Fijak *et al.*, 2018).

Deficits in androgen and sperm production may also result from systemic autoimmune diseases (Silva *et al.*, 2014).

1.6.2. Experimental autoimmune orchitis (EAO)

EAO in rodents is a well-established model of chronic testicular inflammation, which aims to understand the pathomechanisms of immunological male infertility and mimics the associated pathological changes. Autoimmune orchitis can be induced by immunisation with preparations of testicular antigens, such as testicular homogenate (TH) (Tung and Menge, 1985; Tung and Teuscher, 1995; Fijak *et al.*, 2018).

In the rete testis, where a great number of spermatozoa with newly adapted surface molecules are transported towards the epididymis, the BTB is incomplete, leading to an increased predisposition of this region to development of autoimmune orchitis. In line with this finding, induction of EAO by injection with TH in mice was shown to result in infiltrations of lymphocytes first in the tunica albuginea in close proximity to the rete testis and the tubuli recti. From these sites they subsequently spread to the interstitial space (Fijak and Meinhardt, 2006; Itoh *et al.*, 2005; Itoh *et al.*, 1995).

The active immunisation with homogenised testicular tissue suspended in an adjuvant was also shown in other rodent models to successfully induce the disease (Tung and Teuscher, 1995; Doncel *et al.*, 1989). Other experimental approaches for the induction of EAO include the injection of viable syngeneic testicular germ cells without using any adjuvant, the passive transfer of T lymphocytes previously sensitised with sperm antigens into naïve animals (passive EAO), and immune system manipulations, such as thymectomy at day 3 after birth (Guazzone *et al.*, 2009; Fijak *et al.*, 2018; Naito *et al.*, 2012a; Tung *et al.*, 1987).

The initial inflammatory phase of EAO in rodents features the production of testicular auto-antibodies, including autoantibodies against heat shock proteins 60 (Hsp60) and 70 (Hsp70), disulphide isomerase ER 60 and sperm outer dense fibre major protein 2 (ODF-2), elevated levels of inflammatory mediators such as TNF, MCP-1 and activin A, and interstitial infiltration by different types of immune cells, among them $CD4^+$, $CD4^+CD8^+$ and $CD4^+CD25^+$ T cells and macrophages (Fijak *et al.*, 2005; Guazzone *et al.*, 2003; Naito *et al.*, 2012b; Nicolas *et al.*, 2017a; Yule and Tung, 1993; Kohno *et al.*, 1983; Fijak *et al.*, 2018). A complex cluster of pro-inflammatory cytokines enters the seminiferous tubules and affects BTB permeability, resulting in the disruption of the BTB. Hence, development and progression of the disease results in a degeneration of the testicular architecture with severe lesions of the seminiferous tubules, fibrosis development and germ cell apoptosis in late and chronic stages of EAO (Theas *et al.*, 2008; Nicolas *et al.*, 2017a; Fijak *et al.*, 2018; Naito *et al.*, 2012b). Late stage EAO in mice resembles the histopathology of some cases of human male idiopathic spermatogenic disturbances (Suominen and Soderstrom, 1982). In studies of EAO in mice, Nicolas and colleagues (Nicolas *et al.*, 2017a) found that 30 days after the first immunisation with TH in complete Freund's adjuvant (CFA) with *Bordetella pertussis* toxin, only one third of mice showed histological symptoms of EAO, while at 50 and 80 days all animals developed the testicular inflammation. Their study also demonstrated an evident increase in the number of testicular macrophages at day 50 after the first immunisation in EAO. Notably, those macrophages did not express the CD206 marker suggesting that the main population in EAO comprises the pro-inflammatory M1-type macrophages (Nicolas *et al.*, 2017a). Increased serum levels of FSH and LH, and decreased levels of testosterone are further characteristics of the disease (Fijak *et al.*, 2011; Suescun *et al.*, 1994).

1.7. Fibrosis

Fibrosis is characterised by activation of fibroblasts and excessive production and deposition of ECM components, including collagen, resulting in distorted organ architecture and function. It is often a result of failure to resolve active inflammation and based on the establishment of a chronic inflammatory response, which leads to stiffness and/or scar formation in a variety of tissues. Among the fibrotic diseases, which also include idiopathic pulmonary fibrosis, liver cirrhosis, systemic sclerosis, progressive kidney disease, and cardiovascular fibrosis, liver fibrosis is the most frequent. Fibrosis which results from chronic inflammation can be induced by a variety of stimuli including persistent infections, autoimmune reactions, allergic responses and tissue injury. Millions of people are affected by fibrotic diseases, which are chronic, progressive and often lethal.

Hence, fibrosis represents a major health problem worldwide (Zeisberg and Neilson, 2010; Zhang and Stefanovic, 2016; Wynn, 2008; Xia *et al.*, 2013).

The principal cell type driving fibrosis is the myofibroblast, which, when activated, leads to the excessive deposition of ECM proteins, including predominantly collagen I, collagen III, and fibronectin. As myofibroblasts acquire a phenotype intermediate between fibroblasts and smooth muscle cells, they also express αSMA and exhibit contractile activity (Zhang *et al.*, 1994; Bonnans *et al.*, 2014). αSMA is a contractile actin isoform which typically is expressed in vascular smooth muscle cells under physiological conditions or newly expressed in myofibroblasts during healing processes after injury and during fibrosis (Hinz *et al.*, 2001; Hung *et al.*, 2006; Sun *et al.*, 2016).

Myofibroblasts can evolve from a variety of progenitor cell types including resident fibroblasts, epithelial and endothelial cells in processes named epithelial- and endothelial-mesenchymal transition (EMT/EndMT), as well as from circulating bone-marrow derived fibrocytes **(Fig. 7)** (Quan *et al.*, 2006; Willis *et al.*, 2006; Kalluri and Neilson, 2003; Ebihara *et al.*, 2006; Bucala *et al.*, 1994; Brittan *et al.*, 2002; Direkze *et al.*, 2003; Forbes *et al.*, 2004; Xia *et al.*, 2013).

Fibrocytes can also originate from a subpopulation of monocytes via monocyte-to-fibroblast transition (Dong *et al.*, 2016; Yang *et al.*, 2013). After their recruitment and activation, monocyte-derived fibrocytes express mesenchymal markers such as collagen type I, and haematopoietic markers such as CD45 and CD11b (Xia *et al.*, 2013).

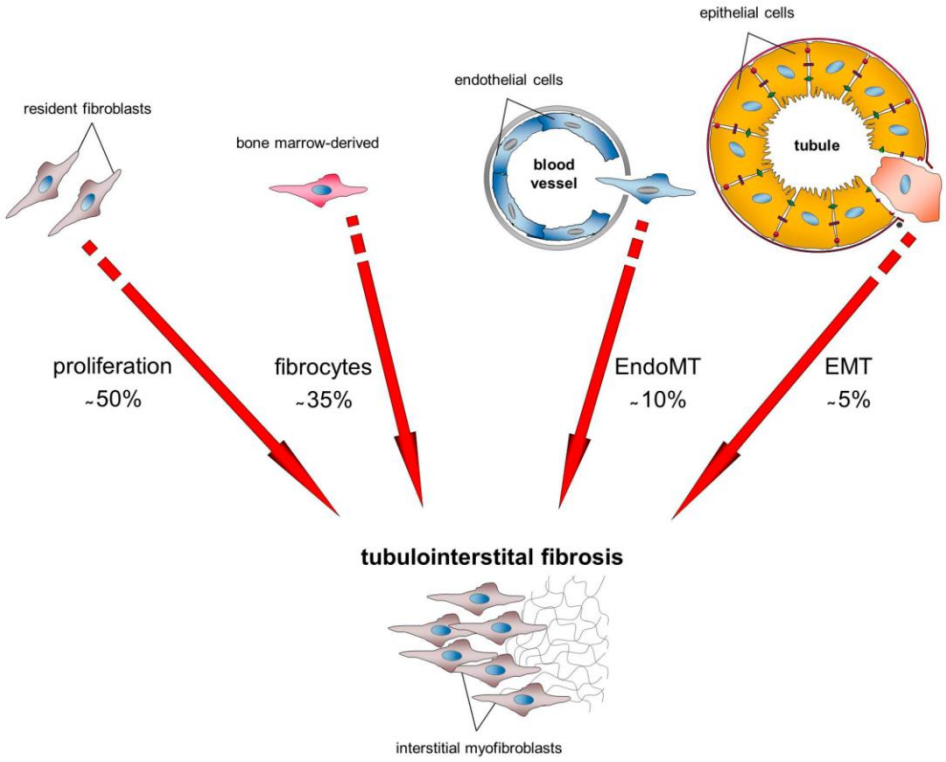

Figure 7: Possible origins of myofibroblasts in fibrosis. Myofibroblasts are a heterogeneous population of cells which can be derived from different sources; through the activation and differentiation of resident fibroblasts, recruitment and differentiation of fibrocytes and the activation and proliferation of endothelial and epithelial cells, via endothelial to mesenchymal transition (EnMT) or via epithelial to mesenchymal transition (EMT). Percentages indicate the contribution of different cell types to kidney fibrosis. Picture taken from (Loeffler and Wolf, 2015).

The activation of myofibroblasts can occur through a variety of mechanisms. These include paracrine signals derived from lymphocytes and macrophages and autocrine factors secreted by myofibroblasts themselves. Moreover, cytokines (including IL-4 and IL-13), chemokines (MCP-1) and various mediators, including activin A, have been identified as important regulators of fibrosis and are being investigated as potential targets of anti-fibrotic drugs (Wynn, 2004; Ong *et al.*, 1999; Buttner *et al.*, 1997; Yamashita *et al.*, 2004; Borthwick *et al.*, 2013; Chiaramonte *et al.*, 1999; Tokuda *et al.*, 2000; Lloyd *et al.*, 1997).

An estimated 80% – 90% of the proteins in the fibrotic ECM consist of collagen type I (Zhang and Stefanovic, 2016). Excessive deposition of collagen type I disrupts normal tissue architecture, leading to a disturbed function of the affected organ. Hence, the assessment of fibrosis severity is usually determined by the amount of collagen type I production. However, collagen type III is also present as a minor component in the fibrotic ECM, as well as traces of other collagens (type V, XI, XXIV and XXVII) (Zhang and Stefanovic, 2016).

1.7.1. Testicular fibrosis in human infertility

Interstitial and peritubular fibrosis is frequent in male infertile patients with disturbed spermatogenesis, including mixed atrophy, SCO and germ cell arrest syndrome. While interstitial fibrosis is mainly composed of collagen type I and fibronectin, peritubular fibrosis is marked by a thickened lamina propria of the seminiferous tubules due to an increase in collagen types I, III and IV, and fibronectin between the cellular layers (Adam *et al.*, 2012; Davidoff *et al.*, 1990; Takaba, 1990; Bonnans *et al.*, 2014). Based on their observations, Davidoff and colleagues (Davidoff *et al.*, 1990) hypothesised that myofibroblasts in the fibrotic testis lose their myoid characteristics to participate in the secretion of high amounts of ECM proteins. This in turn may block the conduction of signals between the interstitium and the seminiferous epithelium by the lamina propria (Davidoff *et al.*, 1990).

1.7.2. ECM proteins

1.7.2.1. Fibronectin

Fibronectins are multidomain, high-molecular weight glycoproteins. Due to their complex structure and extensive presence in various tissues and fluids of the body, fibronectins have been implicated in a wide array of fundamental biological processes. As a component of the ECM, they provide essential connections to cells through integrins and other receptors, thereby regulating cell adhesion, migration, and differentiation. Due to alternative splicing events, fibronectins are secreted as large dimers that are joined by disulphide bonds at the carboxyl-termini with subunits ranging in size from 230 - 270 kDa, and consisting of variable combinations of three different types of homologous repeating domains termed types I, II and III. Its variable composition and diversity of interactions suggests a role for these proteins beyond the structural considerations of the ECM. However, fibronectin is of particular importance for the polymerisation of collagen type I and III, and its continuous presence is also required for matrix integrity *in vivo* (Kawelke *et al.*, 2011; Velling *et al.*, 2002; Aziz-Seible and Casey, 2011).

The domain at the amino-terminal end is composed of type I homologous repeats that can bind to a range of substrates including matrix heparin, cell-surface glycosphingolipids as well as to bacteria. Adjacent to the amino-terminal domain, a highly glycosylated collagen/gelatine binding site of the molecule is located. Fibronectin can bind to the different types of collagen in their native forms and also adheres effectively to the unfolded regions of the denatured collagen triple helix.

The carboxyl-terminal end consists of type III repeats along with a variable segment that is determined by the tissue of origin and is the major heparin binding domain.

Under physiological conditions, fibronectin exists as one of two major forms, soluble plasma fibronectin and cellular fibronectin. The latter form is produced locally in tissues, predominantly by resident fibroblasts and endothelial cells, and is secreted by those cells into the pericellular matrices. Although collagen is the major ECM component of fibrotic tissue, cellular fibronectin levels become significantly elevated prior to collagen deposition during injury and disease (Aziz-Seible and Casey, 2011; Schwarzbauer and DeSimone, 2011). Hence, fibronectin is often used as marker for fibrosis in various organs, including liver and kidney (Soylemezoglu *et al.*, 1997; Liu *et al.*, 2016).

Other studies demonstrated that a blockage of fibronectin deposition resulted in decreased accumulation of collagen and improved signs of fibrosis, suggesting that fibronectin inhibition may offer a therapeutic benefit in fibrotic conditions (Altrock *et al.*, 2015).

1.7.2.2. Collagens

Collagens are the most abundant proteins in the vertebrates, comprising approximately 30% of total body proteins in humans. From the 28 collagen types identified to date, four main types (I, II, III and V) comprise the majority in the ECM. Bones, tendons, skin and muscles are also mainly composed by these four types (Smith and Rennie, 2007).

In human testes, collagens type I, III and IV are located in the tubular basement membrane under normal conditions. Collagen types I and III are also expressed in the interstitial ECM. In the pathological testes from patients with idiopathic male infertility, the layers of the basement membrane of both thickened and obstructed tubules were positive for collagen types I and III, while collagen type IV was localised in the inner layer of the tubular basement membrane and the PTC layers. A thickening of the lamina propria is often observed in chronic inflammation with fibrosis. The ECM in the interstitium, including collagen types I and III, was significantly increased in specimens from men with idiopathic male infertility compared with normal testes (Takaba, 1990).

Of all the collagens, collagen type I is the major fibrillar protein of the ECM and also the main component of tendons, skin, ligaments and many interstitial connective tissues. The protein has a long half-life, averaging 60 – 70 days, depending on the tissue (Rucklidge *et al.*, 1992). Hence, its constitutive synthesis occurs at a low rate. However, during fibrosis, a several hundred-fold increase of collagen type I production can often be observed.

This dramatic increase is accomplished at several levels of regulation, whereby the major regulation occurs on the post-transcriptional level and includes the processing, transport, stabilisation and translation of mRNAs (Zhang and Stefanovic, 2016).

Collagen type I is composed of two α1 and one α2 polypeptide chains, which are synthesised as procollagen polypeptides, join together with the C-terminal globular domains and fold into a triple helix. The assembly in the lumen of the endoplasmic reticulum is followed by the secretion of the procollagen triple helix into the extracellular space, where the globular domains are cleaved and the procollagen triple helix is polymerised into fibrils (Gelse *et al.*, 2003; Canty and Kadler, 2005; van der Rest and Garrone, 1991; Zhang and Stefanovic, 2016).

1.8. Hypothesis and aim of study

As male subfertility and infertility is an important health problem worldwide, research on this topic is of scientific and clinical importance. A tight control of the testicular immune environment is absolutely crucial for the maintenance of normal spermatogenesis and disruption of the immune homeostasis may lead to chronic inflammation of the testis, resulting in fertility disturbances. Hence, understanding the pathogenesis of orchitis may help to develop new therapies and diagnostics for infertility resulting from inflammation.

Although much research on pathologies affecting male infertility has been undertaken, still little is known about the mechanisms underlying many infertility disorders and specifically in understanding chronic inflammation of the testis and autoimmune orchitis.

Activin A is known to be a crucial regulator in a range of inflammatory and fibrotic diseases and is also widely expressed in the testis under physiological conditions (Nicolas *et al.*, 2017a; Hedger and Winnall, 2012). Previous results demonstrated significantly elevated levels of intra-testicular *Inhba* (βa subunit) mRNA levels and upregulation of activin A protein expression in EAO testis. These increases were accompanied by significantly elevated numbers of leukocytes in testes of mice with severe EAO, and an upregulation of *Tnf* mRNA levels in EAO at days 50 and 80 after the first TH-immunisation (Nicolas *et al.*, 2017a). Moreover, severe EAO was characterised by a strong fibrotic response.

Based on these data, we hypothesise that activin A contributes to fibrosis development in EAO by upregulating the fibrotic response in testicular cells.

Hence, the primary aim of the studies described in this thesis was to identify the cell types possibly responsible for fibrosis development in EAO and to assess the influence of activin A on the fibrotic response of these cell types.

Furthermore, the clinical relevance of the EAO mouse model to study human chronic testicular inflammation was examined by comparing multiple disease parameters such as localisation of fibrotic areas as well as *Inhba* and *CD45* expression.

In addition, we hypothesised that, as SC are the main producers of activin A under normal conditions and an upregulation of activin A expression by SC following treatment with inflammatory stimuli was observed, SC are responsible for the production of the activin A, which is contributing to the development and severity of EAO testis. Hence, another aim of the studies was to investigate the influence of activin A produced by SC on inflammatory and fibrotic responses in EAO mouse testis, by generating mice with a SC-specific knock-down of activin A followed by immunisation with testicular homogenate (TH).

2. MATERIALS AND METHODS

2.1. Materials

All materials, buffers, antibodies and primers used are listed in the **Appendix**.

2.1.1. Human testicular biopsies

Bouin's-fixed and paraffin-embedded human testicular biopsies were kindly provided by the Giessen Testicular Biopsy Repository. Samples were obtained from men diagnosed with non-obstructive azoospermia (SCO) and with focal inflammatory lesions associated with impaired spermatogenesis (Schuppe and Bergmann, 2013). Biopsies with qualitatively and quantitatively intact spermatogenesis, without any signs of inflammation or tubular atrophy, from patients with obstructive azoospermia, served as controls. The study was approved by the ethics committee of the medical faculty of the Justus Liebig University, Giessen (AZ 26/11). Written informed consent was obtained from all men undergoing testicular biopsy.

2.1.2. Animals

EAO study wild-type (WT) mice: Adult 10-12 week-old C57BL/6JCrl mice were purchased from Charles River Laboratories (Sulzfeld, Germany). All animal experiments were approved by local ethics committee on animal care (Regierungspraesidium Giessen GI 58/2014 – Nr. 735-GP).

EAO study mice with therapeutic elevation of circulating follistatin levels: Adult 6 week-old C57BL/6J mice were purchased from the Monash Animal Research Platform (MARP) of Monash University, Clayton campus. All animal experiments were approved by the responsible Monash University Animal Ethics committee (MMCB/2015/16).

EAO study *Inhba*SCKO mice: Adult 6-14 week-old male mice, produced as described in **Chapter 2.2.2.**, were bred in the Monash Medical Centre Animal Facility (MMCAF), Clayton. All animal experiments were approved by the responsible Monash University Animal Ethics committee (MMCB2017/40).

In vitro study: For the isolation and treatment of SC and PTC, immature 21-day-old male C57BL/6JCrl mice were purchased from Charles River Laboratories (Sulzfeld, Germany).

All experiments in Germany were carried out in strict accordance with guidelines for care and use of experimental animals of the German law of welfare. Animal experiments conducted in Australia were carried out in strict accordance with the requirements from the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th Edition 2013 and relevant Victorian State legislation.

For all experiments, animals were kept in specific pathogen free (SPF) conditions (12 h light/ dark cycle, 20~22 °C), with access to standard food pellets and water *ad libitum*.

2.2. Methods

2.2.1. Induction of EAO

2.2.1.1. Preparation of testicular homogenate (TH)

Adult male syngeneic or semi-allogeneic mice (C57BL/6) were used for the preparation of total testis homogenates. Mice were killed with CO_2 or isoflurane and the testes collected. Decapsulated testes were mixed 1:1 with sterile 0.9% NaCl and then homogenised using a Potter homogeniser. TH preparations were stored at -20°C.

2.2.1.2. Induction of EAO

EAO was induced by active immunisation with TH in adjuvant using *Bordetella pertussis* toxin as co-adjuvant, according to previously described protocols (Nicolas *et al.*, 2017a, Nicolas *et al.*, 2017b).

EAO induction in WT mice: TH was mixed 1:1 with complete Freund's adjuvant and injected *s.c.* 3 times every 14 days. The immunisation was accompanied by *i.p.* injection of 100 ng *Bordetella pertussis* toxin in 100 µl Munõz Buffer (see **Appendix 10.2.5.** for recipe). Age-matched adjuvant controls, which received 0.9% NaCl in adjuvant (1:1) instead of TH, and untreated mice were also included in all experiments. Animals were sacrificed 30, 50 and 80 days after first immunisation. At day 30 after the first TH-immunisation 33% of mice showed signs of EAO, while 100% animals developed EAO 50 and 80 days after first immunisation.

EAO induction in mice with increased follistatin levels: For investigation of the influence of increased follistatin levels during the course of testicular inflammation, the circulating form of follistatin (FST315) was over-expressed in 6 weeks-old C57BL/6J mice. For this purpose, mice were transfected with a non-replicative recombinant adeno-associated viral vector serotype 6 carrying a gene cassette of follistatin (rAAV-FST315) by injection in the anterior tibialis muscle, as previously described (Nicolas *et al.*, 2017b). 30 days after the injection of the rAAV-FST315 vector or empty vector, which served as control, mice were actively immunised as described above with the following variation: instead of using complete Freund's adjuvant for all three immunisations, mice were immunised only once with TH in complete Freund's adjuvant, followed by two immunisations with TH in incomplete Freund's adjuvant, each accompanied by injections of the *Bordetella pertussis* toxin (Nicolas *et al.*, 2017b).

A development of mild to severe EAO was observed in approximately 75% of mice immunised by this protocol at day 50 after the first TH-immunisation. In the mice treated with rAAV-FST315, serum follistatin levels were increased by 5-fold (about 50 ng/ml) over normal (about 10 ng/ml) at the time of the first immunisation. This increase was consistent among the animals transduced with the FST315 vector.

EAO induction in *Inhba*SCKO mice: In order to investigate the effect of SC-derived activin A on the development of fibrosis during chronic testicular inflammation, EAO was induced in adult *Inhba*SCKO mice using both complete and incomplete Freund's adjuvant, as described above for the mice with increased follistatin levels. Mice, which received 0.9% NaCl in adjuvant (1:1) instead of TH (adjuvant controls) were included as controls. Littermates containing the floxed allele of *Inhba* served as controls. Animals were sacrificed 50 days after the first immunisation.

In all experiments, mice received analgesia in a form of 2.5 mg/ml Tramadol in the drinking water, starting 24 h before each immunisation and until two days post-immunisation. During the TH-immunisation, animals were anaesthetised by inhalation of 3 - 5% isoflurane. For organ collection, animals were euthanatized by inhalation of CO_2 or isoflurane followed by cervical dislocation.

Blood was collected to extract serum and testes were removed and either snap frozen in liquid nitrogen or fixed in Bouin's solution for embedding in paraffin.

2.2.2. Generation of SC specific conditional knock-down model for activin A

To study the influence of SC-derived activin A on the testicular immune environment, as well as the development of inflammation and fibrosis, a knock-down mouse model deficient for activin A derived from SC (*Inhba*SCKO) was generated **(Fig. 8)**.

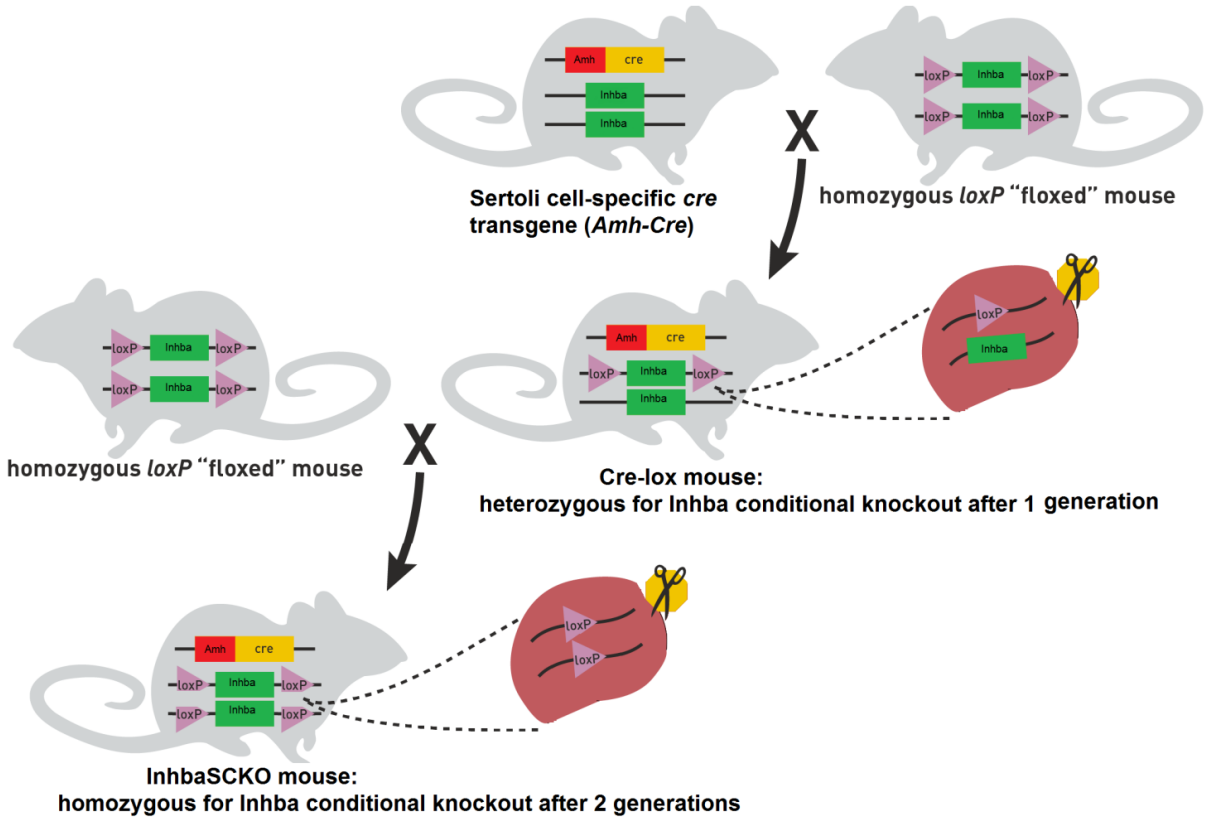

Figure 8: Breeding scheme for generating Cre/lox SC-specific *Inhba* knock-down mice (*Inhba*SCKO). To generate mice that are homozygous for the *Inhba* loxP-flanked *Inhba* allele and heterozygous for the *Amh-Cre* transgene, a homozygous *Inhba* loxP-flanked mouse is mated to an *Amh-Cre* transgenic mouse strain. About 50% of the progeny is heterozygous for the *Inhba* loxP allele and for the *Amh-Cre* transgene. These mice are backcrossed to the homozygous *Inhba* loxP-flanked mice. About 25% of the offspring from this mating will be the experimental male *Inhba*SCKO mice (homozygous for the *Inhba* loxP-flanked allele and heterozygous for the *Amh-Cre* transgene). Image adapted from https://www.jax.org/news-and-insights/jax-blog/2011/september/cre-lox-breeding-for-dummies 18.08.2018.

Mice on a C57-B6/SJL background, harbouring the *Amh-Cre* transgene, express the cre-recombinase enzyme under control of the SC-specific promoter elements of the anti-Mullerian hormone (*Amh*) gene. In these mice, cre-recombinase activity is observed in the SC, with no evidence of cre expression in other tissues (Lecureuil *et al.*, 2002).

Heterozygous *Amh-Cre* transgenic mice (*Amh-Cre*$^{+/-}$) were crossed with mixed 129S6/SvEv/C57BL/6J mice containing two copies of the loxP-flanked *Inhba* gene (*Inhba*$^{loxP/loxP}$) (Pangas *et al.*, 2007), to generate mice with Cre-mediated deletion of both alleles of the floxed *Inhba* specifically in SC (*Amh-Cre*$^{+/-}$/*Inhba*$^{loxP/loxP}$). **Fig. 8** shows the crossing scheme.

This crossing also resulted in the generation of genotype-matched littermates homozygous for the *Inhba* loxP-flanked allele but not harbouring the *Amh-Cre* transgene (*Inhba*FLOX) **(Fig. 9)**, which served as controls in the EAO induction experiments and in the initial phenotypic characterisation of the *Inhba*SCKO mice.

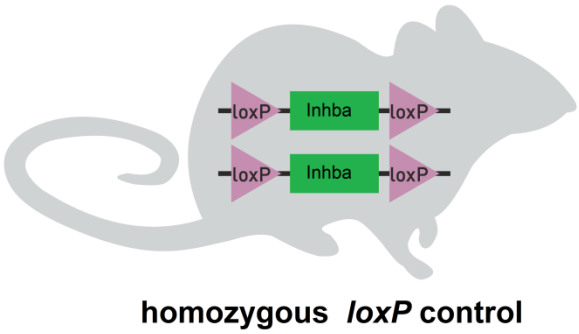

Figure 9: *Inhba*FLOX mice generated through breeding served as controls in the EAO experiment and in the initial characterisation of the *Inhba*SCKO mouse strain. To verify that flanking of *Inhba* by loxP sites did not contribute to a confounding phenotype of interest, littermates with loxP-flanked *Inhba* alleles without *Amh-Cre* were used as a control. Image adapted from https://www.jax.org/news-and-insights/jax-blog/2011/september/cre-lox-breeding-for-dummies18.08.2018.

2.2.3. Cell cultures and treatments

2.2.3.1. Isolation of peritubular cells (PTC) and Sertoli cells (SC) from immature mouse testis

To study the effects of activin A and TNF on the inflammatory and fibrotic responses of PTC and SC, and on the production of activin A in SC, somatic cells were isolated from testes of eight 21 day old C57BL/6J mice. The isolation process was modified and optimised from established protocols for isolation of testicular cells from rodents (Bhushan *et al.*, 2016; Fillion *et al.*, 1994; Okuma *et al.*, 2006; Zeyse *et al.*, 2000).

For this purpose, animals were euthanized and testes collected into sterile Dulbecco's modified Eagle's medium (DMEM)/F12 medium. Subsequently, testes were decapsulated and the seminiferous tubules dissociated. After transfer into a 50 ml Falcon tube, the tissue was digested in 5 ml DMEM/F12 + GlutaMAX containing 1 mg/ml collagenase A and 6 µg/ml DNase I in a 34°C shaking water bath at 60 cycles/min for 20 min to separate the seminiferous tubules from interstitial cells **(Fig. 10A)**. To inhibit the enzymatic activity of the collagenase, 30 ml of DMEM/F12 medium was then added to the tubules and tubules allowed to settle at the bottom for 5 min, before the supernatant was discarded carefully for elimination of interstitial cells. For release of PTC, an enzymatic digestion of the remaining seminiferous tubules in 5 ml 1 mg/ml trypsin in DMEM/F12 + GlutaMAX containing 20 µg/ml DNase I was performed in a 34°C shaking water bath at 60 cycles/min for 20 min **(Fig. 10B)**. For inactivation of the activity of trypsin, 30 ml of DMEM medium containing 10% fetal calf serum (FCS) was added to the seminiferous tubules and the tubules allowed to settle at the bottom for 5 min before the supernatant, containing the PTC, was transferred into a new 50 ml Falcon tube.

The remaining tubules were washed twice with DMEM/F12 medium and each time the supernatant was added to the tube containing the PTC, which then was centrifuged at 400 x g for 10 min at RT, followed by removal of the supernatant and resuspension of the pellet (containing the PTC) in 10 ml DMEM + GlutaMAX with 10% FCS. Afterwards, the suspension was filtered through a 100 µm cell strainer and seeded in a 75 cm^2 flask after addition of further 10 ml DMEM + GlutaMAX containing 10% FCS to the suspension. Isolated cells were cultured for 48 - 72 h at 37°C and 5% CO_2 atmosphere in order to become 80 - 90% confluent **(Fig. 10D)**.

For the purification of SC, remaining seminiferous tubules were digested in 5 ml 1 mg/ml hyaluronidase in DMEM/F12 + GlutaMAX containing 6 µg/ml DNase I for 15 min in a 34°C shaking water bath at 60 cycles/min **(Fig. 10C)**. Tubules were then dissociated by aspirating and releasing them for 10 times using an 18 G needle, and DMEM/F12 added to deactivate the hyaluronidase, before they were centrifuged at 400 x g for 3 min. After removal of the supernatant, the pellet was resuspended in DMEM/F12 + GlutaMAX, and filtered through a 100 µm cell strainer into a new tube to remove cell aggregates. Next, the cell suspension was centrifuged at 400 x g for 3 min and the supernatant discarded. The cells were resuspended in 1 ml DMEM + GlutaMAX containing 0.1% BSA and stained with trypan blue for cell counting using a Neubauer improved counting chamber. Finally, cells were resuspended to a concentration of 1 x 10^6 cells/ml and 500 µl of cell suspension seeded in a 24-well plate.

SC were cultured for 48 h at 34°C and 5% CO_2 atmosphere before they were treated by hypotonic shock to remove contaminating germ cells. Hereby, medium was removed from each well and cells then incubated for 1.5 min with 300 µl ice cold 20 mM Tris-HCl solution. SC were washed once and afterwards allowed to rest for 24 h in DMEM + GlutaMAX containing 0.1% BSA before commencing treatment.

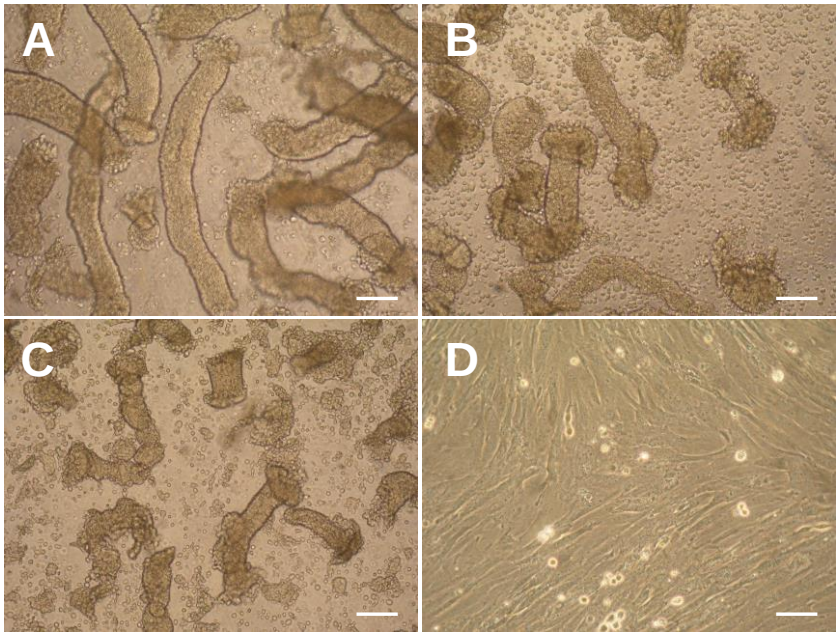

Figure 10: Isolation of PTC from mouse testes. Enzymatic digestion of immature C57BL/6J mouse testes with collagenase leads to dissociation of the seminiferous tubules. Subsequent washing steps are performed to remove interstitial cells **(A)**. Digestion with trypsin results in release of PTC into the supernatant **(B)**. After harvesting of PTC, fragments of seminiferous tubules were further digested by hyaluronidase resulting in very small tubular fragments **(C)**. Isolated PTC were cultured at 37°C and 5% CO_2 atmosphere till confluency **(D)**. Scale bar = 100 µm.

2.2.3.2. Evaluation of SC and PTC purity by double immunofluorescence staining with SOX9 and αSMA

For the estimation of the purity of isolated SC and PTC, an immunofluorescence staining was performed using the cell type specific markers SOX9 (SRY-box9) and αSMA for SC and PTC, respectively **(Fig. 11)**. SC and PTC were seeded in a density of 1.25 x 10^5 cells/well in 8 well chamber culture slides, hypotonic shock performed on SC after 48 h and cells cultured for further 24 h.

Cells were washed with PBS and fixed for 10 min in ice cold methanol. Subsequently, cells were permeabilised in 0.1% Triton X and blocked with 10% normal goat serum in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h. Cells were then washed twice in TBST for 5 min and incubated overnight with the mouse monoclonal FITC conjugated anti-αSMA antibody diluted 1:800 in TBST and/or rabbit polyclonal anti-SOX9 antibody diluted 1:100 in TBST. Cells were then washed twice for 5 min and incubated with secondary goat anti-rabbit IgG AlexaFluor 594 conjugate diluted 1:1000 in TBST for 1 h in the dark. Finally, cells were washed twice with TBST, and slides mounted with ProLong Gold mounting medium containing DAPI.

As the isolation method comprises the separation of the seminiferous tubules from the interstitial cells, allowing the removal of all interstitial cells, no further staining for macrophages was performed. However, the exclusion of a small contamination by germ cells is not possible.

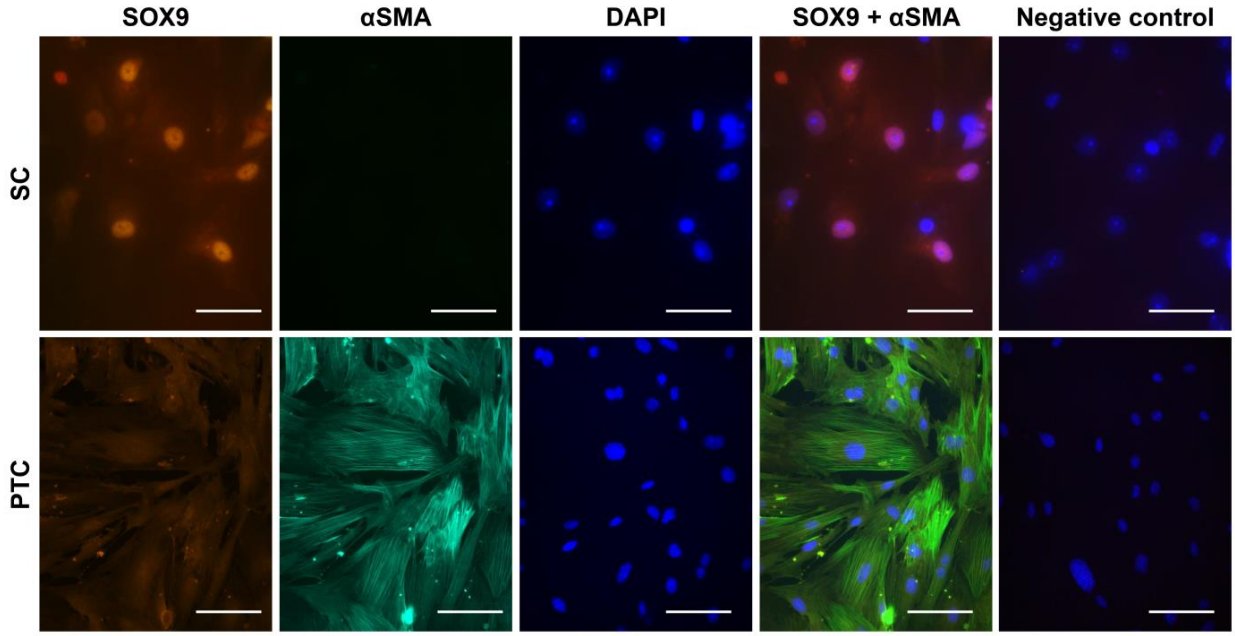

Figure 11: Immunofluorescence staining of isolated SC (upper panel) and PTC (lower panel) shows > 85 % purity of the cultured cells. FITC-αSMA was used as PTC marker and SOX9 (AlexaFluor 594; red) as SC marker. Absence of positive FITC-αSMA (green) cells in the upper panel indicates absence of PTC and hence high purity of isolated SC. while absence of SOX9 in the lower panel shows a high purity of PTC after passaging, with no contamination with SC. No fluorescence was observed when only the secondary goat anti-rabbit IgG AlexaFluor 594 conjugate was used (negative control). Purity of cells was calculated as number of contaminating cells per total number of cells; Scale bars = 50 µm.

2.2.3.3. Treatment of PTC

Prior to treatment of the PTC, isolated, confluent PTC were split in a ratio of 1:1 and passaged into two new 75 cm^2 flasks. This procedure was necessary for the removal of contaminating interstitial and germ cells. Cells were washed twice with PBS and detached using 1 ml 0.05% trypsin for 2 - 3 min at 37°C. The addition of DMEM + GlutaMAX containing 10% FCS inactivated the trypsin and cells were then collected by centrifugation at 400 x g for 10 min at RT. Supernatant was removed and the cell pellet resuspended in 20 ml DMEM medium containing 10% FCS and transferred into two new 75 cm^2 flasks. Subsequently, PTC were grown until confluency and then seeded in a density of 2.5 x 10^5 cells/well (12 well plate) for later protein isolation or 1.25 x 10^5 cells/well (24 well plate) for isolation of RNA. Before beginning of the treatment, the cells were kept under minimal maintenance conditions in DMEM + GlutaMAX with 1% FCS for 24 h.

For the investigation of the effect of recombinant activin A and follistatin 288 (FST288) on fibrotic responses in PTC *in vitro*, cells were treated with recombinant activin A (25 ng/ml and 50 ng/ml for RNA and protein isolations, respectively) and/or 100 ng/ml of human recombinant FST288, while for investigation of the effect of TNF on the inflammatory response, 25 ng/ml recombinant TNF was used; in DMEM + GlutaMAX with 1% FCS either for 4 h (RNA isolation) or 48 h (protein isolation). Measurement of SMAD2 and SMAD3 phosphorylation was performed on isolated protein from cells treated for 30 and 45 min. Media were collected and frozen at -20°C. Experiments were carried out from at least four independent cell isolations. Each treatment was performed in duplicate or triplicate.

2.2.3.4. Treatment of SC

For the analysis of the production of activin A by SC following TNF and FST288 treatment, cells were treated for 24 h with 25 ng/ml and 50 ng/ml TNF and 100 ng/ml FST288 in DMEM + GlutaMAX with 0.1% BSA. For investigation of the inflammatory response of SC, cells were treated with 1 µg/ml and 10 µg/ml LPS. Media were collected and frozen at -20°C for activin A ELISA or qRT-PCR.

2.2.3.5. Cell culture and treatment of NIH 3T3 cells

NIH 3T3 fibroblast cells were obtained from the research group of Dr. Oliver Eickelberg, Department of Internal Medicine, Justus Liebig University Giessen. The cells were cultured in DMEM + GlutaMAX containing 10% FCS and grown at 37°C under 5% CO_2. When grown to a confluency of 80 - 90% (approximately 4 days), cells were passaged into a new 75 cm^2 flask to maintain proper proliferation. Next, cells were washed twice with PBS and detached using 1 ml 0.05% trypsin for 2 min at 37°C. The addition of DMEM + GlutaMAX containing 10% FCS inactivated the trypsin and cells were then collected by centrifugation at 300 x g for 7 min at RT. Supernatant was removed and the cell pellet resuspended in 1 ml DMEM medium containing 10% FCS. Subsequently, 1/10 of the cell suspension was transferred into a new 75 cm^2 flask and 20 ml DMEM + GlutaMAX containing 10% FCS was added.

For the investigation of the effect of activin A and FST288 on the fibrotic response in the NIH 3T3 cells and the analysis of the effect of TNF on the inflammatory response of NIH 3T3 cells, cells were seeded at a density of 2.5 x 10^5 cells/well into 12 well plates when assigned for protein isolation or 1.25 x 10^5 cells/well into 24 well plates for RNA isolation. Cells were allowed to attach for 24 h before the medium was changed to DMEM + GlutaMAX with 1% FCS for 24 h. The cells were then treated with recombinant activin A (25 ng/ml and 50 ng/ml for RNA and protein isolations, respectively) and/or FST288 (100 ng/ml) in DMEM + GlutaMAX with 1% FCS for 24 h (RNA analysis) or 48 h (protein analysis). Cells treated for 30 and 45 min for measurement of SMAD2 and SMAD3 phosphorylation from isolated protein.

2.2.4. Histology

2.2.4.1. Mouse histology

Assessment of the EAO damage score ranging from 0 (no development of EAO) to 5 (fully developed and severe EAO) was based on levels of inflammatory mediators, extent of immune cell infiltration, testicular damage and fibrosis, as previously described (Nicolas *et al.*, 2017b). The scores were assigned as follows:

- Score 0: Normal testicular histology
- Score 1: Normal testicular histology, but with significantly increased mRNA levels of inflammatory mediators within the testis, assessed by qRT-PCR

- Score 2: Focal leukocytic infiltrates accompanied by significant damage and reduced diameter of some seminiferous tubules, normal spermatogenesis in most seminiferous tubules, and significantly increased mRNA levels of inflammatory mediators
- Score 3: Widespread destruction of the seminiferous epithelium with loss of germ cells, accompanied by numerous leukocytic infiltrates and significantly increased mRNA levels of inflammatory mediators
- Score 4: Significantly reduced testis weight, severely disrupted spermatogenesis, a reduced diameter of majority of the seminiferous tubules, numerous leukocytic infiltrates and significantly increased mRNA levels of inflammatory mediators
- Score 5: Significantly reduced testis weight with complete loss of normal testicular morphology characterised by absence of germ cells, atrophy of the seminiferous epithelium, an extensive infiltration of the entire testis by immune cells and significantly increased mRNA levels of inflammatory mediators

2.2.4.2. Human histology

Human testicular biopsies were stained with haematoxylin and eosin (Bergmann and Kliesch, 2010) according to standard protocols. The overall descriptive histopathological assessment was combined with a score count evaluation of spermatogenesis (scores ranging from 10 to 0, identified as number of tubules containing any elongated spermatids divided by 100 as the total number of tubules examined, multiplied by 10) (Bergmann and Kliesch, 2010).

2.2.4.3. Paraffin-embedding

Collected testes were fixed in Bouin's solution for 5 h and subsequently dehydrated in increasing ethanol series starting from 70% (min. 12 h) and followed by 95% (2 - 6 h) and 100% (2 - 6 h), with the solutions being changed several times. Afterwards, testes were transferred into pure benzoic acid methyl ester for 2 h, followed by an incubation in xylene for 2 h before transferring them into a xylene and paraffin (1:1) mixture for 1 h at 60°C. Finally, testis samples were transferred to pre-warmed liquid paraffin at 60°C overnight and embedded in paraffin on the next day.

2.2.4.4. Haematoxylin and eosin (H&E) staining

Haematoxylin and eosin staining was used to assess testicular morphology and the EAO damage score as well as to identify the areas of lymphocytic infiltrates in 5 µm thick Bouin's-fixed and paraffin-embedded human and mouse testis sections. Haematoxylin and eosin stains nuclei dark blue/purple, while cytoplasmic components are stained in varying shades of pink. 5 µm paraffin embedded sections were heated in an oven maintained at 60°C for 20 min. Subsequently, sections were dewaxed in xylene 3 times for 2 min each, rehydrated in 100% ethanol 3 times and 70% ethanol once for 2 min each and rinsed in running tap water for 30 s. Afterwards, sections were immersed in Harris's haematoxylin solution for 7 min and rinsed in running tap water until water runs clear. To achieve differentiation, sections were immersed in acid alcohol solution for 1 s. Following differentiation, sections were rinsed in running tap water for 1 min prior to 'blueing' in Scott's tap water substitute (ammonia water) for 1 min and then washed further 1 min in running tap water. To ensure adequate nuclear staining, slides were examined using a light microscope. Over-staining required the re-immersion of the sections in acid alcohol, while insufficient staining required a further incubation in haematoxylin solution. Slides were then submerged in 1% alcoholic Eosin Y solution for 7 min, followed by dehydration in 100% ethanol 3 times for 2 min and cleaning in xylene 3 times for 2 min before being mounted with coverslips using Entellan new mounting medium.

2.2.4.5. Azo-carmine and aniline blue (azan) staining

Azan staining was performed for the evaluation of fibrosis in 5 µm paraffin sections of human and mouse testis.

The sections were deparaffinised in xylene 3 times for 5 min, rehydrated through a series of decreasing ethanol concentrations (100%, 96% and 70%) for 5 min each, rinsed in distilled water and then stained in aniline alcohol for 5 min. Subsequently, sections were stained with preheated (56°C) 0.1% azo-carmine G solution for 15 min, rinsed in distilled water and nuclei differentiated in aniline alcohol. The sections were again rinsed in distilled water before the connective tissue was stained in 5% phosphotungstic acid for 2 h. After a quick rinse in distilled water, the sections were stained in aniline blue-orange solution for 1 h, followed by a differentiation of tissue constituents in 96% ethanol. Afterwards, the sections were dehydrated in isopropanol for 5 min and treated with xylene 3 times for 5 min before they were mounted in Entellan new mounting medium.

In the stained sections collagen fibres were visualised in blue, connective tissue, chromatin and erythrocytes in red (Kikui and Miki, 1995; Goldner, 1938).

2.2.4.6. Masson's trichrome staining

Similar to azan staining, Masson's trichrome staining is one of the most widely used trichrome stains for visualisation of connective tissues, particularly collagen, in tissue sections. Following a standard procedure, collagen is stained blue, nuclei are stained dark brown, and cytoplasm is stained pink (Leonard *et al.*, 2018).

5 µm paraffin embedded sections were heated in an oven maintained at 60°C for 20 min, de-paraffinised in xylene 3 times for 2 min, rehydrated in 100% ethanol 3 times, 70% ethanol 1 time, and 50% ethanol 1 time for 2 min each and rinsed in running tap water for 30 s. Sections were stained for 5 min in celestin blue solution, washed in tap water for 2 min and then stained in Weigert's iron haematoxylin solution for 30 min. After subsequent washing in running tap water for 5 min, tissue components were differentiated in acidic alcohol solution for 1 s and sections washed again in running tap water for 10 min. Afterwards, sections were stained with Biebrich scarlette-acid fuchsine solution for 5 min, washed with distilled water for 1 min and immersed in 5% phosphotungstic acid for 5 min. Sections were washed again for 5 min before they were stained in aniline blue solution for 5 min and washed in 1% acetic acid for 3 min. Finally, sections were dehydrated in a series of increasing ethanol concentrations (50%, 70%, 100%), cleared in xylene and cover slipped using DPX mounting medium.

2.2.4.7. Morphometrical measurement of seminiferous tubule and lumen diameter

The morphometrical determination of average tubular and luminar diameters per animal was performed by measuring 100 Haematoxylin and eosin stained, randomly selected cross sections of seminiferous tubules presenting a round contour at 100x magnification. By using the closed polygon tool of the OLYMPUS cellSens Dimension software, the tubule or lumen contour of each seminiferous tubule cross section was traced and marked, and from this, the mean diameter for the tubule or lumen was calculated by the software.

2.2.5. Protein analysis

2.2.5.1. Preparation of protein extracts

Testis samples were homogenised with RIPA buffer containing a protease inhibitor cocktail (1:100; mix of AEBSF, Aprotinin, Bestatin, E64, Leupeptin, and Pepstatin A) using a Potter S tissue homogeniser for subsequent use in Western blot or with cold PBS buffer containing a protease inhibitor cocktail (1:100) by using 5 mm stainless steel beads and a Precellys tissue homogeniser at 40 oscillations/s for 1 min for ELISA analysis. Cells were lysed with RIPA buffer containing a protease inhibitor cocktail (1:100) and, in case of intended use for detection of phosphorylated proteins, a phosphatase inhibitor cocktail (1:100), and incubated on ice for 5 min before being scraped with a cell scraper and transferred into an Eppendorf tube. Subsequently, homogenised tissue samples and cell lysates were centrifuged at 14,000 x g for 10 min at 4°C and transferred into a new Eppendorf tube to remove insoluble components. All samples were stored at -20°C.

2.2.5.2. Measurement of protein concentration

Measurement of total protein concentration was conducted using the Pierce BCA Protein Assay according to the manufacturer's instructions. Briefly, samples were diluted 1:10 or 1:5 in lysis buffer. As a standard, concentrations of the provided BSA ranging between 0 and 2000 µg/ml were used and prepared in lysis buffer. 10 µl of each standard and each sample were pipetted as duplicates into a well of a 96 well microplate and 200 µl of the BCA working reagent (1 part of reagent B and 50 parts of reagent A) added to each well. Subsequently, the plate was incubated for 30 min at 37°C and the absorbance measured by a spectrophotometer at either 570 nm (TriStar LB 941, Justus-Liebig University) or 492 nm (Multiskan RC, Monash University). Finally, the protein concentrations were determined based on a standard curve.

2.2.5.3. Sodium-dodecyl-sulphate (SDS) polyacrylamide gel electrophoresis (PAGE)

SDS PAGE (Laemmli, 1970) was performed in order to separate proteins according to their size. This is possible as SDS confers an overall net negative charge on proteins in proportion to their molecular mass. A 7.5% - 12.5% resolving gel solution was poured into the assembled gel mould between two glass plates separated by 1 mm spacers. After polymerisation of the resolving gel, the stacking gel solution was poured on top. See **Appendix 10.2.2.** for gel and buffer recipes.

Lysed NIH 3T3 fibroblast or PTC cell samples as well as testicular extracts were prepared in 4 x Laemmli sample buffer containing 20% β-mercaptoethanol and boiled for 5 min at 95°C to denature proteins. The fully polymerised gel was mounted in the electrophoresis chamber and the chamber filled with 1X SDS Running Buffer. Total protein (30 µg) was loaded into each lane onto 7.5%, 10% or 12.5% SDS-polyacrylamide gels, depending on the size of the protein of interest. As a standard, 5 µl of BlueEasy Prestained protein marker was loaded. Electrophoresis was performed at constant 85 V – 125 V.

2.2.5.4. Western blotting

After performance of the electrophoresis, proteins were transferred into a nitrocellulose membrane using a wet or semi-dry electro blotter, blocked with 5% non-fat milk in TBST for 1 h at RT, the membrane subsequently washed 3 times for 5 min in TBST and incubated with primary antibodies in 5% BSA in TBST overnight at 4°C. See **Appendix 10.4.** for primary and secondary antibodies used, and dilutions. Membranes were washed 3 times for 5 min in TBST, incubated for 1 h with secondary antibodies in 5% non-fat milk in TBST and washed again 3 times for 10 min in TBST. Membranes were probed using SuperSignal West Pico chemiluminescent substrate and subsequently developed with Fusion FX Western Blot & Chemi imaging system. The results were analysed with the Vilber Lourmat FusionCapt Advance Solo 4 16.07 software.

If necessary, the blots were stripped with stripping buffer for 2 min in a 60°C water bath, subsequently washed 3 times for 5 min in TBST and probed again.

2.2.5.5. Quantification of Western Blots

Western blots were quantified with the Vilber Lourmat FusionCapt Advance Solo 4 16.07 software. β-actin was used as a loading control for all Western blotting experiments. The intensity value for the protein of interest was normalised to the intensity value of β-actin for each lane on each gel and expressed as a percentage. Western blots were repeated at least 3 times and the values from each gel were averaged for statistical analysis.

2.2.5.6. Activin A enzyme-linked immunosorbent assay (ELISA)

Total activin A content in the supernatants of SC was measured using a specific ELISA (Knight *et al.*, 1996) according to the manufacturer's instructions (Oxford Bio-Innovations, Cherwell, Oxfordshire, UK) with some modifications as previously described (Okuma *et al.*, 2006). Notably, this assay is not affected by the presence of follistatin. See **Appendix 10.2.6.** for buffer and solution recipes.

Briefly, a Corning 96 well plate was coated with 100 µl/well anti-βA subunit antibody (E4) at a concentration of 650 ng/ml in carbonate buffer, and the plate subsequently incubated overnight in a humidified box at RT.

On the next day, the plate was washed 4 times with an automated plate washer with Wash Buffer and afterwards blocked with 300 µl of Blocking Buffer for 1 - 2 h at RT.

Human recombinant activin A was used as standard, diluted in Assay Buffer ranging from 8.23 - 2000 pg/ml. 125 µl of standard or sample and 125 µl of a 6% SDS solution were added to borosilicate tubes and the tubes boiled for 3 min. The samples were allowed to cool before the addition of H_2O_2 to a final concentration of 2% (v/v) and subsequent incubation for 30 min at RT. The plates were washed 4 times before the addition of 25 µl 20% BSA/Tris/Triton buffer to each well and incubated for 1 h at RT shaking before addition of 25 µl biotinylated-E4 detection antibody (1:600) prepared in 5% BSA/Tris/Triton buffer to the wells and incubated overnight in a humidified box at RT.

On day 3, the plates were washed 3 times with Wash Buffer before addition of 100 µl of streptavidin poly-HRP (1:4000) in 1% casein colloid/PBS solution to all wells and incubation for 1 h shaking on a microplate shaker at 600 rpm at RT. After further 3 times wash with Wash Buffer, 100 µl TMB Single Solution was added and the plates incubated for 15 min before addition of 50 µl 0.3M HCl to each well. Subsequently, the plates were read at 450 nm with a 630 nm reference filter on a Multiskan RC plate reader and data processed using the Genesis Lite EIA software.

The mean sensitivity of the activin A ELISA was 11 pg/mL – 2000pg/ml (n = 6 independent experiments) with an average intra-assay coefficient of variability (CV) of 3.3 % and an inter-plate CV of 7%.

2.2.6. DNA and RNA analysis

2.2.6.1. RNA extraction from snap frozen tissues and cells

Total RNA was isolated from snap frozen mouse und human testes, NIH 3T3 cells, SC and PTC using the RNeasy Mini kit according to manufacturer's instructions. To ensure the complete removal of genomic DNA (gDNA) contamination an on column DNase I digestion was performed for 15 min at RT.

2.2.6.2. RNA extraction from paraffin embedded human testicular biopsies

Total RNA was isolated from paraffin-embedded human testicular biopsies using the RNeasy FFPE kit following manufacturer's instructions.

Briefly, three 10 µm sections were prepared and transferred into a sterile 2 ml vial. Subsequently, 1 ml xylene was added, the samples mixed and centrifuged for 2 min at > 14,000 x g. After disposal of the supernatant 1 ml 100% ethanol was added. The samples were then mixed and centrifuged for 2 min at > 14,000 x g. The supernatant was discarded and the pellet dried at 37°C. Afterwards the pellet was resuspended in 240 µl PKD buffer and 10 µl proteinase K, mixed, and incubated at 55°C in a water bath for 30 min, followed by incubation at 80°C in a water bath for 15 min, to remove formalin linkages in the tissue. To remove any contaminating gDNA, the extracted mRNA was incubated with RNase-free DNase I for 15 min at RT. After addition of 500 µl RBC buffer and 1200 µl 100% ethanol, the samples were mixed and 700 µl transferred onto the spin column membrane. This step was followed by centrifugation of the columns for 15 s at > 10,000 x g and disposal of the flow-through. Subsequently, 500 µl RPE buffer were added to the column, the samples centrifuged for 15 s at > 10,000 x g and the flow-through discarded. Addition of 500 µl RPE buffer was followed by centrifugation of the samples for 2 min at > 10,000 x g and disposal of the flow-through. To ensure the complete drying of the membrane, the spin column was placed in a new 2 ml vial and centrifuged with the lid open for 5 min at > 14,000 x g. Flow-through was discarded and the spin column placed in a new 1.7 ml vial. To elute RNA, 15 µl RNase free water was added, the samples centrifuged for 1 min at RT and > 14,000 x g and finally the RNA stored at -80°C.

2.2.6.3. Measurement of RNA concentration and purity

After extraction, RNA quantity and purity were determined by a NanoDrop ND 2000 Spectrophotometer using 1.0 µl total RNA. The optical density (OD; OD of 1 was equated to 40 µg/ml RNA) at 260 nm (absorption maxima of nucleic acids) was used to measure the concentration. Measurements at 280 nm (absorption maxima of proteins), and 230 nm (absorption of contaminants) were performed to verify the purity of the isolated RNA. Ratios of OD 260/280 ≥ 2.0 and OD 260/230 = 2.0 - 2.2 indicate a sufficient purity of the isolated RNA for downstream analysis, such as qRT-PCR. A lower OD260/280 ratio indicates the presence of protein, while a lower OD260/230 ratio may indicate the presence of other contaminants, which absorb at 230 nm.

2.2.6.4. Complementary deoxyribonucleic acid (cDNA) synthesis

cDNA synthesis was performed according to established protocols (Fijak *et al.*, 2015; Pleuger *et al.*, 2017).

RNA from mouse and human testes as well as isolated testicular cells was reverse transcribed using 0.5 - 1.2 µg of total RNA. RNA was mixed with 2 µl oligo-dT_{15} primer and pre-incubated at 70°C for 10 min. The qRT-PCR mixture (containing 1 µl recombinant RNasin ribonuclease inhibitor [200 U], 8 µl 5x M-MLV buffer, 2 µl 10 mM of dNTP and 18 µl RNase free water) was pre-incubated at 42°C for 2 min. Subsequently, the qRT-PCR mixture and 1 µl (200 units) M-MLV reverse transcriptase were added to the mRNA/oligo-dT_{15} primer mix and reverse transcribed at 42°C for 75 min. Further incubation at 70°C for 15 min was performed for the enzymatic inactivation.

Reverse transcription of testicular RNA from the EAO induction in *Inhba*SCKO mice was performed using the SuperScript III First-Strand Synthesis System and 800 ng total RNA, diluted with RNase free water to total volume of 8 µl. RNA was mixed with 1 µl random hexamers (50 ng/µl) and 1 µl dNTPs (10 mM) and pre-incubated at 65°C for 5 min. Subsequently, RT mixture (containing 2 µl 10X RT buffer, 4 µl MgCl2 [25 mM], 2 µl DTT [0.1 M], 1 µl RNaseOUT [40 U/µl] and 1 µl of SuperScript III reverse transcriptase [200 U/µl]) was added to each sample and the samples incubated at 25°C for 10 min. Afterwards, samples were heated to 50°C for 50 min, followed by 85°C for 5 min. Finally, 1 µl *E. Coli* RNase H (2 U/µl) was added to each sample and samples incubated at 37°C for 20 min.

By following the same reverse transcription procedure, but omitting the SuperScript III reverse transcriptase (200 U/µl) from the RT mixture, the absence of an amplified PCR product after RT-PCR verified the lack of gDNA contamination in the RNA samples.

All cDNA samples were stored at -20°C.

2.2.6.5. Verification of absence of genomic DNA contamination by β-actin polymerase chain reaction (PCR) amplification

The β-actin (*Actb*) transcript was amplified using the isolated RNA sample as a template. Primers are listed in the **Appendix 10.5.1.**, PCR conditions and reaction set up are listed in **Table 1** and **Table 2**, respectively.

Table 1: Reaction setup for the amplification of β-actin.

Component	Volume per 25 µl reaction
Green Go Taq Flexi buffer (5x)	5.0 µl
$MgCl_2$ (25 mM)	2.0 µl
Forward Primer (10 pM)	0.5 µl
Reverse Primer (10 pM)	0.5 µl
dNTP (10 mM)	0.5 µl
Go Taq G2 Flexi DNA polymerase (5u/µl)	0.25 µl
RNA template	1 µl
ddH_2O	15.25 µl
Total reaction mix volume	25µl

Table 2: PCR conditions for β-actin amplification.

94°C	4 min	
94°C	40 s	25 cycles
55°C	40 s	
72°C	40 s	
72°C	10 min	
4°C	∞	

The PCR product was visualised on a 1% agarose gel as described in **Chapter 2.2.6.6**. The cDNA positive control showed a β-actin band at the expected size of 156 bp. This band was missing in the RNA samples in the absence of gDNA contamination and the negative control containing water instead of RNA.

2.2.6.6. Agarose gel electrophoresis

Agarose gel electrophoresis was performed to analyse DNA according to its molecular weight. To analyse PCR products, samples were mixed 1:10 with 10X loading dye and run on a 1% (w/v) agarose containing SYBR Safe DNA gel stain. The gel was prepared and run with 1X TAE buffer, which was also used to run the gel at constant 110 V (2 - 10 V/cm gel). For estimation of the size and amount of DNA in the applied sample, a DNA marker was run in parallel. Bands were visualised by UV-light using the Universal Hood II GelDoc System and the Quantity One 1-D Analysis Software. Agarose gels were photographed using a Gel Jet Imager 2000 documentation system.

2.2.6.7. Genotyping of transgenic mice

gDNA was isolated from mouse-tail biopsies. Tail tips were incubated in 200 µl of lysis buffer I at 95°C for 1 h. Subsequently, an equal amount of neutralisation buffer II was added to the tissue lysate, the lysate mixed by inverting the tubes several times and centrifuged at full speed for 1 min. Buffer recipes are described in **Appendix 10.2.1**.

Extracted gDNA was then used as template DNA for PCR amplification. Primers are listed in the **Appendix 10.5.2.**, PCR conditions and reaction set up are listed in **Table 3** and **Table 4**, respectively.

The PCR product was visualised on a 1% agarose gel as described in **Chapter 2.2.6.6**.

Table 3: PCR conditions for genotyping of transgenic mice.

95°C	3 min	
95°C	30 s	35 cycles
62°C	30 s	
72°C	30 s	
72°C	5 min	
4°C	∞	

Table 4: Reaction setup for genotyping PCR amplification using *Taq* DNA polymerase.

Component	Volume per 25 µl reaction
Taq DNA Polymerase buffer (10X)	2.5 µl
$MgCl_2$ (50 mM)	1.0 µl
Forward Primer (20 µM)	0.5 µl
Reverse Primer (20 µM)	0.5 µl
dNTP (10 mM)	0.5 µl
Taq DNA Polymerase (5 U/µL)	0.2 µl
DNA template	2.0 µl
RNase free H_2O	18.5 µl
Total reaction mix volume	25µl

2.2.6.8. Quantitative real-time PCR (qRT-PCR)

For quantitative analyses of mRNA expression of a target gene, PCR amplification under real time conditions was performed, whereby the fluorescent dye SYBR Green, which only intercalates double-stranded DNA, was used for the detection of the amplified PCR products (Arya *et al.*, 2005). The quantity of a specific gene is calculated as the relative amount after normalisation to stable expressed housekeeping genes.

For samples from WT mice testes (EAO study), human testicular biopsies and isolated testicular cells, qRT-PCR was performed using CFX TouchTM Real-Time PCR detection system and iTaq Universal SYBR Green Supermix in a total volume of 20 µl, while for cDNA from the EAO experiment on *Inhba*SCKO mice the Fast Real-Time PCR 7900HT and Power SYBR green PCR master mix in a total volume of 10 µl was used. Both reaction set ups are described in **Table 5**. As reference, mRNA expression analysis of hypoxanthin-guanin-phosphoribosyltransferase (*Hprt*), beta-2 microglobulin (*B2m*) and 18S rRNA was used in mouse samples, and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) in human samples. The selection of the housekeeping genes was based on their stable expression under all treatment conditions.

For each target gene, technical duplicates or triplicates were performed, which then were averaged for further calculations. For details of the PCR conditions see **Table 6**. The primer sequences are shown in **Appendix 10.5.1**. All primers were designed with an annealing temperature of 55°C or 60°C.

Table 5: PCR conditions used for qRT-PCR of WT (C57BL/6JCrl) mouse testes, human testicular biopsies and isolated testicular cell samples, using the iTaq Universal SYBR Green Supermix, and for mouse testes from the *Inhba*SCKO EAO study, using the Power SYBR green PCR master mix.

iTaq Universal SYBR Green Supermix		**Power SYBR green PCR master mix**		
95°C	1 min			
95°C	15 s	95°C	10 min	
55°C/60°C	30 s (45 cycles)	95°C	15 s (40 cycles)	
72°C	30 s	55°C/60°C	1 min	
	Data collection	95°C	15 s	Dissociation stage
50°C	10 s (melt curve)	60°C	15 s	
20°C	∞	95°C	15 s	

Table 6: Reaction setup for qRT-PCR of WT (C57BL/6JCrl) mouse testes, human testicular biopsies and isolated testicular cell samples, using the iTaq Universal SYBR Green Supermix, and of mouse testes from *Inhba*SCKO EAO study, using the Power SYBR green PCR master mix.

iTaq Universal SYBR Green Supermix		**Power SYBR green PCR master mix**	
Component	Volume per 20 µl reaction	Component	Volume per 10 µl reaction
iTaq Universal SYBR Green supermix (2x)	10 µl	Power SYBR green PCR master mix	5 µl
Forward Primer (10 µM)	0.5 µl	Forward Primer (10 µM)	0.5 µl
Reverse Primer (10 µM)	0.5 µl	Reverse Primer (10 µM)	0.5 µl
DNA template	1 µl	DNA template	1 µl
RNase free H_2O	8 µl	RNase free H_2O	3.5 µl
Total reaction mix volume	20 µl	*Total reaction mix volume*	10 µl

Each qRT-PCR was followed by a melt curve analysis to check the formation of a single, specific PCR product. With increasing temperature, the denaturing double-stranded DNA becomes single-stranded. Since the intercalating dyes fluoresce only when they are bound to double-stranded DNA, the presence of single-stranded DNA results in the release of the intercalated fluorescent dye and decrease of the fluorescent signal. The melt curve is then obtained by plotting the change in slope of this fluorescence curve as a function of temperature. A single peak shows a single, specific amplicon, while the occurrence of more than one peak represents nonspecific amplification. A representative melt curve is depicted in **Fig. 12**.

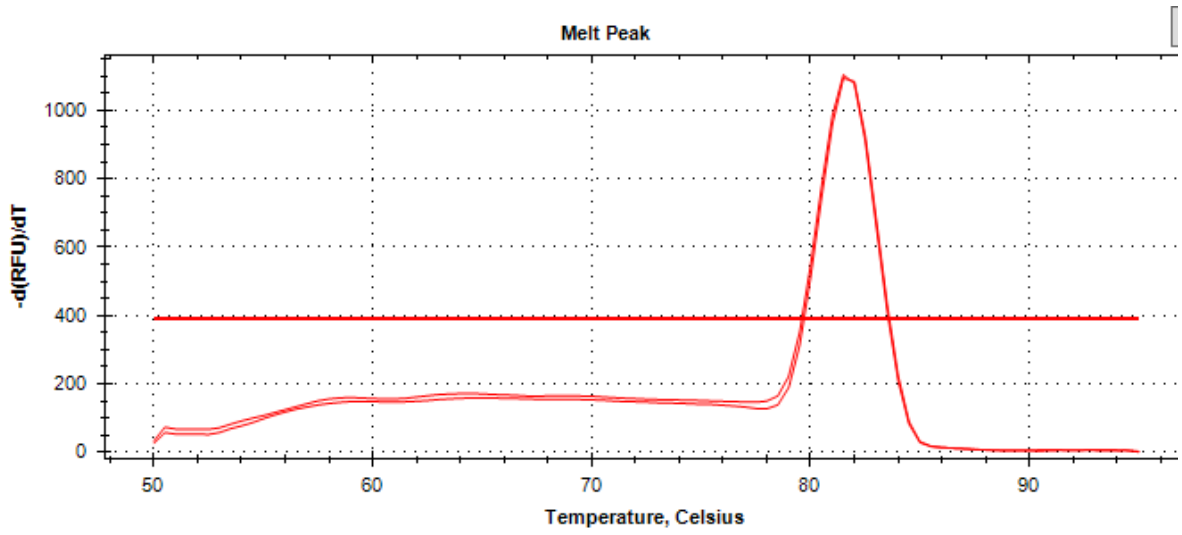

Figure 12: Representative melt curve from qRT-PCR of a 18S rRNA transcript performed in duplicates. The single amplicons from both 18S rRNA duplicates show single peaks at 81.5°C following melt curve analysis.

2.2.7. Immunohistochemistry

2.2.7.1. Double immunofluorescence staining of F4/80 + collagen type I and CD45 + collagen type I

Double immunofluorescent staining was performed on 8 µm cryosections of mouse testis to investigate the co-localisation of F4/80 or CD45 with collagen type I.

Cryosections were rehydrated 10 min in TBST, fixed for 10 min in ice cold methanol (-20°C), permeabilised in 0.1% Triton X-100 in TBS for 15 min and blocked with 10% normal goat serum in 1% BSA/TBST for 1 h.

Sections were then washed twice in TBST for 5 min and incubated overnight at 4°C with a rat monoclonal anti-mouse F4/80 antibody diluted 1:200 and a rabbit polyclonal anti-mouse collagen I antibody diluted 1:200 in 1% goat serum in TBST. Sections were then washed twice with TBST for 2 min and incubated with a secondary goat anti-rabbit IgG AlexaFluor488 and goat anti-rat IgG AlexaFluor546 conjugated antibodies diluted 1:1000 in TBST for 1 h in the dark. Finally, sections were washed twice with TBST for 5 min, and slides mounted with ProLong Gold mounting medium containing DAPI.

2.2.7.2. Activin A immunohistochemistry staining on mouse testis sections

To assess the knock-down of *Inhba* in the SC in the *Amh-Cre*$^{+/-}$ *Inhba*$^{loxP/loxP}$ mice, activin A immunohistochemistry staining on mouse testicular sections was performed using the Mouse on Mouse (M.O.M.) Basic Kit and the VECTASTAIN Elite ABC HRP Kit according to Manufacturer's instructions.

Briefly, 5 µm tissue sections from Bouin's-fixed and paraffin-embedded mouse testes were deparaffinised in histosol twice for 5 min each, rehydrated through decreasing ethanol concentrations (twice 100%, followed by 70% for 5 min each), rinsed in distilled water and further rehydrated for 5 min in distilled water. For antigen retrieval, sections were then boiled in a microwave in citrate buffer (see **Appendix 10.2.3.** for recipe) for 3 min at 1000 W followed by 7 min at 450 W, left to cool down in the citrate buffer for 20 min and washed in TBS for 5 min. To quench any endogenous peroxidase activity, sections then were immersed in 3% (v/v) H_2O_2 in distilled water for 5 min and washed twice for 2 min. Blocking of non-specific binding sites was achieved by incubation for 1 h with the working solution of the mouse IgG blocking reagent at RT. This step was followed by washing in TBS twice and pre-incubation in the working solution of the M.O.M. diluent for 5 min. After draining the excess liquid, the sections were covered with the mouse monoclonal anti-mouse E4 activin βA antibody diluted 1:300 in 0.1% BSA in TBS at 4°C overnight. One section of each sample served as negative control and was incubated with the isotype IgG in 0.1% BSA in TBS instead.

On the next day, sections were washed twice in TBS for 2 min and then incubated with the working solution of the M.O.M. biotinylated anti-mouse IgG reagent for 1 h at RT.

Following this, sections were washed twice in TBS for 2 min and incubated with the avidin-biotin-peroxidase complex solution for 30 min at RT. Sections were washed twice in TBS for 5 min and then incubated with the liquid DAB + substrate chromogen system to visualise antibody binding.

The colour development was monitored under the microscope and stopped after 45 s by immersion of sections twice for 5 min in distilled water. Sections were then counterstained in Harris' Haematoxylin for about 10 - 15 s, washed in distilled water to remove excess stain and dipped 3 times in Scott's tap water. After washing in distilled water for 5 min, sections were dehydrated through a series of alcohols; 70% for 3 min, twice 100% for 5 min and finally twice histosol for 5 min before being mounted and cover slipped in DPX.

2.2.7.3. Immunofluorescence staining for the detection of fibronectin

5 µm tissue sections from Bouin's-fixed and paraffin-embedded human and mouse testes were deparaffinised in xylene twice for 10 min each, rehydrated through a series of decreasing ethanol concentrations (twice 100% for 10 min, followed by 95%, 70% and 50% for 1 min each), rinsed in distilled water and further rehydrated for 10 min in TBST. For antigen retrieval, sections were then boiled in a microwave in citrate buffer for 4 min at full power followed by 4 min defrost program, left to cool down in the citrate buffer for 15 min and washed in TBST for 3 times 5 min each. The slides were then blocked with 10% goat serum in TBST at RT for 1 h and incubated with a rabbit polyclonal anti-mouse fibronectin antibody diluted 1:300 **(**see **Appendix 10.4.**) in TBST at 4°C overnight. After incubation, samples were washed 3 times with TBST and incubated with a secondary goat anti-rabbit Alexa Fluor 488 conjugated IgG antibody in TBST (dilution). Afterwards, sections were washed 3 times in TBST and mounted in ProLong Gold mounting medium containing DAPI for nuclear staining. Fluorescence was detected with a Zeiss Axioplan 2 Imaging Microscope.

2.2.8. Quantification of total collagen content

Quantitative estimation of total collagen content in testis tissue was performed by chromogenic determination of hydroxyproline concentration from paraffin sections using Quickzyme Total Collagen Assay and normalised to total protein content measured by Quickzyme Total Protein Assay. Both assays were performed according to manufacturer's instructions.

Twelve 10 µm sections of paraffin embedded mouse testes and four 30 µm sections of human testicular biopsies were collected in screw-capped tubes, 150 µl 6 M HCl added and the suspension incubated in a thermoblock for 20 h at 95°C. After incubation, tubes were cooled to RT and centrifuged for 10 min at 13,000 x g before transfer of the supernatant to a new tube.

For subsequent use in the total collagen assay, hydrolysed samples were diluted 1:0.5 in demineralised water, resulting in a concentration of 4 M HCl in the sample. A standard ranging from 0 to 300 µg/ml, diluted in 4 M HCl, was also hydrolysed for 20 h at 95 °C, centrifuged for 10 min at 13,000 x g and the supernatant transferred into a new tube. 35 µl of standard and sample was pipetted into the appropriate wells of the assay microplate, 75 µl assay buffer added to each well and the plate incubated 20 min at RT, while shaking. The detection reagent was prepared by mixing reagents A and B in a ratio of 2:3 and 75 µl/well added to each well. Then the plate was covered with an adhesive plate seal, mixed well and incubated for 1 h at 60°C in a water bath. Afterwards, the plate was cooled to RT, the plate seal removed and the plate read at 570 nm by using the microplate reader TriStar LB 941. The collagen concentration was calculated by averaging the duplicate values for each standard or sample, followed by subtracting the average blank from all readings and creation of a standard curve by plotting the reading mean (minus blank) on the y-axis against the collagen content on the x-axis. The drawn best-fit linearised curve was used to convert the absorption readings of the samples to collagen content.

For the total protein assay, 15 µl of hydrolysed samples and pre-hydrolysed standard were directly pipetted into the appropriated wells. The standard dilutions were made with 6 M HCl and ranged from 0 to 3.0 mg/ml. 120 µl of assay buffer were added to each well, the contents mixed by shaking the plate and 15 µl colour reagent working solution (1 volume colour reagent stock solution and 9 volumes reagent buffer) pipetted into each well. The plate was sealed with an adhesive plate seal and mixed before incubation for 1 h at 85°C in a water bath. Afterwards, the plate was cooled down to RT, the seal removed and the plate read at 570 nm. The calculation of the protein concentration was performed the same way as the calculation of the collagen concentration. The total collagen content of a sample was then calculated as a ratio by dividing the collagen concentration by the total protein concentration.

2.2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software. Results are expressed as mean ± S.E.M. The data sets were tested for normal distribution using the Kolmogorov-Smirnov and for equal variances using the F-test for two groups and the Bartlett's test for more than two groups. Differences of two groups were determined by Student's t-test, or Mann Whitney test when applicable. When more than two groups were compared, one-way ANOVA followed by Tukey's multiple comparisons test, or Friedman test followed by Dunn's multiple comparison test were used. Pearson's correlation coefficient "r" was used to show the linear relationship between two sets of data in correlation analyses. r = 1 represents a positive correlation, while r = -1 represents a negative and r = 0 no correlation. A P value of < 0.05 was considered statistically significant.

3. RESULTS

3.1. Investigating fibrosis and the role of activin A in the testis of mouse and human

This study involved assessment and comparison of the expression and distribution of fibrotic markers in the testes of a mouse model of EAO, with and without elevated levels of the circulating follistatin, and in human testicular biopsies with impaired spermatogenesis and leukocytic infiltrates to their respective controls.

For verification of the clinical relevance of the EAO mouse model, analysis of activin A subunit *INHBA*, and *PTPRC* (CD45) mRNA in human testicular biopsies with impaired spermatogenesis and leukocytic infiltrates was performed.

3.1.1. Total collagen content is increased in testes of mice with EAO and in human testicular biopsies with impaired spermatogenesis and leukocytic infiltrates

As chronic testicular inflammation in EAO mice is accompanied by a strong fibrotic response, visible as thickening of the lamina propria and interstitial deposition of ECM components (Nicolas *et al.*, 2017a), a hydroxyproline assay was performed to quantitatively assess the total collagen content in the testis. Collagens are the major components of the ECM and hence commonly used as indicator for fibrosis. Total collagen was significantly increased in the testes of mice with EAO at day 80 after the first TH-immunisation compared to untreated and adjuvant control testes **(Fig. 13A)**. Note that the testes of mice immunised with TH are referred to as “EAO testes” in all subsequent studies, regardless of whether they have evidence of orchitis or not. In the EAO testes of mice at day 30 and 50 after the first TH-immunisation, no changes in total collagen content were detectable. Notably, the collagen type I α-2 mRNA levels were significantly increased in EAO testes at day 50 after the first immunisation **(Fig. 14)**. This time point was chosen as it is the progressed and “active” stage of the disease. All processes leading to disease development already take place before 80 days EAO, which is the chronic stage.

In line with the data from the mouse model of EAO, a significant increase in total collagen was also detected in human testicular biopsies with impaired spermatogenesis and leukocytic infiltrates **(Fig. 13B)**.

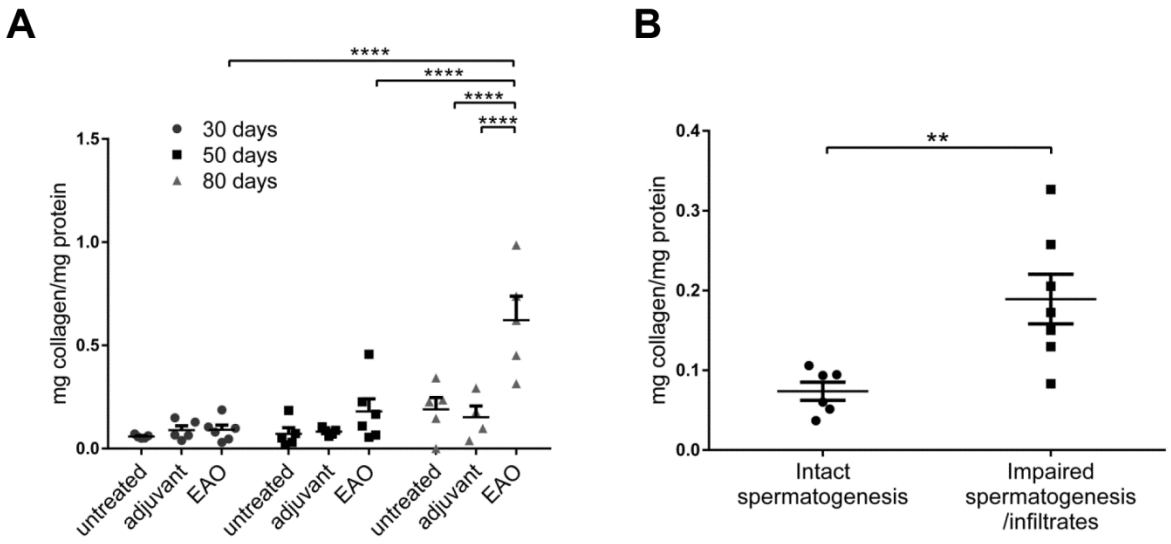

Figure 13: Total collagen was elevated in EAO mouse testis 80 days after the first testis homogenate (TH) immunisation and in human testis with impaired spermatogenesis and leukocytic infiltrates. Total collagen measured by hydroxyproline assay in untreated, adjuvant control and EAO mouse testes at 30, 50 and 80 days after first TH-immunisation **(A)** and in human testicular biopsies with intact spermatogenesis without any signs of inflammation, or impaired spermatogenesis with leukocytic infiltrates **(B)**. Data are mean ± SEM, n = 4 – 6 animals **(A)** and 6 - 7 patients **(B)** per group; **P < 0.01, **** P < 0.0001.

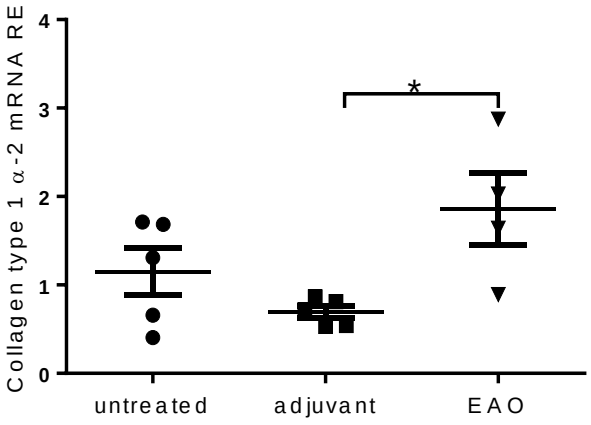

Figure 14: Relative collagen type 1 α-2 (*Col1a2*) mRNA expression levels were increased in EAO mouse testes. Relative expression (RE) of collagen type I α-2 mRNA levels in untreated, adjuvant control and EAO mouse testes 50 days after first TH-immunisation were measured by qRT-PCR and normalised to 18S rRNA and hypoxanthin-guanin-phosphoribosyltransferase. Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison. Data are shown as mean ± SEM; n = 4 – 6 animals per group; * P < 0.05.

3.1.2. *INHBA* mRNA levels in human testicular biopsies with impaired spermatogenesis and leukocytic infiltrates are increased and correlate with the extent of infiltrates and fibrosis

Previous results showed an increase in the levels of activin A and number of leukocytes in mouse testis with severe EAO (Nicolas *et al.*, 2017a). In order to investigate the clinical relevance of these findings, the relative mRNA levels of *INHBA* (gene encoding for the βA subunit of activin A) and *PTPRC* (CD45, leukocyte common antigen), as an index of leukocyte recruitment to the testis, were assessed in human testicular biopsies from SCO patients with inflammatory lesions associated with impaired spermatogenesis **(Fig. 15A, 15B)**.

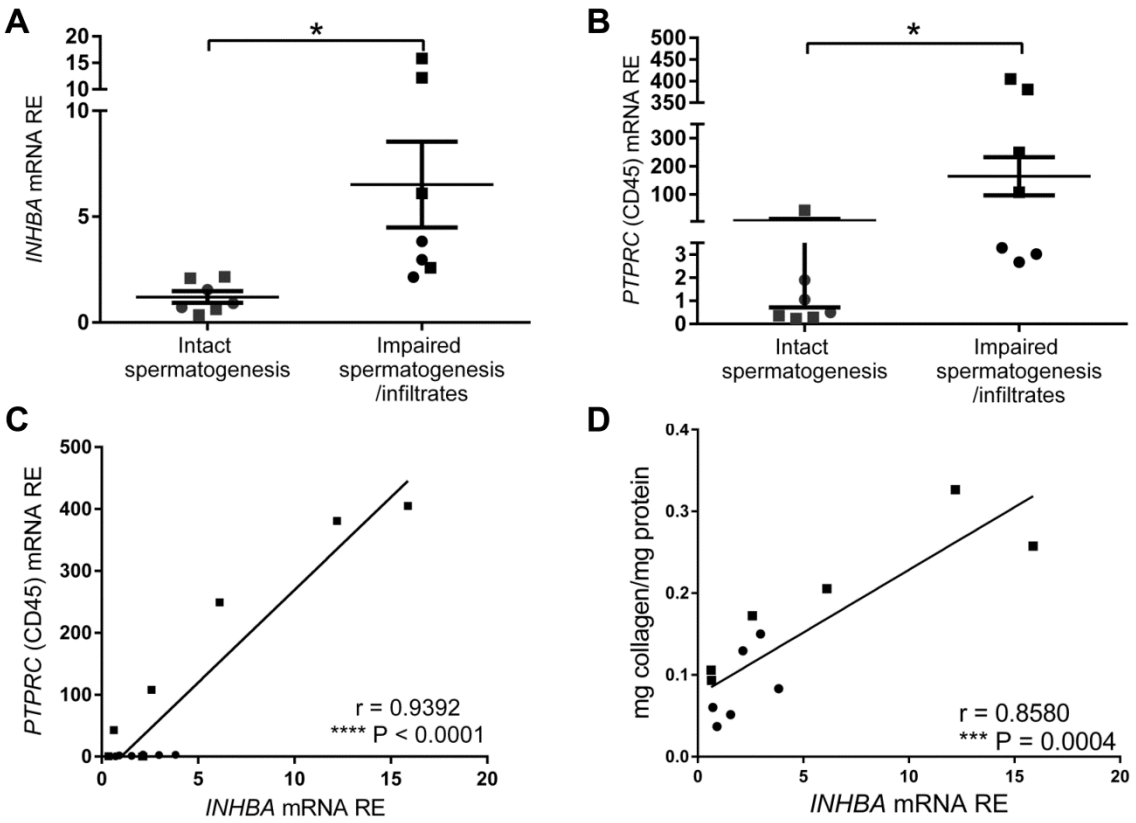

Figure 15: I*NHBA* and *PTPRC* (CD45) mRNA expression levels were elevated in human testicular biopsies with impaired spermatogenesis and leukocytic infiltrates compared to biopsies with intact spermatogenesis. **Relative *INHBA* mRNA expression positively correlated with *PTPRC* mRNA expression and total collagen content.** *PTPRC* **(A)** and *INHBA* **(B)** RE was measured by qRT-PCR. *INHBA* mRNA levels were plotted against *PTPRC* mRNA levels **(C)** and total collagen **(D)** (data from **Fig. 13B**) in paraffin embedded (■) or frozen (●) testicular biopsies with intact spermatogenesis, and with impaired spermatogenesis and leukocytic infiltrates. Data are mean ± SEM and Pearson's r, n = 7 biopsies/group, except in panel D where n = 12 (2 of 14 samples were under the detection limit of the assay); * P < 0.05, *** P < 0.001, **** P < 0.0001.

In human testicular biopsies with impaired spermatogenesis and leukocytic infiltrates, a significant increase of both *INHBA* **(Fig. 15A)** and *PTPRC* **(Fig. 15B)** mRNA levels was observed compared to biopsies of control patients with normal spermatogenesis. In order to elucidate if there is any causal statistical relationship between the expression activin A and extent of immune cell infiltration, Pearson correlation analysis for these genes was performed. The relative mRNA levels of *INHBA* correlated positively with *PTPRC* mRNA levels **(Fig. 15C)**. In the same patient specimens, *INHBA* mRNA levels also correlated positively with total collagen content **(Fig. 15D)**. Total collagen values from **Fig. 13B** were used for the correlation analysis. The individual patient data is shown in **Appendix 10.6.**

These data indicate a link between the expression of activin A, inflammation and fibrosis in testes of humans with non-obstructive idiopathic impaired spermatogenesis and leukocytic infiltrates.

3.1.3. Expression of fibronectin and collagen is linked to severity of disease in murine EAO and human testes with impaired spermatogenesis and inflammatory lesions

Testicular expression of collagen and fibronectin was investigated during the course of EAO in mice and in human biopsies with impaired spermatogenesis and inflammatory infiltrates. As collagen and fibronectin are major components of the ECM and hence excessively produced during fibrosis (Shi *et al.*, 2010; Singh *et al.*, 2010; Bonnans *et al.*, 2014), their deposition is a marker of the severity of fibrosis.

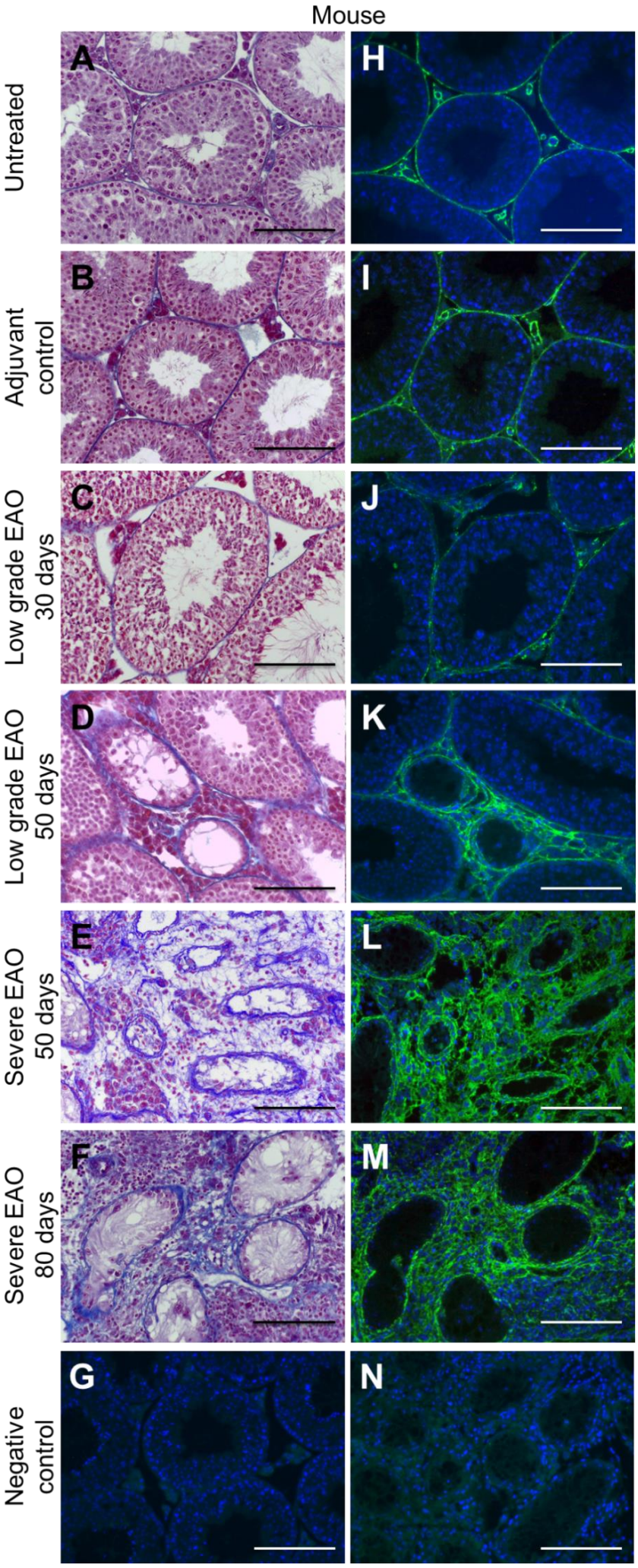

Figure 16: **Extent of collagen deposition (A - F) and fibronectin (G - N) was increased in EAO mouse testes.** Azan staining with blue coloured collagen fibres, and fibronectin (FITC; green) immunofluorescence of untreated **(A, H)** and adjuvant control **(B, I)** mouse testes, as well as mouse testes with low grade EAO at 30 days **(C, J)**, low grade EAO at 50 days **(D, K)**, severe EAO at 50 days **(E, L)** and severe EAO at 80 days **(F, M)** after first TH-immunisation. Representative negative controls (secondary antibody only) of untreated **(G)** and severe EAO testes at 50 days after first TH-immunisation **(N)** showed no FITC immunofluorescence. Scale bars = 100 µm.

Testes from untreated **(Fig. 16A)** and adjuvant control **(Fig. 16B)** mice, as well as human testicular biopsies showing normal spermatogenesis **(Fig. 17A)** exhibited a normal morphology with seminiferous tubules containing germ cells at all stages of spermatogenesis, and collagen fibres mainly localised as a thin layer around the seminiferous tubules **(Fig. 16A, 16B, 17A)**. Fibronectin was also localised as a thin layer within the tubular wall and surrounding blood vessels in the interstitium of all normal mouse **(Fig. 16H, 16I)** and human **(Fig. 17D)** testes.

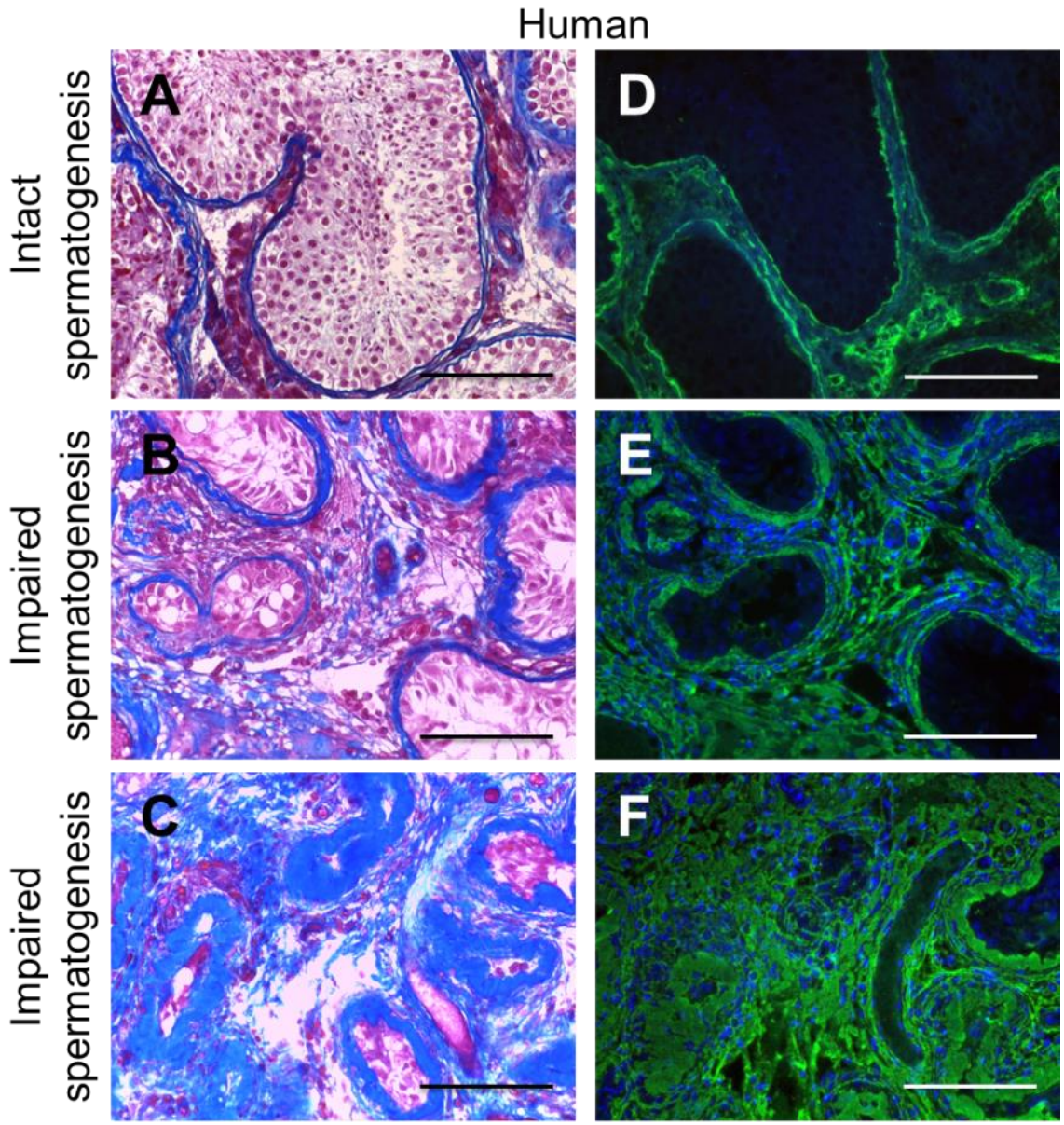

Figure 17: Extent of collagen deposition (A - C) and fibronectin (D - F) was increased in human testes with impaired spermatogenesis and leukocytic infiltrates compared to the controls. Azan staining with blue coloured collagen fibres, and fibronectin (FITC; green) immunofluorescence staining of human testicular biopsies with intact spermatogenesis **(A, D)** and of biopsies from infertile patients with impaired spermatogenesis and leukocytic infiltrates **(Patient 1: B, E; Patient 2: C, F)**. Scale bars = 100 µm.

In contrast, mouse testes with severe EAO **(Fig. 16E, 16F)** and human biopsies showing impaired spermatogenesis and leukocytic infiltrates **(Fig. 17B, 17C)**, displayed a large interstitial deposition of collagen and a thickening of the basal lamina around the seminiferous tubules. As previously described (Nicolas *et al.*, 2017a), these changes were accompanied by tubular atrophy of seminiferous tubules, reduction of the seminiferous epithelium height and germ cell sloughing. Furthermore, extensive interstitial deposition of fibronectin and thickening of the fibronectin layer surrounding the seminiferous tubules was observed **(Fig. 16L, 16M, 17E, 17F)**.

In the analysed human testicular biopsies, mRNA expression of *INHBA* and *PTPRC* (CD45) as well as collagen content and impaired spermatogenesis showed a clear relationship. Histologically, increased collagen and fibronectin expression co-localised with areas of immune cell infiltration in some patient samples **(Fig. 18)**.

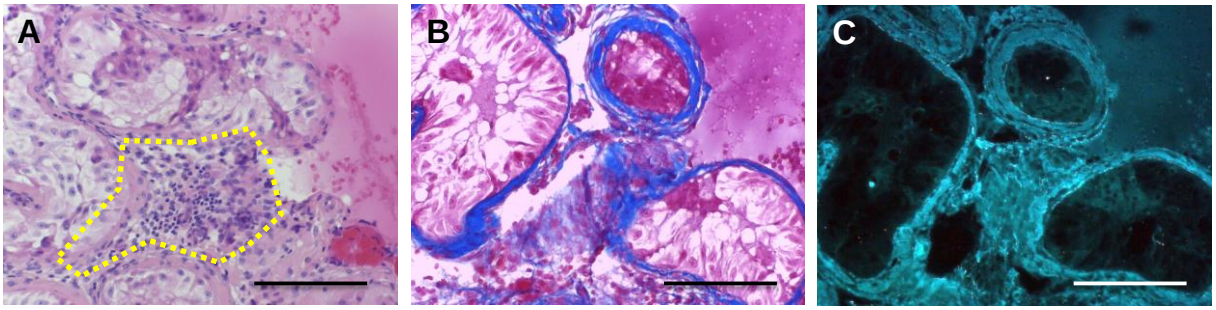

Figure 18: Sites of fibronectin expression co-localised with deposition of collagen and severity of fibrosis in some human testes with leukocytic infiltrates. Haematoxylin and eosin staining with interstitial infiltration by immune cells **(A)**, azan staining with blue coloured collagen fibres **(B)** and immunofluorescence staining of fibronectin **(C)** in representative human testicular biopsy with impaired spermatogenesis and leukocytic infiltrates. Area with infiltrate is indicated by dotted, yellow line. Scale bars = 100 µm.

The increase in fibronectin was accompanied by increased collagen deposition, stronger impairment of spermatogenesis and a greater extent of immune cell infiltration in mouse testes with severe EAO. These results indicate the suitability for the use of fibronectin and collagen as fibrotic markers in EAO.

3.1.4. Fibronectin expression is decreased in TH-immunised testes of mice with elevated circulating levels of follistatin and positively correlates with EAO damage score

In a previous study, the circulating form of follistatin (FST315) was over-expressed in mice prior to immunisation with TH, by injection with a non-replicative recombinant adeno-associated viral vector serotype 6 carrying a gene cassette of follistatin (rAAV-FST315) (Nicolas *et al.*, 2017b). Results of this study demonstrated an overall reduction of the EAO response in animals with increased FST315 levels compared to mice with normal follistatin levels. However, some of these mice still developed severe EAO (Nicolas *et al.*, 2017b). Western blot analysis of fibronectin expression in the testes from untreated, adjuvant control and follistatin vector (FST) or empty vector (EV) pre-treated TH-immunised mice **(Fig. 19A)** from this study was performed, and the quantified and normalised expression of fibronectin **(Fig. 19B)** plotted against the EAO damage score **(Fig. 19C)**.

Western blot analysis revealed a low basal expression of fibronectin in testes of untreated and adjuvant control animals **(Fig. 19A; 19B)**. In TH-immunised testes from follistatin pre-treated animals (FST-immunised), which displayed a damage score of 0, the expression of fibronectin was comparably low to untreated and adjuvant control testes **(Fig. 19A; 19B)**. On the contrary, pre-treatment of TH-immunised animals with an empty vector (EV-TH-immunised) resulted in testes with a damage score of 4 and a significantly increased expression of fibronectin compared to the controls and the FST-TH-immunised group **(Fig. 19A, 19B)**. This result indicates a positive influence of elevated circulating levels of follistatin on EAO by reducing its severity. Fibronectin expression positively correlated with the EAO damage score **(Fig. 19C)** indicating that higher fibronectin expression is associated with greater severity of EAO and fibrosis.

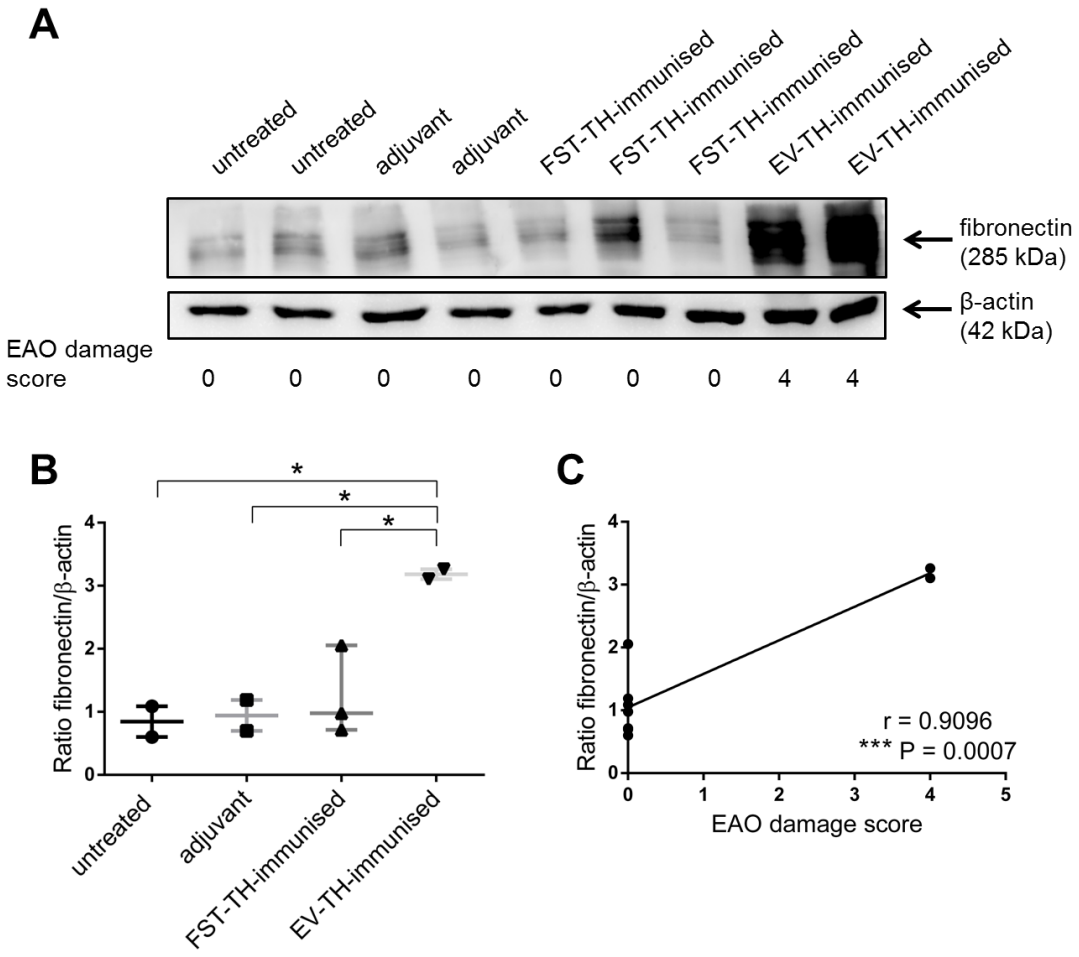

Figure 19: Expression of fibronectin positively correlated with EAO damage score in mouse testes. Western blot with fibronectin expression **(A)** in testis samples from untreated, adjuvant control, and TH-immunised mice pre-treated with rAAV-FST315 (FST) or empty vector (EV). β-actin served as loading control. Respective EAO damage score of each animal was previously assessed and defined from histological examination (Nicolas *et al.*, 2017b). 20 µg total protein was loaded per well. Densitometric quantification of fibronectin expression normalised to β-actin **(B)**, and correlation analysis of fibronectin expression and EAO damage score (regardless of treatment with FST315 vector) **(C)**; Data are shown as mean ± SEM and Pearson's r; * $P < 0.05$.

3.2. Investigation of involvement of leukocytes in the development of fibrosis in EAO testis

3.2.1. Collagen type I positive leukocytes in EAO mouse testis indicate the involvement of immune cells in the fibrotic response

Fibrocytes are bone marrow-derived fibroblast precursors, which are derived from a subpopulation of monocytes via a process called monocyte-to-fibroblast transition (Liang *et al.*, 2017). They have been shown to give rise to myofibroblasts in different diseases including renal fibrosis. These cells express mesenchymal markers, such as collagen I, and hematopoietic markers, such as CD45 or F4/80 (Xia *et al.*, 2013). In order to investigate the possible involvement of leukocytes in the development of fibrosis in EAO mouse testes, co-expression of collagen type I and the leukocyte common marker CD45 was investigated by immunofluorescence staining. The immunostaining revealed the occurrence of collagen type I and CD45 co-expressing cells in low grade and severe EAO testis at day 50 **(Fig. 20B, 20E)** and 80 **(Fig. 20C, 20F)** after the first TH-immunisation, respectively. Double positive cells for collagen type I and CD45 were absent in the testes of control animals **(Fig. 20A, 20D)**. This result indicates possible involvement of immune cells in the formation of ECM following chronic testicular inflammation.

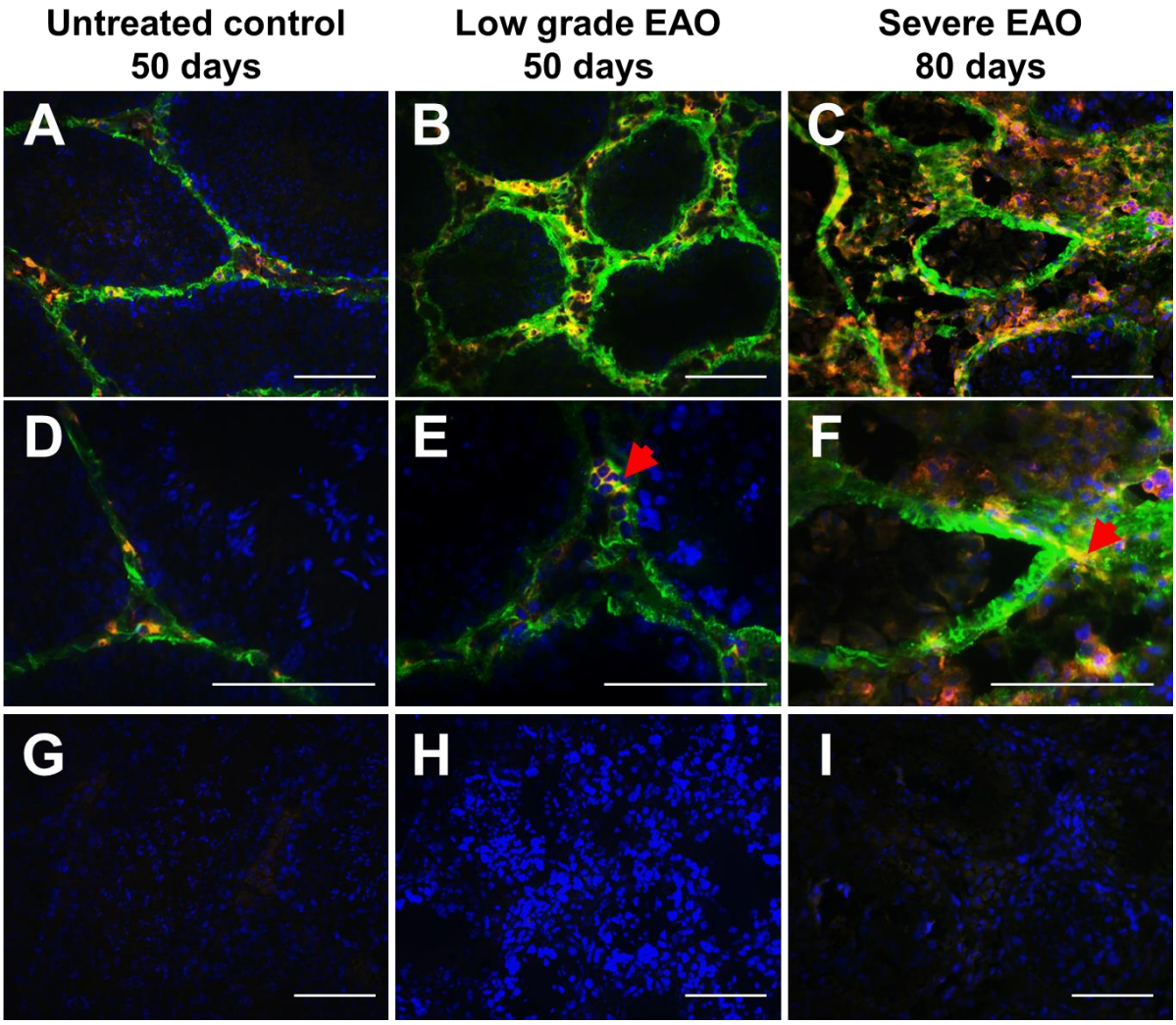

Figure 20: Collagen type I expressing leukocytes in EAO mouse testis indicate the possible involvement of leukocytes in the development of fibrosis following chronic testicular inflammation. Collagen type I (A - F; AlexaFluor 488; green) and leukocyte common marker CD45 **(A - F;** AlexaFluor 546; orange) immunofluorescence staining on cryosections from control **(A; D)**, low grade EAO testes at day 50 **(B; E)** and severe EAO testes at day 80 **(C; F)** after the first immunisation. Double positive cells display a yellow signal (red arrows), which was visible when green and orange fluorescence overlapped. **(G)**, **(H)** and **(I)** represent the respective negative controls; Scale bars = 100 µm.

3.2.2. Collagen type I positive macrophages in EAO testis indicate a possible involvement of monocytes/macrophages in the development of the fibrotic response

To further investigate the possibility that activated fibroblasts originate from bone marrow–derived fibroblast progenitor cells, co-expression of collagen type I and the macrophage marker F4/80 was investigated **(Fig. 21)**.

Immunofluorescence staining showed collagen type I-expressing macrophages in low grade EAO testes at day 30 **(Fig. 21B)**, severe EAO testes at day 50 **(Fig. 21E)**, and severe EAO testes at day 80 **(Fig. 21G)** after the first immunisation. It appears that the severity of the fibrotic response was associated with a higher frequency of double positive (collagen type I + F4/80) cells. The double positive cells were missing in the testes of control animals **(Fig. 21A)**. This result indicates the possibility that monocytes/macrophages are a cell subset that at least partly contributes to the production of ECM in the EAO testis.

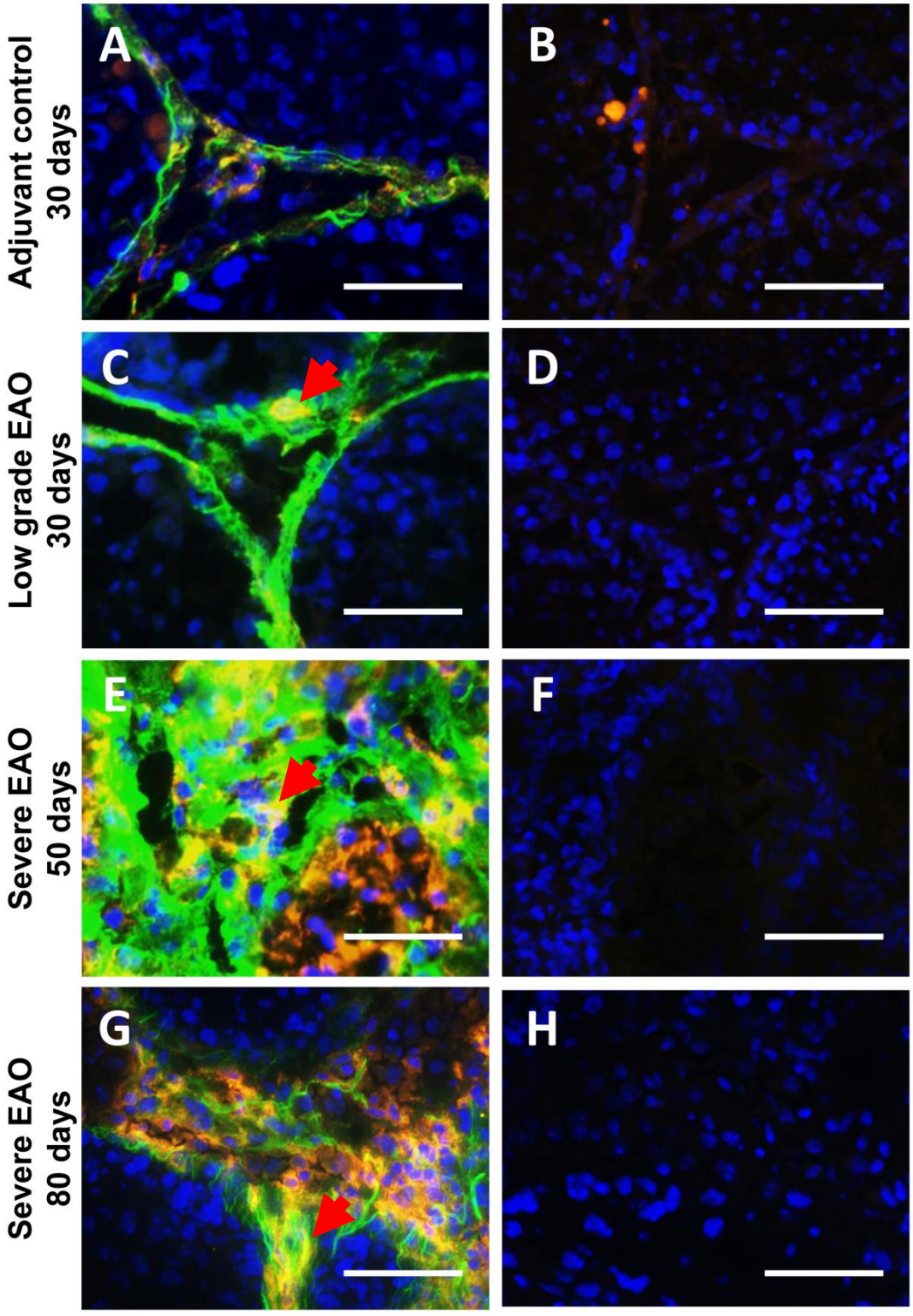

Figure 21: Possible involvement of monocytes/macrophages in development of fibrosis in EAO testis was indicated by co-expression of collagen type I and the macrophage marker F4/80. Double immunofluorescence staining of collagen type I (AlexaFluor 488; green) and F4/80 (AlexaFluor 546; orange) on testicular mouse cryosections from untreated **(A)**, low grade EAO at day 30 **(C)**, severe EAO at day 50 **(E)**, and severe EAO at day 80 **(G)** after first TH-immunisation. Red arrows show areas of collagen type I and F4/80 co-expression. **(B)**, **(D)**, **(F)** and **(H)** represent the respective negative controls; scale bars = 50 µm.

3.3. *In vitro* studies investigating the influence of activin A on the fibrotic response of testicular PTC and NIH 3T3 cells

The involvement of different cell types in fibrosis development in EAO mouse testis and the influence of activin A on the fibrotic response of those cells were investigated.

Previous results revealed that fibrosis in EAO testis initiates around the seminiferous tubules, and is also accompanied by morphological changes in the distribution of αSMA in PTC (Nicolas *et al.*, 2017a). Hence, PTC were considered as a cell type possibly responsible for the fibrotic response. As interstitial fibrosis is also a feature of EAO in mouse testes, and resident fibroblasts are known to contribute to fibrosis development in many tissues, testicular fibroblasts were considered as another cell type possibly involved in the development of fibrosis in EAO (Nicolas *et al.*, 2017a; Kendall and Feghali-Bostwick, 2014). A NIH 3T3 cell line was employed as a surrogate for testicular fibroblasts, which due to their low abundance and the lack of specific marker proteins are difficult to obtain in pure preparations from the testis (Krenning *et al.*, 2010).

Note that in all *in vitro* experiments either 25 ng/ml or 50 ng/ml activin A were used, as both doses were effective. Treatment durations of PTC and NIH 3T3 cells also differed, as the optimal time frame with the highest response was chosen.

3.3.1. Activin A activates SMAD2 and SMAD3 signalling in primary mouse PTC and NIH 3T3 cells

In order to investigate the responsiveness of the tested cell types to activin A, the activation of the activin A signalling pathway was investigated. As activin A transmits its signals through phosphorylation of SMAD2 and SMAD3, and the nuclear accumulation of the SMAD2/3 – SMAD 4 complex, the intensity of SMAD2 and SMAD3 phosphorylation shows the magnitude of signalling pathway activation (Heldin *et al.*, 1997).

A time course experiment, using NIH 3T3 cells treated with 25 ng/ml recombinant activin A for different time intervals, was performed to establish the optimal treatment duration resulting in the strongest phosphorylation of SMAD2 and SMAD3.

Western blot analysis showed that SMAD2 phosphorylation in NIH 3T3 cells was most intensive after 30 min and 45 min **(Fig. 22A)**, while SMAD3 phosphorylation was constantly high in a time frame from 15 to 60 min **(Fig. 22B)** after activin A treatment. Based on these results, activin A treatment durations of 30 min and 45 min were selected for further experiments.

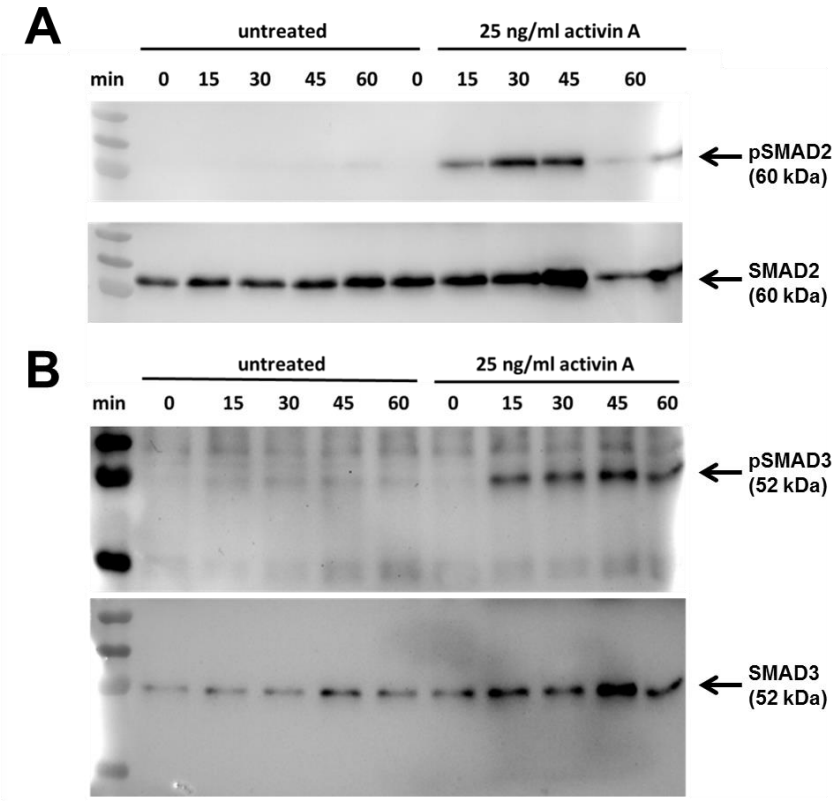

Figure 22: Strongest SMAD2 phosphorylation was observed 30 min and 45 min after activin A treatment, while SMAD3 phosphorylation is constantly high after stimulation of NIH 3T3 cells with activin A. Western blots of phosphorylated SMAD2 (pSMAD2) **(A)** and phosphorylated SMAD3 (pSMAD3) **(B)** in NIH 3T3 cells treated with 25 ng/ml activin A for 0, 15, 30, 45 and 60 min. Total SMAD2 **(A)** and total SMAD3 **(B)** were used as loading control.

In order to investigate the responsiveness of PTC **(Fig. 23A, 23B, 24A)** and NIH 3T3 cells **(Fig. 23C, 23D, 24B)** to activin A, both cell types were treated for 30 min and 45 min with 50 ng/ml activin A, 50 ng/ml activin A together with 100 ng/ml FST288, 100 ng/ml FST288 alone or left untreated. Activin A treatment resulted in phosphorylation of SMAD2 **(Fig. 23A, 23B, 23C, 23D)** as well as SMAD3 **(Fig. 24A, 24B)**. These effects were abolished by the addition of 100 ng/ml FST288, indicating that both SMAD2 and SMAD3 phosphorylation were specifically due to response to activin A. No phosphorylation of SMAD2 or SMAD3 in either cell type was observed when cells were treated with FST288 alone or were untreated **(Fig. 23, 24)**.

Note that, as two time points were analysed, and the increase of pSMAD3 following activin A treatment was very evident for both time points, SMAD3 phosphorylation in primary mouse PTC and NIH 3T3 cell cultures was interpreted qualitatively.

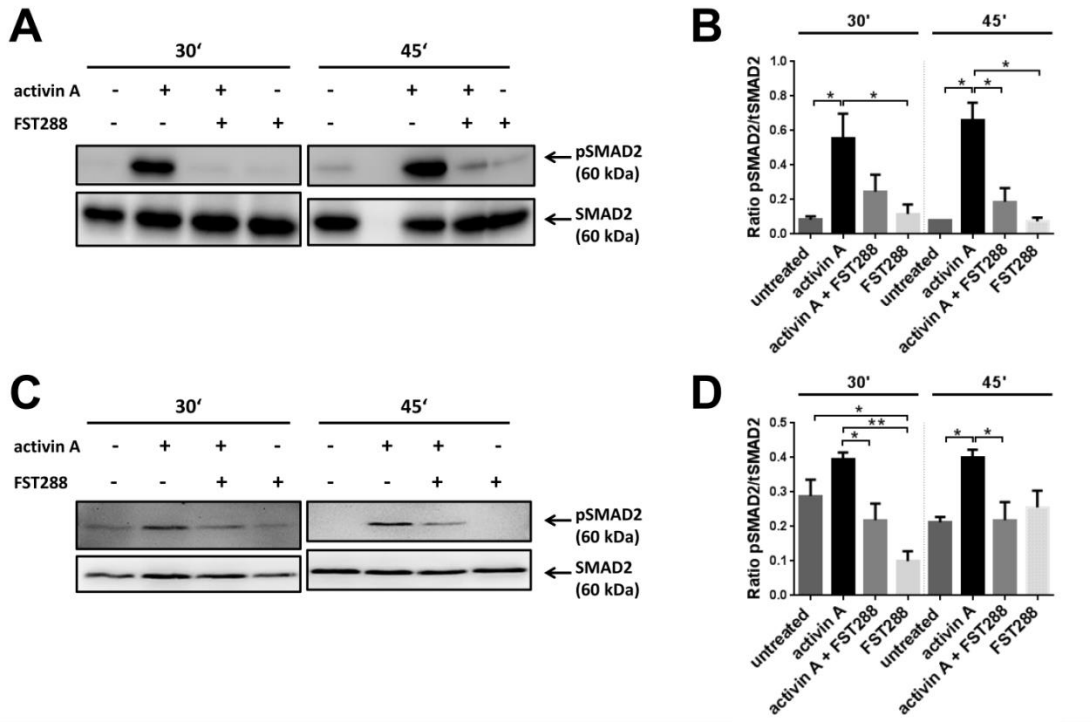

Figure 23: Activin A induced SMAD2 phosphorylation (pSMAD2) in primary mouse PTC (A; B) and NIH 3T3 cell cultures (C; D). Representative Western blots of pSMAD2 in PTC **(A)** and NIH 3T3 cells **(C)** treated with 50 ng/ml activin A, 100 ng/ml FST288, a combination of both or left untreated for 30 min and 45 min. Densitometric quantification of pSMAD2 in PTC **(B)** and NIH 3T3 cells **(D)**. 25 µg total protein was loaded per well. Total SMAD2 served as loading control. Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison. Data are shown as mean ± SEM; n = 3 **(A, B)** and 2 – 3 **(C, D)**; * $P < 0.05$, ** $P < 0.01$.

These results demonstrate the responsiveness of PTC and NIH 3T3 cells to activin A and suggest that they are potential targets of activin A *in vivo*.

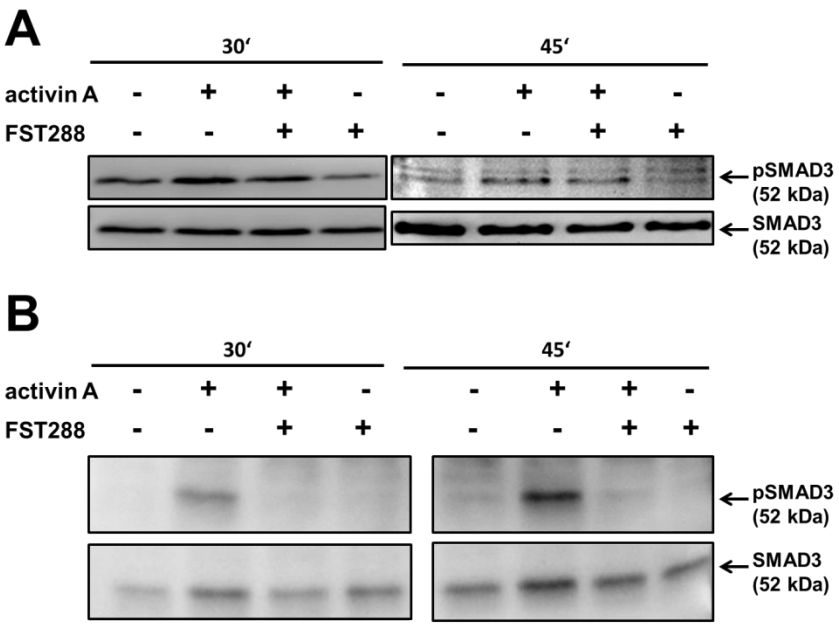

Figure 24: Activin A induced SMAD3 phosphorylation (pSMAD3) in primary mouse PTC and NIH 3T3 cell cultures. Western blots of pSMAD3 in PTC **(A)** and NIH 3T3 cells **(B)** after treatment with 50 ng/ml activin A, 100 ng/ml FST288, a combination of both or left untreated for 30 min and 45 min. 25 µg total protein was loaded per well. Total SMAD3 served as loading control; n = 2 **(A)** and 1 **(B)**.

3.3.2. Activin A stimulates expression of fibrotic genes in primary mouse PTC and NIH 3T3 cells

In order to figure out which cell types could be responsible for fibrosis development in EAO and to investigate the influence of activin A on the fibrotic response of these cells, PTC and NIH 3T3 cells were treated with activin A and the relative mRNA levels and expression of the fibrotic proteins collagen type I, III and IV, fibronectin and αSMA were assessed. Note that protein levels of collagen type IV were too low for detection by Western Blot.

Treatment of PTC with 25 ng/ml activin A resulted in significantly increased relative expression of collagen type IV (*Col4a1*) and fibronectin (*Fn*) mRNA, while there was no effect of activin A on αSMA (*Acta2*), collagen type I (*Col1a2*) and III (*Col3a1*) mRNA levels **(Fig. 25A)**.

RESULTS

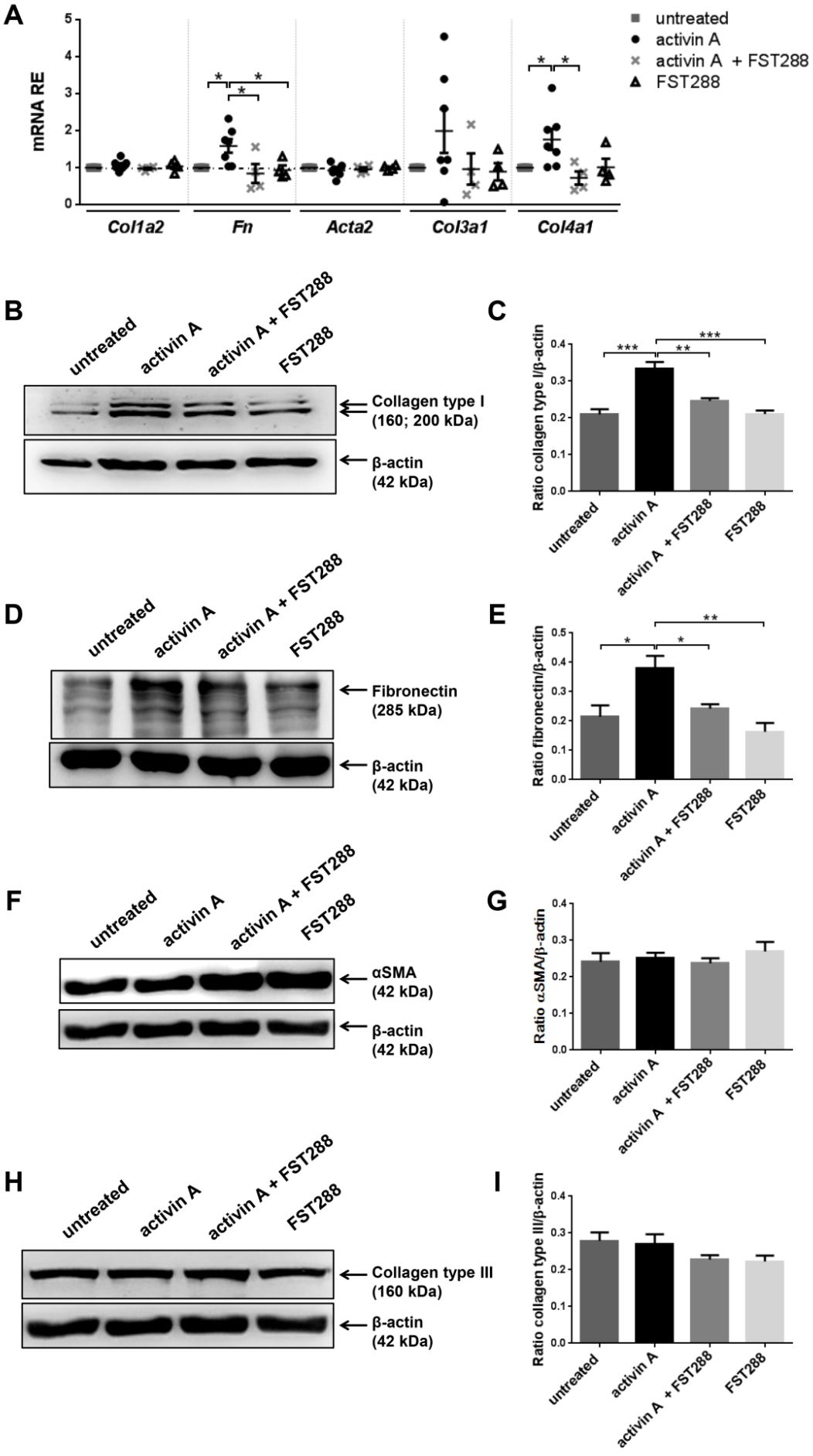
A
mRNA RE
untreated
activin A
activin A + FST288
FST288
Col1a2
Fn
Acta2
Col3a1
Col4a1
B
untreated
activin A
activin A + FST288
FST288
Collagen type I
(160; 200 kDa)
β-actin
(42 kDa)
C
Ratio collagen type I/β-actin
D
Fibronectin
(285 kDa)
E
Ratio fibronectin/β-actin
F
αSMA
(42 kDa)
G
Ratio αSMA/β-actin
H
Collagen type III
(160 kDa)
I
Ratio collagen type III/β-actin

Figure 25: Collagen type I, IV, and fibronectin expression in primary mouse PTC cultures was increased following activin A treatment. Relative mRNA levels of collagen type I (*Col1a2*), fibronectin (*Fn*), αSMA (*Acta2*), collagen type III (*Col3a1*) and collagen type IV (*Col4a1*) in PTC treated (4 h) with 25 ng/ml activin A, 25 ng/ml activin A and 100 ng/ml FST288, 100 ng/ml FST288 or left untreated, were measured by qRT-PCR (n = 4 - 7) and normalised to 18S rRNA, hypoxanthin-guanin-phosphoribosyltransferase and beta-2 microglobulin **(A)**. Representative Western blots of collagen type I **(B)**, fibronectin **(D)**, αSMA **(F)** and collagen type III **(H)** expression in PTC treated (48 h) with 50 ng/ml activin A, 50 ng/ml activin A and 100 ng/ml FST288, 100 ng/ml FST288 or remained untreated. β-actin served as a loading control. 30 µg total protein was loaded per well. Densitometric quantification of collagen type I **(C)**, fibronectin **(E)**, αSMA **(G)** and collagen type III **(I)** normalised to β-actin. Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison. Data are shown as mean ± SEM; n = 3 – 5 replicate experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The addition of the activin A binding protein FST288 (100 ng/ml) abolished the elevating effect of activin A **(Fig. 25A)**. None of the investigated genes were altered when cells were treated with FST288 alone **(Fig. 25A)**.

At the protein level, treatment of PTC with activin A resulted in significantly elevated expression of collagen type I **(Fig. 25B; 25C)** and fibronectin **(Fig. 25D; 25E)**. No changes of αSMA **(Fig. 25F; 25G)** and collagen type III **(Fig. 25H; 25I)** expression were observed. Combined treatment activin A and treatment with FST288 alone had no effect on the expression of any of the investigated proteins **(Fig. 25B - I)**.

In NIH 3T3 cells, αSMA and fibronectin mRNA levels were elevated, while collagen type I, III and IV mRNA expression was not affected by activin A treatment **(Fig. 26A)**.

At the protein level, activin A stimulated the expression of collagen type I **(Fig. 26B, 26C)** and fibronectin **(Fig. 26D, 26E)** as well as αSMA **(Fig. 26F, 26G)**, while it had no effect on the expression of collagen type III **(Fig. 26H; 26I)**. Moreover, the addition of FST288 inhibited the effects of activin A on the expression of all investigated proteins **(Fig. 26B - I)**. Treatment with FST288 alone seemed to have a suppressive effect on the expression of fibronectin in NIH 3T3 cells **(Fig. 26D, 26E)**.

These data indicate possible involvement of both PTC and fibroblasts in the fibrotic response in testis and highlight the possibility that this response might be mediated by activin A.

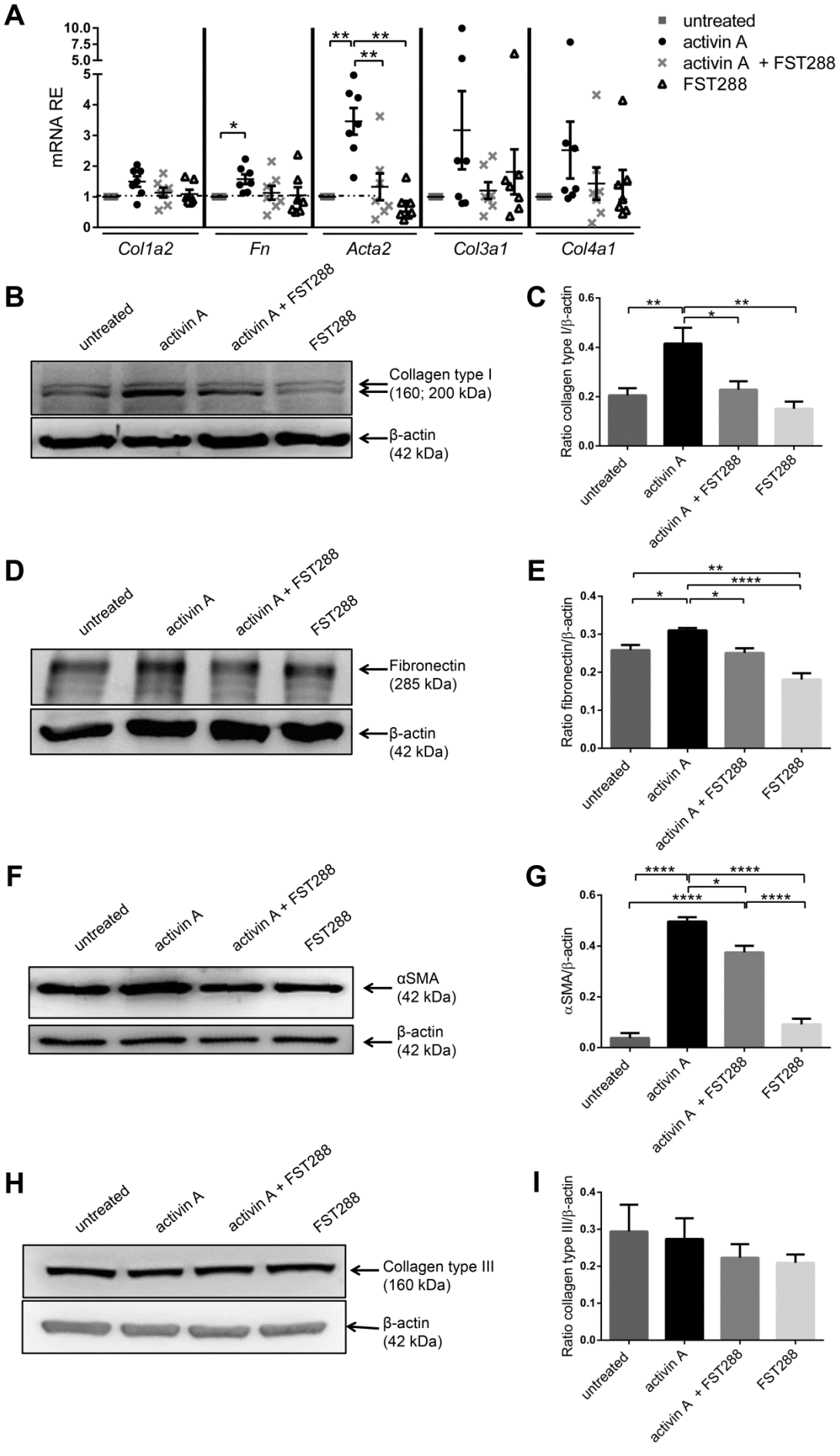
A
mRNA RE
untreated
activin A
activin A + FST288
FST288
Col1a2
Fn
Acta2
Col3a1
Col4a1
B
Collagen type I
(160; 200 kDa)
β-actin
(42 kDa)
C
Ratio collagen type I/β-actin
D
Fibronectin
(285 kDa)
E
Ratio fibronectin/β-actin
F
αSMA
(42 kDa)
G
αSMA/β-actin
H
Collagen type III
(160 kDa)
I
Ratio collagen type III/β-actin

Figure 26: Expression of collagen type I, αSMA and fibronectin was increased in NIH 3T3 fibroblast cultures following activin A treatment. Collagen type I (*Col1a2*), fibronectin (*Fn*), αSMA actin (*Acta2*), collagen type III (*Col3a1*) and collagen type IV (*Col4a1*) mRNA RE in mouse NIH 3T3 fibroblasts treated for 24 h with 25 ng/ml activin A, 25 ng/ml activin A and 100 ng/ml FST288, 100 ng/ml FST288 or remained untreated was measured by qRT-PCR (n = 8) and normalised to 18S rRNA, hypoxanthin-guanin-phosphoribosyltransferase and beta-2 microglobulin **(A)**. Representative Western blots of collagen type I **(B)**, fibronectin **(D)**, αSMA **(F)** and collagen type III **(H)** expression in NIH 3T3 cells treated with 50 ng/ml activin A, 50 ng/ml activin A and 100 ng/ml FST288, 100 ng/ml FST288 or left untreated for 48 h. β-actin served as a loading control. 30 µg total protein was loaded per well. Densitometric quantification of collagen type I **(C)**, fibronectin **(E)**, αSMA **(G)** and collagen type III **(I)**. Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison or Friedman test followed by Dunn's multiple comparison test. Data are shown as mean ± SEM; n = 3 – 5 replicate experiments; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

3.4. *In vitro* investigation of the influence of inflammatory mediators on the inflammatory response in SC and PTC

In order to get a more detailed insight into the establishment and mechanism of testicular inflammation in the mouse EAO model, the influence of activin A and TNF, which were both found to be elevated in EAO mouse testes (Nicolas *et al.*, 2017a), on the inflammatory response of primary mouse SC and PTC was investigated. Moreover, the effect of TNF on the production of activin A by primary SC was tested *in vitro*.

3.4.1. Lipopolysaccharide (LPS) induces elevation of inflammatory response by primary SC *in vitro*

In order to test the inflammatory responsiveness of mouse primary SC, the inflammatory conditions were mimicked *in vitro* by treating SC with LPS, a major component of the outer membrane of Gram-negative bacteria, which can stimulate the release of inflammatory cytokines in cell types that express TLR4. These cell types include all leukocytes and epithelial cells, such as the SC (Ngkelo *et al.*, 2012). After 24 h treatment with 1 or 10 µg/ml LPS the relative mRNA levels of TNF (*Tnf*), MCP-1 (*Ccl2*), activin A (*Inhba*) and IL-1α (*Il1a*) in SC were significantly increased **(Fig. 27)**. Although IL-6 (*Il6*) mRNA levels were not significantly changed, an increase of IL-6 mRNA was seen after treatments with both doses of LPS, and presumably would reach statistical significance with a higher number of experimental repetitions. This result demonstrates the responsiveness of SC to an inflammatory stimulus.

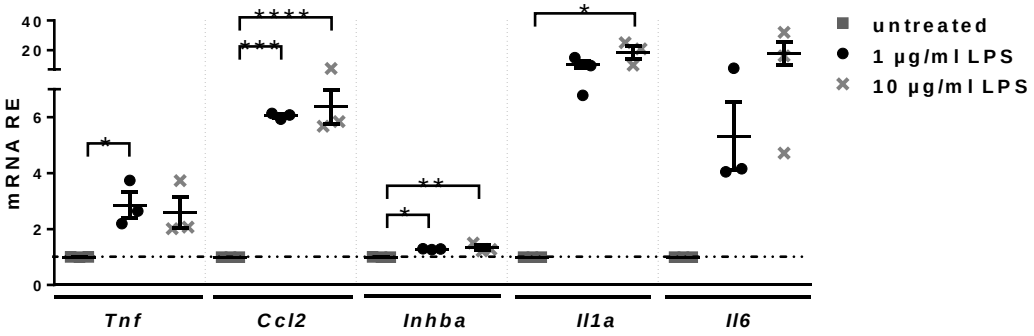

Figure 27: LPS treatment increased the expression of TNF, MCP-1, activin A, IL-1α and IL-6 mRNA in primary mouse SC. TNF (*Tnf*), MCP-1 (*Ccl2*), activin A (*Inhba*), IL 1α (*Il1a*) and IL-6 (*Il6*) mRNA RE in mouse SC (n = 3) treated for 24 h with 1 µg/ml LPS, 10 µg/ml LPS or remained untreated. RE levels were measured by qRT-PCR and normalised to 18S rRNA, hypoxanthin-guanin-phosphoribosyltransferase and beta-2 microglobulin. Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison. Data are shown as mean ± SEM; n = 3 replicate experiments; * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

3.4.2. Influence of activin A on the inflammatory response of SC and PTC *in vitro*

Treatment with 25 ng/ml activin A showed no significant effect on the expression of inflammatory genes, such as TNF, MCP-1, activin A, IL-1α and IL-6, in primary SC **(Fig. 28A)** and primary PTC **(Fig. 28B)**. However, treatment with FST288 alone as well as combined treatment with activin A and FST288 decreased the basal expression of MCP-1 and IL-6 in SC **(Fig. 28A)** and IL-6 in PTC **(Fig. 28B)**. These data suggest an immunosuppressive role of follistatin.

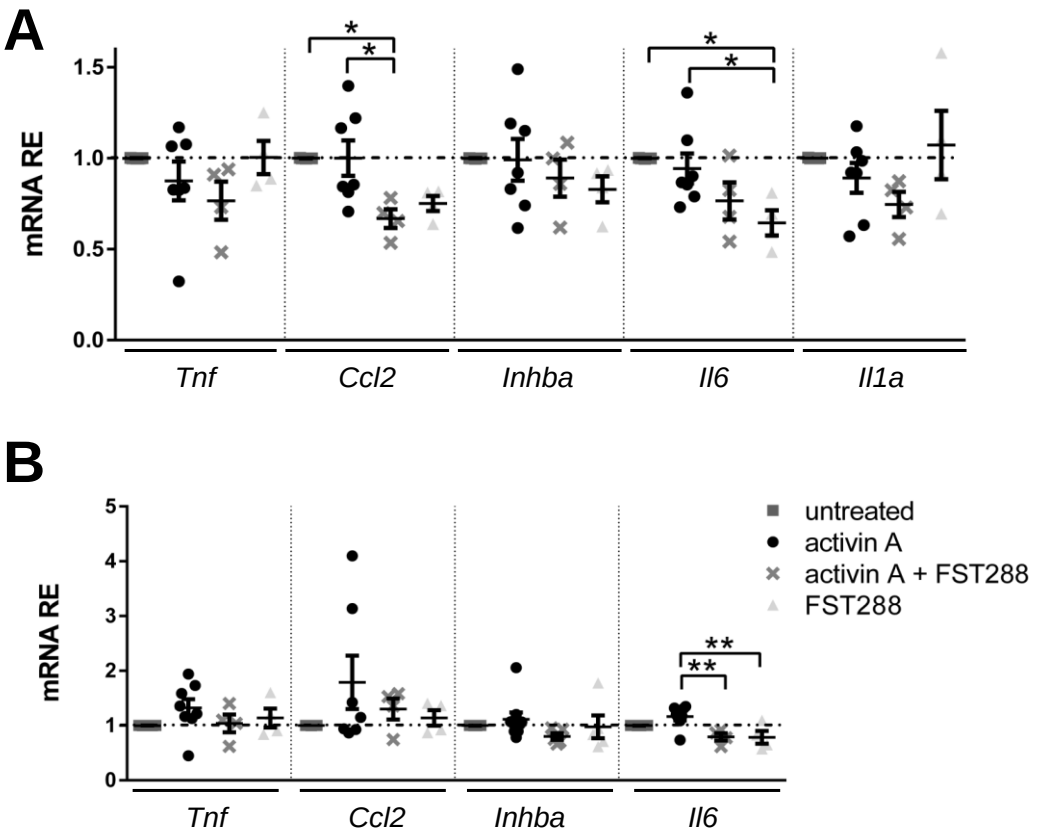

Figure 28: Activin A had no effect on mRNA expression of TNF, MCP-1, activin A, IL-1α and IL-6 in primary mouse SC (A) and PTC (B). FST288 decreases basal expression of MCP-1 and IL-6 mRNA levels in SC and IL-6 mRNA levels in PTC. TNF (*Tnf*), MCP-1 (*Ccl2*), activin A (*Inhba*), IL-1α (*Il1a*) and IL-6 (*Il6*) mRNA RE in mouse SC treated for 24 h **(A)** and TNF, MCP-1, activin A and IL-6 mRNA RE in mouse PTC treated for 4 h **(B)** with 25 ng/ml activin A, 25 ng/ml activin A and 100 ng/ml FST288, 100 ng/ml FST288 or left untreated. RE levels were measured by qRT-PCR and normalised to 18S rRNA, hypoxanthin-guanin-phosphoribosyltransferase and beta-2 microglobulin. Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison. Data are shown as mean ± SEM; n = 5 - 9 **(A)** and 4 – 8 **(B)** replicate experiments; * $P < 0.05$, ** $P < 0.01$.

3.4.3. Influence of TNF on the inflammatory response of SC and PTC *in vitro*

Treatment with 25 ng/ml recombinant TNF resulted in a significant increase of relative mRNA levels of MCP-1, IL-1α and IL-6 in SC **(Fig. 29A)** and TNF, MCP-1 and IL-6 in PTC **(Fig. 29B)**. Interestingly, TNF decreased TNF RE in SC **(Fig. 29A)** suggesting the existence of a negative feedback loop on its own expression *in vivo*. In contrast, no measurable effect of TNF on *Inhba* mRNA levels was seen.

These results suggest the importance of TNF in the initiation and maintenance of the inflammatory response in EAO mouse testis.

Note that treatment durations of SC and PTC differed, as they were dependent on the optimal response strength tested in precedent time course experiments.

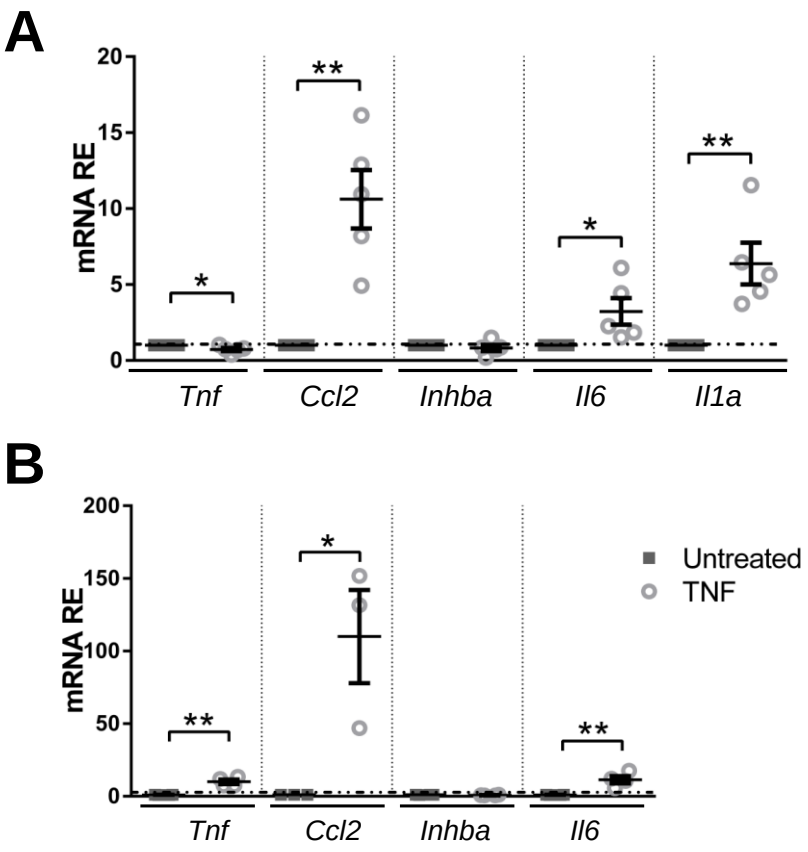

Figure 29: In primary mouse SC (A) TNF treatment led to downregulation the mRNA expression of TNF itself and upregulation the levels of MCP-1, IL-1 α and IL-6 mRNA, whereas in primary mouse PTC (B), TNF stimulated TNF, MCP-1 and IL-6 mRNA expression. TNF (*Tnf*), MCP-1 (*Ccl2*), activin A (*Inhba*), IL-1α (*Il1a*) and IL-6 (*Il6*) mRNA RE in mouse SC treated for 24 h **(A)** and TNF, MCP-1, activin A and IL-6 mRNA RE in mouse PTC treated for 4 h **(B)** with 25 ng/ml TNF or remained untreated. RE levels were measured by qRT-PCR and normalised to 18S rRNA, hypoxanthin-guanin-phosphoribosyltransferase and beta-2 microglobulin. Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison. Data are shown as mean ± SEM; n = 5 **(A)** and 3 – 4 **(B)** replicate experiments; * $P < 0.05$, ** $P < 0.01$.

3.4.4. Activin A secretion by murine SC is stimulated by TNF

Previous studies demonstrated that many testicular cells in the adult testis produce activin A constitutively, including the spermatogenic cells, Leydig cells, macrophages and capsular mast cells. However, the SC have been implicated as the main producers of activin A in the adult testis, particularly in response to inflammation (Hedger and Winnall, 2012; Nicolas *et al.*, 2017a). Studies have also shown that activin A expression in rat SC is stimulated by many inflammatory mediators, including TNF and IL-1α, (Kazutaka *et al.*, 2011). To further investigate the role of SC in the inflammatory process and its contribution to the development of fibrosis through increased production of activin A by TNF, which is elevated in the chronically inflamed mouse testis (Nicolas *et al.*, 2017a), primary SC were cultured for 24 h in the presence of 25 ng/ml TNF and 50 ng/ml TNF as well as 100 ng/ml FST288 or remained untreated, and the production of activin A was measured by ELISA.

SC secrete activin A in culture under unstimulated conditions A **(Fig. 30)**. Addition of 100 ng/ml FST288 reduced the amount of activin A released by SC almost to undetectable levels (assay sensitivity = 0.11 ng/ml) **(Fig. 30)**, indicating a basal expression of activin A in SC under physiological conditions.

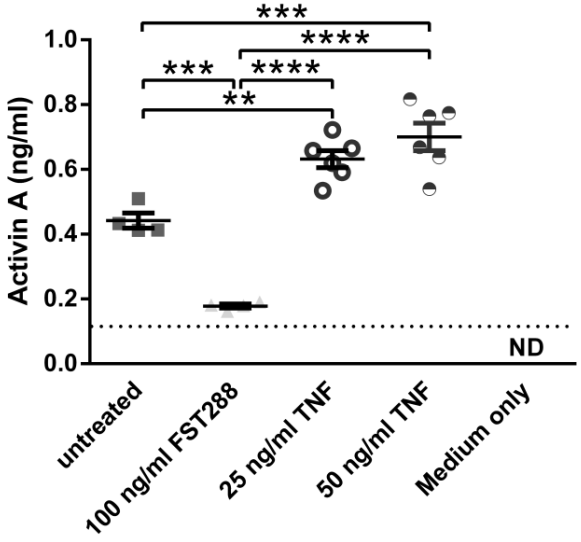

Figure 30: Activin A secretion in primary mouse SC was stimulated by TNF. Activin A concentration was measured in supernatants of SC cultured for 24 h with 25 ng/ml TNF, 50 ng/ml TNF, 100 ng/ml FST288 or left untreated, as well as the medium only by ELISA. The assay sensitivity was 0.11 ng/ml (dashed line). A representative result from six cell preparations is shown. Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison. Data are shown as mean ± SEM; n = 4 - 6 replicate wells; ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Activin A concentration in the supernatant of SC treated with 25 and 50 ng/ml recombinant TNF was increased approximately 1.5-fold compared to untreated SC in 6 independent experiments **(Fig. 30)**, demonstrating that the production of activin A by SC can be stimulated by an inflammatory stimulus in the murine testis.

3.5. Investigation of influence of activin A produced by SC on testicular inflammatory and fibrotic responses

To evaluate the role of activin A derived from SC in chronic testicular inflammation and development of fibrosis, mice with a SC-specific knock-down of activin A (*Inhba*SCKO) were generated by crossing transgenic mice expressing the activin βA gene (*Inhba*) between two *loxP* sites (activin βA-floxed) with mice expressing cre-recombinase under control of the Anti-Müllerian hormone (*Amh*) promoter (*Amh-Cre*). As the *Amh-Cre* transgene is expressed exclusively in SC from 15 days post coitum on, and also occurs in every SC (De Gendt *et al.*, 2004), activin A production should only be knocked down in this cell type. Littermates produced by the crossing procedures that did not express the cre-recombinase, but had two activin βA-floxed copies (*Inhba*FLOX) were also generated and served as controls **(Fig. 9)**. This was necessary to control for the different genetic backgrounds of the *Amh-Cre* and *Inhba*^{loxPloxP} mice, which produced offspring of mixed 129S6/SvEv/C57BL6J/SJL genetic backgrounds. Testis weights, EAO induction rate, histopathological changes, inflammatory and fibrotic responses, as well as testicular activin A levels were analysed at 50 days after the first immunisation. Note that the number of animals with the SC conditional knockout genotype (*Amh*$^{cre/+}$*Inhba*$^{loxP/loxP}$) available for study was limited within the time-frame available for the research. Furthermore, due to this limitation, mice with ages ranging between 52 – 101 days were used.

3.5.1. Genotyping of transgenic mice

To screen for mice with the appropriate genotypes, gDNA isolated from mouse-tail biopsies was used as template DNA for PCR amplification. A typical agarose gel is shown as an example in **Fig. 31**.

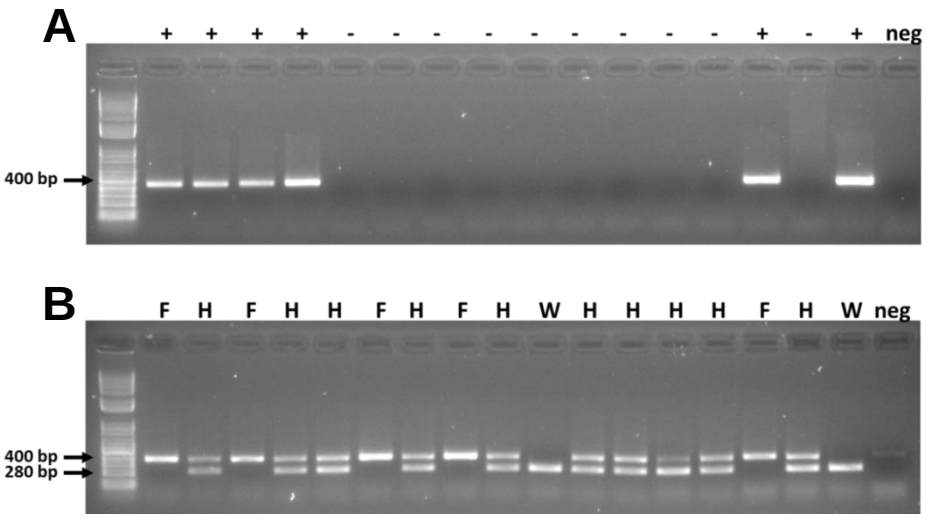

Figure 31: Example agarose gel showing genotyping analysis for *Amh-Cre* (A) and *Inhba* floxed (B) alleles. DNA of mice harbouring one or two alleles of *Amh-Cre* (+) shows a band at 400 bp, while mice without at least one allele demonstrate no transcript (-). Mice harbouring the WT alleles of *Inhba* (+/+; W) reveal a band of 280 bp, while mice homozygous for the floxed *Inhba* (Flox/Flox; F) show a 400 bp product, and mice heterozygous for this allele (Flox/+; H), have both bands. Neg = Water only control. Only mice showing an *Amh-Cre* band and the 400 bp floxed *Inhba* DNA fragment are carrying the required *Inhba*SCKO genotype (lanes 1, 3 and 14). Mice which had no *Amh-Cre* band and the 400 bp floxed *Inhba* DNA fragment (*Inhba*FLOX) were used as controls (lanes 6 and 8), while mice with both 280 bp and 400 bp fragments (heterozygotes) were not included in the experiments.

3.5.2. Verification of activin A knock-down in SC

3.5.2.1. *Inhba* mRNA levels in testis of *Inhba*SCKO mice were decreased compared to the *Inhba*FLOX control

In order to verify the knock-down of activin A expression in SC, *Inhba* mRNA levels in the testes of *Inhba*SCKO and *Inhba*FLOX mice were assessed by qRT-PCR and activin A levels were measured by ELISA **(Fig. 32)**. While no significant changes of activin A protein in the testis of *Inhba*SCKO mice compared to the *Inhba*FLOX controls were found **(Fig. 32B)**, *Inhba* mRNA levels were significantly decreased by an average of 50% **(Fig. 32A)**. These data indicate that the conditional knock-down of *Inhba* in the SC did not suppress total activin A levels in the testis, presumably due to compensatory production by other cells types, in the absence of inflammation.

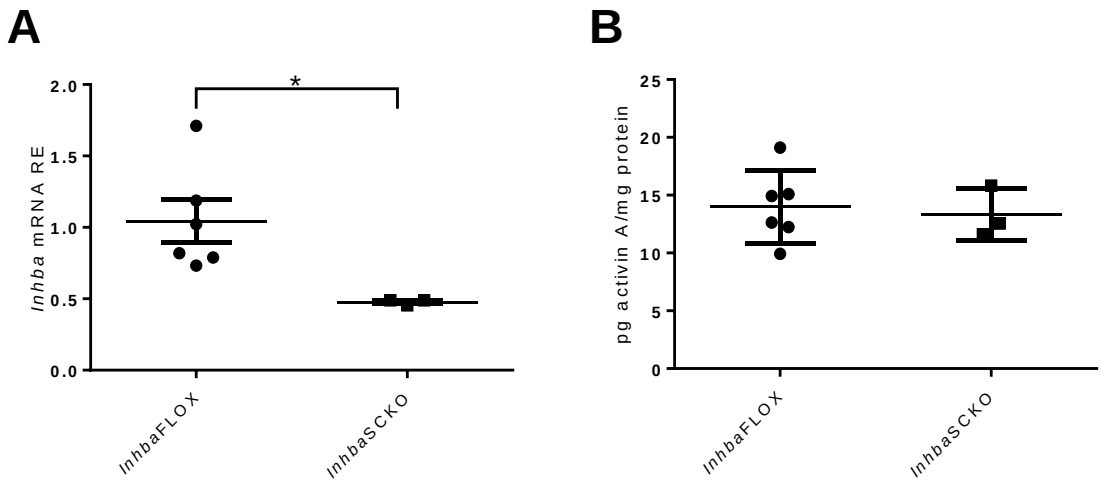

Figure 32: Testicular *Inhba* mRNA levels were decreased in *Inhba*SCKO mice compared to the *Inhba*FLOX control. No changes in testicular activin A levels were detected. Relative *Inhba* mRNA levels **(A)** and activin A concentration **(B)** were measured in TH of *Inhba*SCKO and *Inhba*FLOX adjuvant controls by qRT-PCR and ELISA, respectively. The ELISA assay sensitivity was 9.0 pg/ml. Activin A levels were normalised to the total protein concentration and *Inhba* mRNA levels to 18S rRNA and hypoxanthin-guanin-phosphoribosyltransferase. Significance was tested using student´s t test. Data are shown as mean ± SEM; n = 3 - 6 animals per group; * $P < 0.05$.

3.5.2.2. Effect of knock-down of activin A produced by SC on distribution of activin A in *Inhba*SCKO mice

To further assess the knock-down of activin A in the SC, activin A staining was performed on testicular sections of 56 days old *Inhba*SCKO mice **(Fig. 33B, D)** and *Inhba*FLOX controls **(Fig. 33A, C)**. In all groups, activin A staining was detected in the interstitial compartment (principally macrophages) as well as inside the seminiferous tubules in the spermatids and residual cytoplasm **(Fig. 33)**. Staining within the actual cytoplasm of the SC (as opposed to the residual cytoplasm, residual bodies and intracytoplasmic vesicles) was clearly evident in the *Inhba*FLOX mice **(Fig. 33A, C)**, but appeared to be reduced or absent in the *Inhba*SCKO testes **(Fig. 33B, D)**. These data indicate that production of activin A by the spermatogenic cells is minimally affected in the *Inhba*SCKO mice, and that activin A in the SC of these mice may be derived from the spermatogenic cells rather than production by the SC themselves.

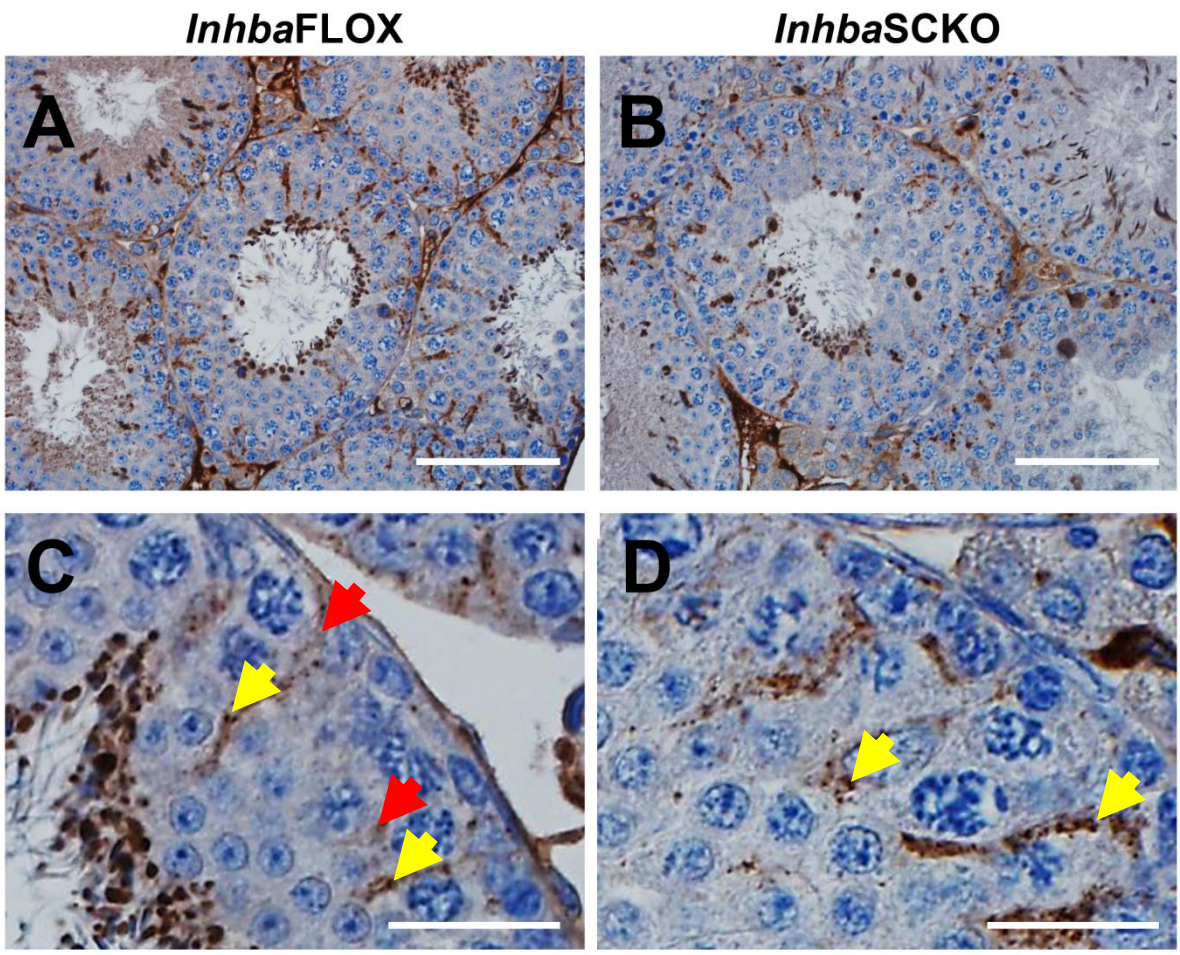

Figure 33: While in SC of *Inhba*FLOX mice (A, C) activin A was found in vesicles (yellow arrows) and in the cytoplasm (red arrows), in some *Inhba*SCKO animals (B, D), activin A was often restricted to vesicles and was less pronounced in the cytoplasm of the SC. Immunohistochemistry staining showing expression of activin A in testicular sections from *Inhba*FLOX **(A, C)** and *Inhba*SCKO **(B, D)** mice. Scale bars represent 50 µm in **(A, B)** and 20 µm in **(C, D)**.

3.5.3. Phenotype characterisation of mice with SC-specific knock-down of activin A

In order to assess the effect of the reduced production of activin A by SC and the possible subsequent phenotypical changes in the testes of *Inhba*SCKO mice, histological and morphometrical examination was performed on Bouin's-fixed and paraffin-embedded sections of 56 days old *Inhba*SCKO mice and their *Inhba*FLOX controls.

3.5.3.1. The lumen diameter of the seminiferous tubules is reduced in *Inhba*SCKO mice

The histological phenotype of the *Inhba*SCKO testis was assessed by haematoxylin and eosin staining of three untreated *Inhba*SCKO and five *Inhba*FLOX mice.

While testes of *Inhba*SCKO mice grossly showed a normal morphology with seminiferous tubules containing SC as well as germ cells at all stages of spermatogenesis, 2 of 3 animals had areas with reduced lumen diameters of the seminiferous tubules **(Fig. 34B)**. This was not observed in testes of *Inhba*FLOX mice **(Fig 34A)**. Notably, despite the reduced luminar diameters, no differences were observed in the testis weights of *Inhba*SCKO and *Inhba*FLOX mice **(Fig. 36)**.

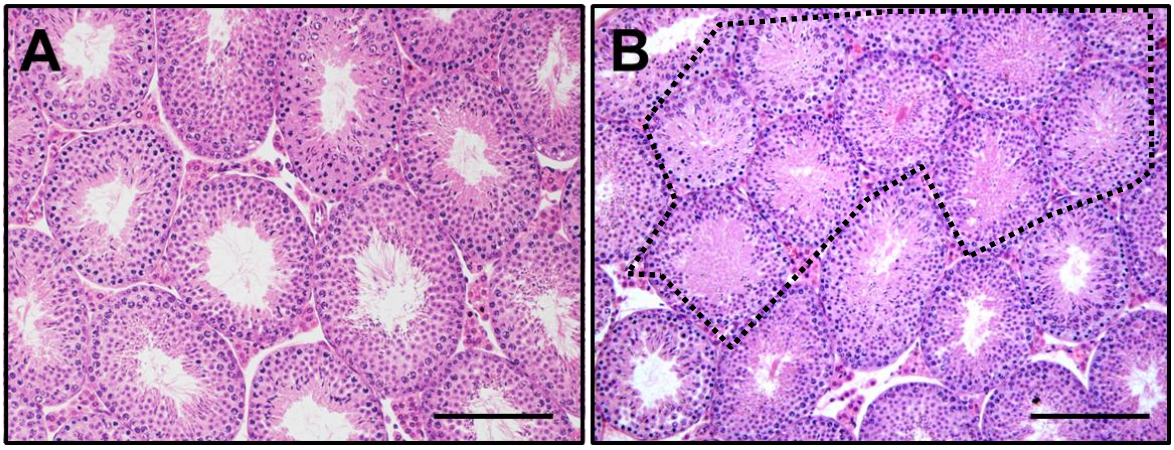

Figure 34: Formation of lumen in seminiferous tubules in the testes of *Inhba*SCKO mice was reduced in some areas (dotted line). Haematoxylin and eosin staining in paraffin sections showing representative testicular histology of *Inhba*FLOX **(A)** and *Inhba*SCKO **(B)** mice. In *Inhba*SCKO mice, the lumen diameter of the seminiferous tubules is visibly reduced compared to the *Inhba*FLOX control. Scale bars = 100 µm.

In order to quantitatively assess the diameter of the seminiferous tubules and lumen, morphometrical measurement was performed. While the mean diameter of seminiferous tubules in mice of both groups was about 200 µm **(Fig. 35A)**, the mean lumen diameter in *Inhba*SCKO mice (63.70 µm) was significantly smaller compared to *Inhba*FLOX (74.15 µm) mice **(Fig. 35B)**, suggesting that impairment of activin A production by SC has an effect on the function of SC resulting in reduction of the tubular lumen.

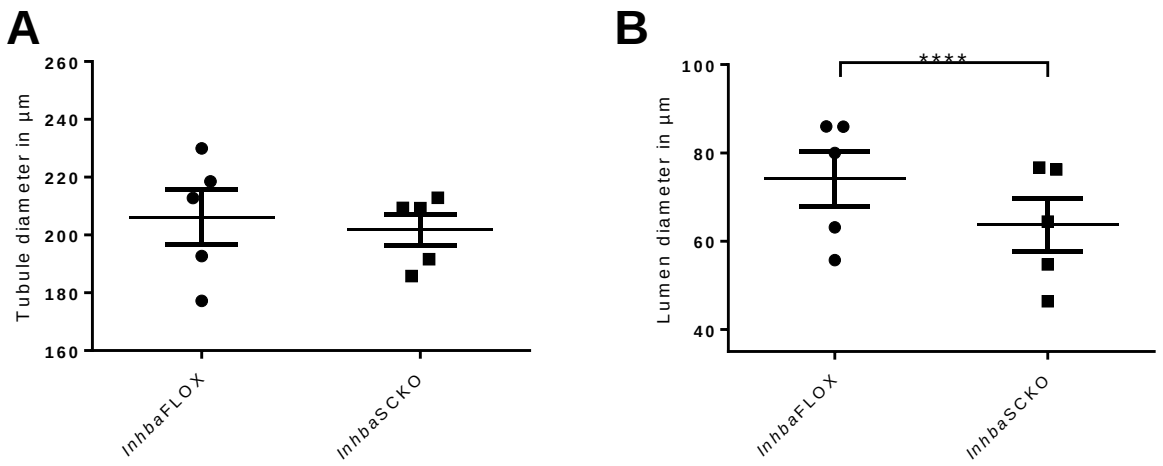

Figure 35: Lumen diameter was reduced in *Inhba*SCKO mice compared to *Inhba*FLOX controls. Tubule **(A)** and lumen **(B)** diameter was measured in a minimum of 100 seminiferous tubules from *Inhba*SCKO and *Inhba*FLOX mice testis and averaged. No difference in tubule diameters between the groups was observed, while lumen diameter in *Inhba*SCKO mice was significantly smaller compared to *Inhba*FLOX mice. Significance was tested using student´s t test. Data are shown as mean ± SEM; n = 3 - 5 animals per group; **** $P < 0.0001$.

3.5.4. EAO induction in mice with SC-specific knock-down of activin A

The aim for the studies on induction of EAO in *Inhba*SCKO mice was to evaluate the effect of activin A produced by SC on the development of chronic testicular inflammation and fibrosis. Therefore, the induction rate of EAO, testicular activin A levels, histopathological changes as well as inflammatory and fibrotic responses were analysed at 50 days after the first TH-immunisation. An experimental time frame of 50 days was chosen based on previous experiments, because 30 days were too short for full development of EAO in WT mice, while an experimental duration of 80 days was not resulting in additional benefits compared to 50 days.

The successful induction of EAO was determined based on parameters including testis weight, alteration of the testicular architecture, leukocytic infiltration, and the expression levels of inflammatory mediators including TNF, MCP-1/CCL2, IL-6 and activin A (results summarised in **Table 7**).

3.5.4.1. Analysis of mean testis weight in *Inhba*SCKO and *Inhba*FLOX mice

Previous EAO experiments on C57BL/6J mice revealed a significant reduction of the testis weights of TH-immunised animals at day 50 after the first immunisation compared to the untreated and adjuvant controls (Nicolas *et al.*, 2017b).

As, in the present study, evidence of unilateral damage, determined based on differences in the testis weights of an animal, was only detected in one animal, for analyses of all animals and treatment groups paired testis weights were used. No significant differences in the paired testis weights were seen between the adjuvant control and the TH-immunised animals **(Fig. 36)**. However, reduction of the testis weight was seen in one TH-immunised *Inhba*SCKO mouse, which also developed severe EAO. Interestingly, paired testis weights of TH-immunised testes of *Inhba*SCKO exhibited an overall decrease compared to the TH-immunised *Inhba*FLOX testes **(Fig. 36)**, and no reduction of the testis weights was seen in TH-immunised *Inhba*FLOX animals, which developed EAO. Unexpectedly, the testis weights of one *Inhba*FLOX mouse with an EAO damage score of 3 were increased compared to the testis weights of other TH-immunised *Inhba*FLOX mice. These results indicate an effect of *Inhba* knock-down on the testis weight. It also points out the possibility that floxing of *Inhba* or the mixed genetic background of the mouse strain might have an influence on the development of EAO.

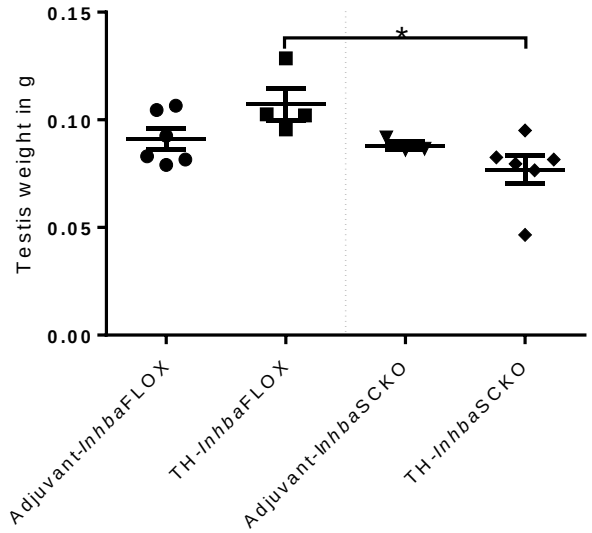

Figure 36: Paired testis weights of *Inhba*FLOX and *Inhba*SCKO mice immunised with NaCl + adjuvant or TH + adjuvant at day 50 day after the first immunisation. Statistical significance was tested using one-way ANOVA followed by Tukey's multiple comparison. Data are shown as mean ± SEM; n = 3 - 6 paired testes per group; * $P < 0.05$.

Furthermore, current research using the *Inhba* null mouse, showed a reduction of the testis weights, attributable to lower SC number (Mendis *et al.*, 2011). These data suggest the probability of a reduction of *Inhba*SCKO testes weights. However, the mean testis weight of the adjuvant treated *Inhba*SCKO and *Inhba*FLOX mice was similar **(Fig. 36)**.

3.5.4.2. Knock-down of SC-derived activin A has no effect on the number of leukocytes in *Inhba*SCKO mice

In order to assess the possible effect of activin A derived from SC on the leukocyte population and the recruitment of leukocytes in EAO mouse testes, the levels of CD45 (leukocyte common antigen) mRNA were measured by qRT-PCR **(Fig. 37)**, and the histopathological changes and number of leukocytic infiltrates in the testes of TH-immunised and adjuvant control *Inhba*SCKO and *Inhba*FLOX mice were examined histologically **(Fig. 38)**. No differences were detectable between the testes of adjuvant control *Inhba*SCKO and *Inhba*FLOX mice indicating that activin A produced by SC has no effect on the number of leukocytes in the testis **(Fig. 37)**.

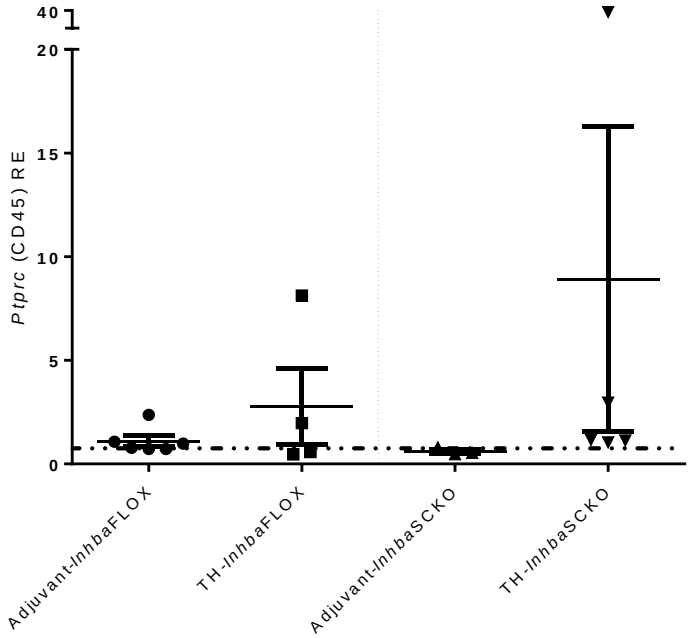

Figure 37: Relative levels of CD45 *mRNA* in *Inhba*SCKO and *Inhba*FLOX mice. Relative mRNA expression of *Ptprc* (CD45) in the testes of TH-immunised and adjuvant control *Inhba*SCKO and *Inhba*FLOX mice 50 days after first TH-immunisation analysed by qRT-PCR, and normalised to 18S rRNA and hypoxanthin-guanin-phosphoribosyltransferase. Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison for treatment groups and for comparison of *Inhba*SCKO and *Inhba*FLOX. Data are shown as mean ± SEM; n = 3 – 6 animals per group.

Moreover, no significant differences in the levels of CD45 were detected in the TH-immunised testes of *Inhba*SCKO and *Inhba*FLOX mice **(Fig. 37)**. This result was expected, as the overall number of animals was small and large individual animal variation was observed in the response to TH-immunisation.

Increase of CD45 expression was restricted to testes of animals with EAO **(Table 7; Fig. 37; Fig. 38)**, which only developed in 50% of TH immunised mice (3 of 6 *Inhba*SCKO; 2 of 4

*Inhba*FLOX mice). This result was consistent with the histological examination of the testes **(Fig. 38)**.

As the number of animals was not big enough for reasonable analysis and comparison of the mice regarding their EAO damage score, TH-immunised mice that developed EAO were subdivided into groups showing severe symptoms of the disease (severe EAO), mild symptoms of EAO (low grade EAO) and no symptoms of the disease. The findings are summarised in **Table 7**.

In both TH-immunised *Inhba*SCKO and *Inhba*FLOX mouse testes, massive leukocytic infiltrates were found in severe EAO, while no or little infiltrates were present in low grade EAO **(Fig. 38)**. The lack of obvious differences in the number of leukocytes between *Inhba*SCKO and *Inhba*FLOX mice suggests that knock-down of activin A derived from SC has no effect on the recruitment of leukocytes in EAO. However, for a reliable conclusion, study of a greater number of animals will be required.

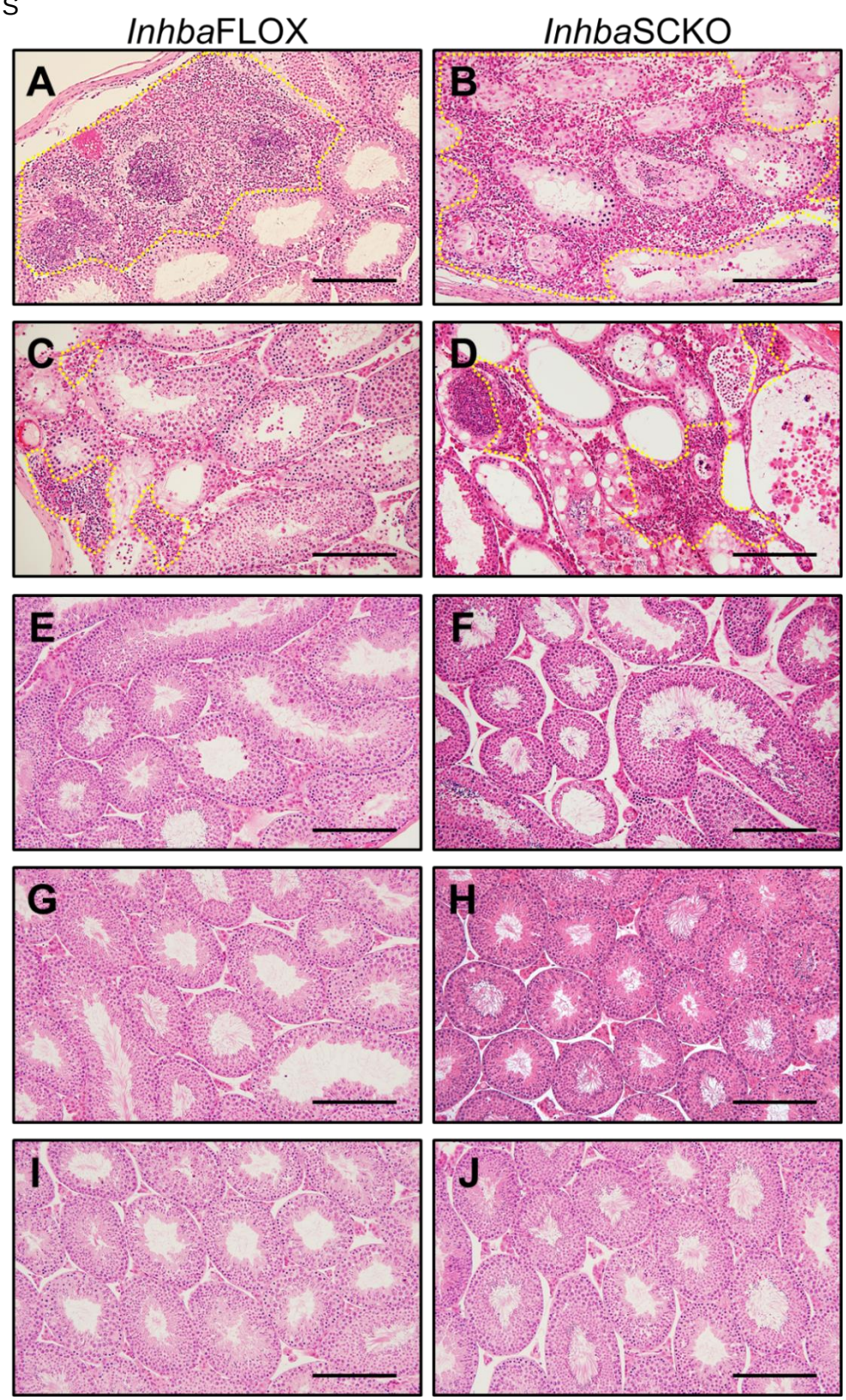

Figure 38: Leukocyte infiltration coincides with severity of EAO in testes of *Inhba*FLOX (A, C, E, G, I) and *Inhba*SCKO (B, D, F, H, J) mice 50 days after the first TH-immunisation. Leukocytes were qualitatively assessed by haematoxylin and eosin staining on testicular Bouin's-fixed paraffin sections from TH-immunised *Inhba*FLOX and *Inhba*SCKO mice. Representative pictures of leukocytic infiltrates in testes from animals with severe EAO **(A, B, C)**, low grade EAO **(D, E, F)**, TH-immunised testes without development of EAO **(G, H)** and adjuvant control testes **(I, J)**. Areas with infiltrates are indicated by dotted, yellow line. Scale bar = 100 µm.

3.5.4.3. Testes of adjuvant control *Inhba*SCKO mice may be more susceptible to inflammation

Histological examination of haematoxylin and eosin stained paraffin sections revealed the presence of leukocytic infiltrates in two of three examined testes of adjuvant control *Inhba*SCKO mice **(Table 7; Fig. 39A, B)**. No infiltrates were found in the testes of the six examined adjuvant control *Inhba*FLOX mice **(Table 7)** nor in any previous WT EAO experiments (Nicolas *et al.*, 2017a, Nicolas *et al.*, 2017b). This suggests that the *Inhba*SCKO mice may be susceptible to spontaneous autoimmune orchitis, which may be triggered by activation of the immune system through the action of adjuvant and *B. pertussis* toxin alone.

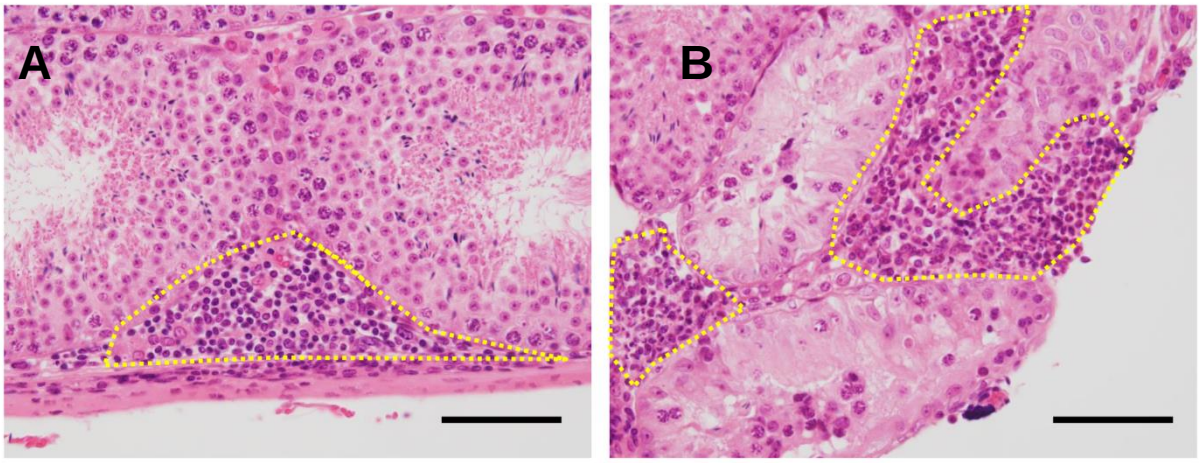

Figure 39: Leukocytic infiltrates were present in 2 of 3 adjuvant control *Inhba*SCKO mouse testes. Haematoxylin and eosin staining on paraffin sections showing testicular histology with leukocyte infiltrates of two *Inhba*SCKO mice **(A, B)**. Areas with infiltrates are indicated by dotted, yellow line. Scale bar = 50 µm.

3.5.4.4. Knock-down of SC-derived activin A has no effect on fibrosis development in EAO testes of *Inhba*SCKO mice

In order to assess the effect of activin A derived from SC on the development of fibrosis in EAO, relative collagen type I (*Col1a2*) mRNA levels were measured by qRT-PCR **(Fig. 40)** and collagen deposition was identified by Masson's trichrome staining **(Fig. 41)** in TH-immunised and adjuvant control testes of *Inhba*SCKO and *Inhba*FLOX mice.

No significant changes in the relative levels of collagen type I were detectable in the testes of TH-immunised and adjuvant control *Inhba*SCKO and *Inhba*FLOX mice **(Fig. 40)**.

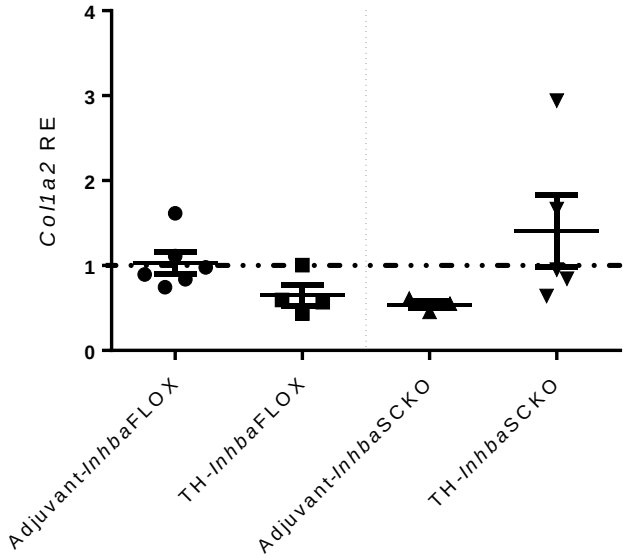

Figure 40: Relative mRNA levels of collagen type I α2 (*Col1a2)* in testes of *Inhba*FLOX mice and *Inhba*SCKO were not significantly different. Relative mRNA expression levels of *Col1a2* in the testes of TH-immunised and adjuvant control *Inhba*SCKO and *Inhba*FLOX mice at day 50 after the first immunisation analysed by qRT-PCR and normalised to 18S rRNA and hypoxanthin-guanin-phosphoribosyltransferase. Statistical analysis was done using one-way ANOVA followed by Tukey's multiple comparison for all treatment groups and for comparison of *Inhba*SCKO mice with *Inhba*FLOX controls. Data are shown as mean ± SEM; n = 3 – 6 animals per group.

Although not quantified, the Masson's trichrome staining revealed a relationship between the development of fibrosis and the degree of EAO. A strong fibrotic response, observed as an increase in collagen fibres, was present in severe EAO testes of *Inhba*SCKO and *Inhba*FLOX mice **(Fig. 41A, B)**, while very little or no fibrosis was seen in low grade EAO testes of both groups **(Fig. 41C, D)**. No fibrosis was found in TH-immunised testes without histological signs of EAO or in the adjuvant controls **(Fig. 41E, F, G, H)**. As the expression of collagen type I mRNA levels was similar in the testes of *Inhba*SCKO and *Inhba*FLOX mice **(Fig. 41)** and no differences in the development of fibrosis in EAO testes were found between the groups **(Fig. 41)**, these preliminary results indicate that activin A produced by SC has no effect on the development of fibrosis in chronic testicular inflammation.

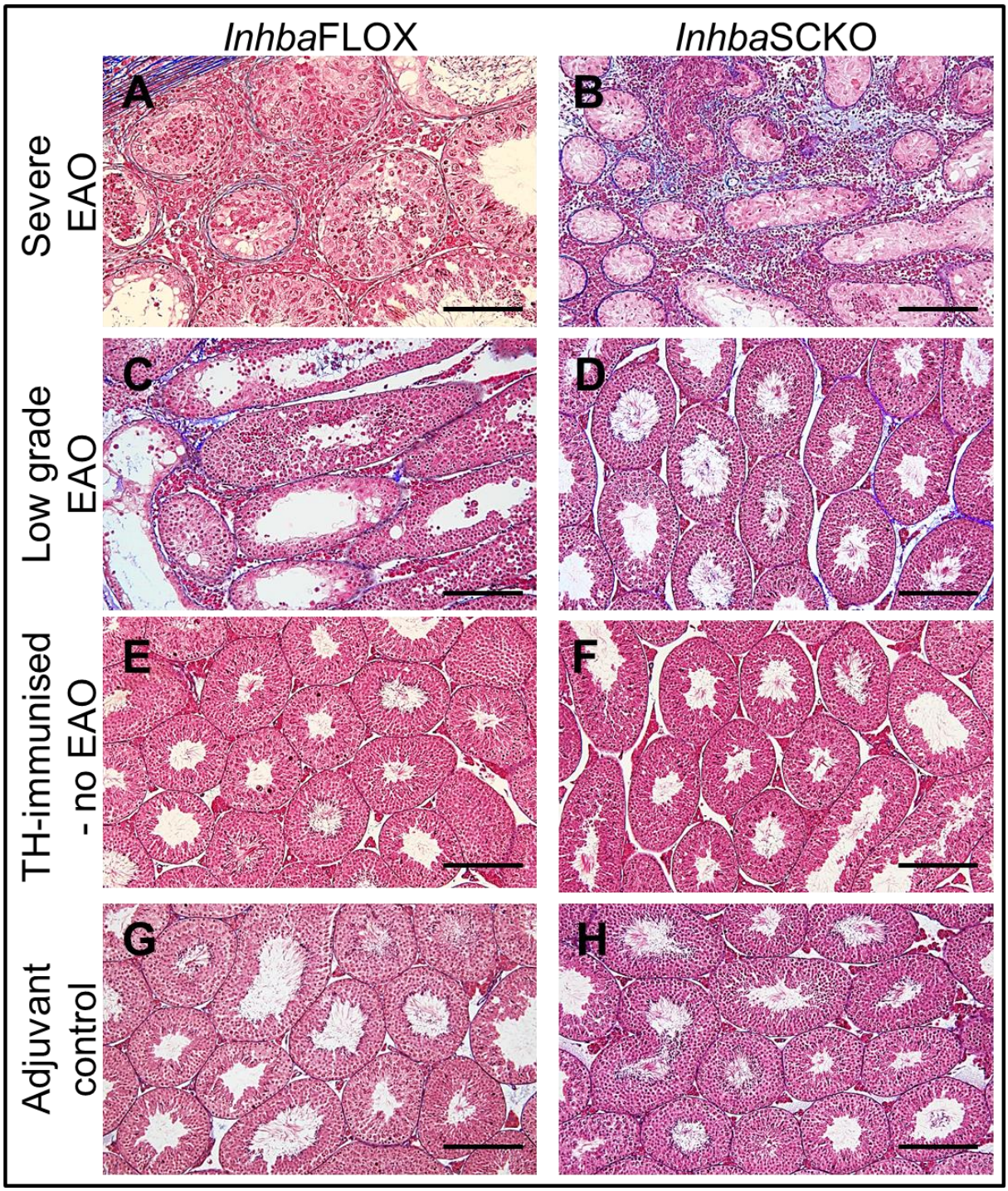

Figure 41: Fibrosis development in testes of *Inhba*FLOX (A, C, E, G) and *Inhba*SCKO (B, D, F, H) mice 50 days after first immunisation. The fibrotic response was evaluated by Masson's trichrome staining of testicular Bouin's-fixed paraffin sections from TH-immunised **(A - F)** and adjuvant control **(G, H)** *Inhba*FLOX **(A, C, E, G)** and *Inhba*SCKO **(B, D, F, H)** mice 50 days after first immunisation. Collagen fibres are stained in blue. Representative pictures of testes from animals with severe EAO **(A, B)**, low grade EAO **(C, D)**, TH-immunised testes without development of EAO **(E, F)** and from adjuvant controls **(G, H)**. Scale bars = 100 µm.

3.5.4.5. EAO scoring of testicular damage and analysis of EAO induction rate in *Inhba*SCKO and *Inhba*FLOX mice

As the testes of TH-immunised mice showed varying grades of EAO development, for reproducible comparison of EAO development between the groups, and to previous experiments, the scoring system of the disease progression was utilised from the previous study (Nicolas *et al.*, 2017b).

Table 7: EAO damage score and parameters used for the scoring assessment of the individual TH-immunised and adjuvant control *Inhba*SCKO and *Inhba*FLOX mice. Immune cell infiltrates, fibrosis development and tubular damage were assessed by histological examination of Bouin's-fixed paraffin sections from TH-immunised and adjuvant control *Inhba*SCKO and *Inhba*FLOX mouse testes. Relative mRNA levels of inflammatory cytokines were measured by qRT-PCR. Both testes of an animal were weighted. The left testis was used for the assessment of the histology and the right one was used for mRNA and protein isolation. ND = insufficient tissue for analysis. The presence of immune cell infiltrates, fibrosis and tubular damage was classified as follows: - no; + focal; ++ widespread; +++ majority; ++++ overall.

Treatment/ Group	Testis weight (histology)	Testis weight (mRNA analysis)	Infil-trates	Fibrosis	Tubular damage	*Tnf* RE	*Il6* RE	*Ccl2* RE	EAO damage score
TH-immunised *Inhba*SCKO	0.041	0.052	++++	++++	++++	100.8	15.4	192.4	4
	0.076	0.089	-	-	-	3.1	ND	ND	1
	0.082	0.081	-	-	-	0.6	0.8	1.0	0
	0.094	0.096	-	-	-	1.1	1.2	0.8	0
	0.079	0.080	-	-	-	0.7	0.6	1.4	0
	0.064	0.089	+	+	+++	2.6	2.5	2.8	3
Adjuvant controls *Inhba*SCKO	0.085	0.088	+	-	-	1.5	0.5	0.8	0
	0.086	0.098	+	++	++++	0.5	0.5	0.3	0
	0.087	0.085	-	-	-	0.5	0.5	0.5	0
TH-immunised *Inhba*FLOX	0.098	0.107	++	+	++	4.2	3.7	5.8	2
	0.093	0.098	-	-	-	0.5	0.9	0.4	0
	0.134	0.123	++	++	++++	10.0	7.6	29.1	3
	0.104	0.100	-	-	-	0.3	0.5	0.3	0
Adjuvant controls *Inhba*FLOX	0.081	0.085	-	-	-	1.2	1.0	0.8	0
	0.103	0.110	-	-	-	1.1	2.5	1.8	0
	0.101	0.108	-	-	-	0.7	0.7	1.0	0
	0.078	0.085	-	-	-	0.8	0.8	0.4	0
	0.076	0.082	-	-	-	0.8	0.7	0.8	0
	0.092	0.093	-	-	-	0.5	1.1	1.0	0

The individual EAO damage scores of the animals and the parameters used for the determination of the scores are listed in **Table 7**. A description of the scoring system is provided in **Chapter 2.2.4.1**.

TH-immunisation induced EAO in 50% (3/6) *Inhba*SCKO and 50% (2/4) *Inhba*FLOX mice 50 days after the first immunisation, suggesting that reduction of activin A in the SC does not prevent EAO from occurring. However, whether this reduces the severity of EAO has to be investigated further. Interestingly, one *Inhba*SCKO mouse from the adjuvant control group showed pathological changes of the testicular morphology with leukocytic infiltrates, fibrosis and overall reduction of the epithelial height **(Table 7)**. These changes do not resemble the histopathology of EAO and reasons for their presence need to be further clarified.

3.5.4.6. Activin A was positively correlated with EAO damage score in *Inhba*SCKO and *Inhba*FLOX mouse testes

Correlation analysis of activin A levels measured by ELISA and EAO damage score revealed that activin A levels increase with the degree of EAO in the testis of *Inhba*FLOX **(Fig. 42A)** and *Inhba*SCKO **(Fig. 42B)** mice. These results are consistent with the hypothesis that activin A plays a role in the development of the disease, and possibly indicate that cells other than the SC are involved in the increase in activin A production in the EAO testis. However, as the number of available animals was small, a certain conclusion is not possible and further investigation will be required.

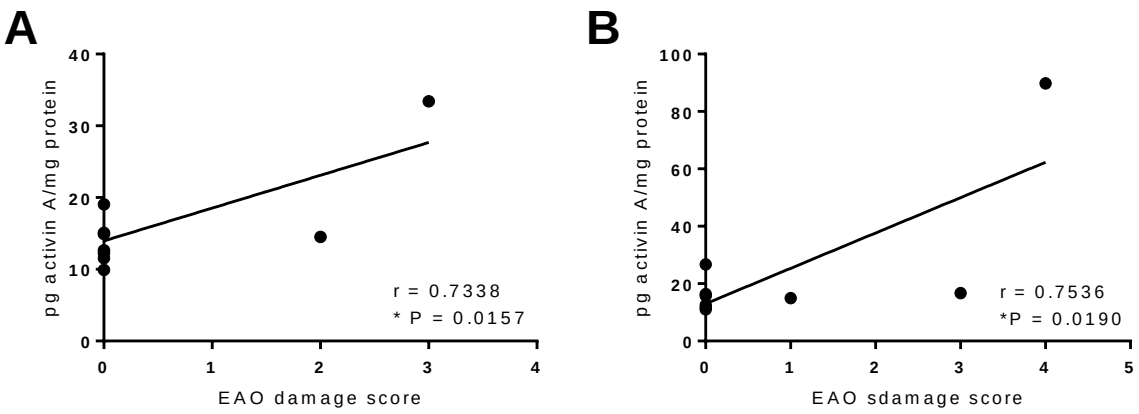

Figure 42: Activin A levels positively correlate with EAO damage score in *Inhba*FLOX (A) and *Inhba*SCKO mice (B). Correlation analysis of the EAO damage score and activin A measured by ELISA in testicular homogenates of TH-immunised and adjuvant control *Inhba*SCKO and *Inhba*FLOX mice at day 50 after the first immunisation. Statistical analysis was done using Pearson's r; n = 10 animals (4 TH-immunised and 6 adjuvant controls) **(A)** and 9 animals (6 TH-immunised and 3 adjuvant controls) **(B)**.

4. DISCUSSION

Although the testis is an immune privileged organ, infection and inflammation are able to overcome the tolerogenic mechanisms in some cases, and induce autoimmune reactions against testicular autoantigens resulting in impaired spermatogenesis and infertility. Murine EAO is a model of human testicular inflammation, useful for elucidating pathogenic mechanisms involved in development of testicular damage. Active immunisation of mice with TH in combination with adjuvant and *Bordetella pertussis* toxin results in the development of EAO, which is characterised by interstitial infiltration by leukocytes, fibrosis and apoptosis of germ cells, leading to damage or complete destruction of the seminiferous epithelium (reviewed in Fijak *et al.*, 2018).

It was shown, in the present thesis, that total collagen was significantly increased in EAO mouse testes 80 days after first TH-immunisation and in human testicular biopsies with impaired spermatogenesis and leukocytic infiltrates. Severity of the disease was also associated with elevation of fibronectin expression in the testes of mice with EAO and in human testicular biopsies with SCO.

These findings are in line with the fact that preformation of a fibronectin matrix is essential for the formation of a collagen network, as collagen deposition cannot occur in the absence of fibronectin and fibronectin guided collagen polymerisation (Shi *et al.*, 2010). Since collagens are the major components of ECM, and excessive ECM deposition is responsible for development of fibrosis, fibronectin and collagens were used as markers of fibrosis in this study. Expression of fibrotic markers was analysed to assess the fibrotic response in TH-immunised mouse testes, human testicular biopsies as well as different cell types *in vitro* for elucidation of their contribution to fibrosis development during chronic testicular inflammation. Furthermore, the effect of activin A on the inflammatory and fibrotic response was investigated *in vitro* and in a mouse model deficient for activin A in SC.

Overall, data from this study provide evidence for the involvement of PTC, fibroblasts and immune cells in the development of testicular fibrosis associated with chronic inflammation and the key role of activin A, which is increased in EAO mouse testis and in human testicular biopsies with impaired spermatogenesis and leukocytic infiltrates, in regulating the fibrotic responses of those cell types.

Furthermore, preliminary results indicate that activin A produced by SC has no effect on the occurrence of EAO as well as number of immune cell infiltrates and fibrosis development. However, *Inhba*SCKO mice may be more susceptible to inflammation. More study is needed to confirm these preliminary observations.

Clinical relevance of EAO mouse model for studying fibrosis in chronic testicular inflammation

Previous studies reported an activation of the SMAD signalling pathway and also an increase in SMAD2 mRNA levels in testicular biopsies of men with SCO, which are often found among patients diagnosed with idiopathic infertility (Sun *et al.*, 2008; Gonzalez *et al.*, 2010; Wijayarathna and de Kretser, 2016). This result suggests the involvement of TGF-β family members, such as activin A, which signal via phosphorylation of SMAD2/SMAD3 in the pathology. In this thesis, we focussed on activin A, as the increase in activin A was shown to be positively correlated with the severity of EAO in mouse testes, and activin A is also known to be a crucial regulator of fibrosis in many tissues (Nicolas *et al.*, 2017a).

In line with these data, results of this study revealed an increased expression of *INHBA* (activin A dimer subunit) mRNA levels in examined human testicular biopsies with idiopathic non-obstructive spermatogenesis impairment (SCO) and leukocytic infiltrates. In addition, collagens and fibronectin were found to be increased, and correlated with the extent of leukocytic infiltrates, *INHBA* mRNA levels and fibrosis development. In agreement with this, the number of leukocytes and levels of activin A were also found to be increased in EAO mouse testes, where they positively correlated with the severity of the disease (Nicolas *et al.*, 2017a). Moreover, we found that the distribution pattern of fibrotic markers was similar in examined human testicular biopsies and mouse testes.

These results indicate the existence of a similar mechanism in the development of fibrosis following chronic inflammation in the mouse and human and suggest a role of activin A as a mediator of this process in both organisms. These data also highlight the suitability of the EAO model to study fibrosis development and inflammation.

Multiple cell types involved in fibrotic response under the control of activin A

The involvement of different cell types, which excessively produce ECM proteins resulting in the development of fibrosis in different tissues, has been demonstrated in several reports. Often fibroblasts are reported as the major players in fibrotic diseases, including kidney and heart fibrosis. Moreover, activin A was shown to activate and induce the expression of αSMA, fibronectin and collagen type I in renal interstitial fibroblasts and αSMA, fibronectin and collagens type I and III in cardiac fibroblasts (Yamashita *et al.*, 2004; Yang *et al.*, 2018; Hu *et al.*, 2016; Wang *et al.*, 2017; Maeshima *et al.*, 2014).

Similar to renal interstitial fibroblasts, treatment of NIH 3T3 cells with activin A, in the present study, resulted in elevation of fibronectin mRNA levels and increased production of collagen type I, αSMA and fibronectin proteins. NIH 3T3 cells were employed as a surrogate for testicular fibroblasts. This was necessary, because isolation of pure testicular fibroblasts is technically impossible due to the lack of specific marker proteins exclusively expressed by testicular fibroblasts (Krenning *et al.*, 2010). Hence, further studies will be required to make a more informed conclusion. However, the elevation of αSMA is in line with the finding that fibroblasts acquire a smooth muscle cell phenotype during their activation (Bonnans *et al.*, 2014).

In liver fibrosis, the hepatic stellate cells, which are specialised pericytes found in the basement membrane of the perisinusoidal space, are the principal cell type involved in fibrotic ECM production (Tsukada *et al.*, 2006; Atzori *et al.*, 2009; Munde, 2014). Hepatic stellate cells and the testicular PTC have the common feature of producing basement membrane proteins. Examples of ECM proteins produced by PTC include several types of collagens, including collagen type I, III and IV, laminin and fibronectin. Under normal conditions in the human testis, collagen type I, III and IV are also located in the tubular basement membrane, further highlighting the similarity between mouse and human. Collagen type I and III are also expressed in the interstitial ECM. In pathological testes from male patients with infertility classified as idiopathic, the basement membrane layers of both thickened and obstructed tubules, which are often observed in chronic inflammation with fibrosis, were positive for collagens type I and IV, whereby collagen type IV was localised in the inner layer of the tubular basement membrane and the PTC layer (Takaba, 1990; Mayerhofer, 2013; Schell *et al.*, 2010). In line with these findings, our results demonstrate that activin A treatment resulted in elevation of fibronectin and collagen type IV mRNA levels and increased production of collagen type I and fibronectin in PTC, while it interestingly had no effect on the αSMA expression in PTC.

During the differentiation to myofibroblasts, an increase in αSMA is expected. We hypothesised that since PTC are myoid cells, which are required for the rhythmic contraction and relaxation of the seminiferous tubules to assure successful transport of the immotile spermatozoa (Mayerhofer, 2013), and hence, generally express this contractile protein for proper execution of function, their expression of αSMA under physiological conditions is already sufficiently high. However, a recent study provides evidence that αSMA is an inconsistent marker of contractile and collagen-producing fibroblasts as only a minority of collagen-producing cells co-express αSMA in the fibrotic lung and kidney (Sun *et al.*, 2016).

In contrast to the thickened basement membrane of fibrotic specimens from men with idiopathic male infertility, collagen type IV expression is not changed in the interstitial fibrotic area, while collagen type I is still increased (Takaba, 1990). We observed the same expression pattern in the NIH 3T3 cells following activin A treatment. Based on this observation, we speculate that possibly both PTC and fibroblasts are involved in the development of testicular fibrosis. While PTC may contribute to the development of tubular fibrosis, resident fibroblasts might be responsible for the interstitial fibrosis.

It is noteworthy that these findings were obtained through the use of an *in vitro* system with mouse PTC, and the clinical relevance of the results requires further validation. Structural and functional differences of the testis, including connective tissue composition, architecture of the tubular wall and differences in the numbers of immune cell classes, between the human and mouse testis exist (Mayerhofer *et al.*, 2018). Hence, the involvement of PTC in the fibrotic response in human testis should be further investigated using a human PTC culture system as well as in *in vivo* models. While the protocol for isolation and culture of adult human PTC is already established (Albrecht *et al.*, 2006), human testicular tissue was not available for the present study. Interestingly, despite the fact that enhanced synthetic activities of human PTC have been implicated in tubular fibrosis in patients with infertility caused by impaired spermatogenesis, studies on mixed atrophy patients showed a reduction of the expression of several contractile marker proteins, such as myosin heavy chain, αSMA, calponin and cGMP-dependent protein kinase 1. This indicates the alteration and impairment of contractile functions of PTC, possibly leading to the impairment of sperm transport (Welter *et al.*, 2013). We were not able to detect changes in αSMA expression in activin A or FST288 treated primary mouse PTC. This lack of regulation of αSMA expression might be due to other factors, not tested in the present study, being responsible for the downregulation of αSMA, or biological differences between the mouse and humans. The latter option is plausible as previous studies on EAO mouse testes demonstrated a change in the distribution, but not the amount of αSMA (Nicolas *et al.*, 2017a). Nonetheless, although we did not see a downregulation of αSMA in PTC after activin A stimulation, we speculate that the excessive production of collagen types I and IV, and fibronectin may lead to stiffening of the tubular wall, which might hamper the contractility of the seminiferous tubules, resulting in infertility.

Interestingly, we were not able to detect changes in the mRNA levels of collagen type I in PTC or NIH 3T3 cells, while in both cell types an upregulation of the protein levels was present. An explanation for this finding might be coming from studies showing that the enormous upregulation of collagen expression in wound healing and fibrosis is mainly due to post-transcriptional mechanisms, including upregulation of collagen mRNA translation and increased stability of the protein product (Lindquist *et al.*, 2000; Zhang and Stefanovic, 2016).

Furthermore, we were not able to detect changes in the expression levels of collagen type III in PTC or NIH 3T3 cells. However, the reason for this might be regulation of collagen type III expression at time points other than those examined in this study.

Another novel finding of this study was the presence of collagen type I and CD45 or F4/80 double positive cells in EAO mouse testes. Those apparently collagen-expressing immune cells were missing in the testes of control animals. This result indicates the possibility that immune cells/macrophages at least partly contribute to the fibrotic response in the EAO testis by monocyte to fibroblast transition. The involvement of bone marrow derived monocytes in fibrosis, as progenitor population of activated fibroblasts, was shown in other diseases, including renal fibrosis, which is seen in progressive chronic kidney diseases (Morishita *et al.*, 2014; Yang *et al.*, 2013). Moreover, Yang and colleagues showed the co-expression of CD45 and collagen type I, as well as the expression of procollagen by macrophages. However, how the immune cells are involved in the induction of fibrosis would need to be investigated further. A possible method would be the measurement of fibrotic marker expression, such as collagen or fibronectin, following stimulation of isolated macrophages with activin A.

Taken together, these data suggest that multiple cell types contribute to fibrosis development in EAO testis, including PTC, resident fibroblasts and macrophages. Also, the involvement of other cell types, not explored in this study, should be considered. Estimations from injury induced renal fibrosis imply that approximately 15% of fibroblasts originate from the bone marrow, 36% arise from local tubular epithelial cells via EMT, and the rest is likely to be contributed by proliferation and activation of resident fibroblasts (Kalluri and Neilson, 2003).

Activin A - a key player in testicular fibrosis

A role of activin A in tissue fibrosis has been established in models of liver, heart, kidney and lung fibrosis (Ohga *et al.*, 1996; Yamashita *et al.*, 2004; Hu *et al.*, 2016). Activin A stimulates the proliferation and differentiation of cardiac fibroblasts (Hu *et al.*, 2016). In rat models of hepatic fibrosis and anti-Thy1 glomerulo-nephritis, a widely used animal model for human mesangial proliferative glomerulonephritis induced by anti-Thy1.1 monoclonal antibody, mRNA and protein levels of activin A were upregulated and coincided with the occurrence of fibrosis (Gaedeke *et al.*, 2005; Sugiyama *et al.*, 1998; Wan *et al.*, 2007).

Moreover, activin A was detected in glomerular mesangial cells and interstitial fibroblasts in normal kidneys, as well as abundantly expressed in interstitial αSMA-positive myofibroblasts in unilateral ureteral obstruction (UUO) kidneys (Maeshima et al. 2014). Other studies also demonstrated that SMAD4 knockdown by siRNAs inhibited the proliferation and differentiation of myofibroblasts and inhibited renal fibrosis (Morishita et al. 2014).

As activin A signals through SMADs these data might be an indication for the involvement of activin A action in renal fibrosis.

In this study, activin A was able to induce the expression of ECM proteins in PTC and NIH 3T3 cells, implicating it as a key mediator of testicular fibrosis in EAO. As activin A activated SMAD2 and SMAD3 signalling in both NIH 3T3 cells and PTC, we propose that it acts directly on the cells to induce the expression of the fibrotic genes.

Whether activin A has an influence on the fibrotic response of bone marrow-derived macrophages remains to be investigated.

Involvement and interactions of testicular cells and inflammatory mediators in the inflammatory response– possible implications for chronic testicular inflammation

Previous studies revealed a strong activin A expression in SC and in cells within the inflammatory infiltrates following induction of EAO in mice (Nicolas *et al.*, 2017a). Testicular inflammation also resulted in increased TNF levels in the mouse testis (Nicolas *et al.*, 2017a). Earlier reports in a rat model of EAO showed that TNF is produced by testicular macrophages from EAO animals, and is particularly relevant for the induction of germ cell apoptosis leading to testicular damage in severe EAO (Theas *et al.*, 2008). Hence, the importance of inflammatory mediators, including TNF and activin A, as well as involvement of different cell types, such as SC and PTC, in the inflammatory response was examined to get a better understanding of mechanisms leading to chronic testicular inflammation.

LPS treatment was used to mimic the effect of inflammatory conditions on mouse SC *in vitro*. SC significantly increased their expression of *Tnf*, *Ccl2*, *Inhba* and *Il1a* mRNA. As *Tnf*, *Ccl2* and *Inhba* were found to be increased in the testes of mice with EAO, this result suggests the importance of SC in the development of inflammation.

Treatment with activin A had no significant effect on the expression of genes encoding inflammatory mediators, such as TNF, MCP-1, activin A, IL-1α and IL-6 in primary SC and PTC, while treatment with FST288 decreased the basal expression of mRNA encoding for MCP-1 and IL-6 in SC and IL-6 in PTC. These data suggests an immunosuppressive role of follistatin.

Treatment with TNF resulted in a significant increase of MCP-1, IL-1α and IL-6 mRNA expression in SC and TNF, MCP-1 and IL-6 in PTC. These results are in agreement with publications on human male infertility, which proposed that human testicular mast cells and macrophages as well as other cell types produce TNF, which may target PTC and stimulate their secretion of IL-6 and MCP-1 upon activation of TNF receptors (Mayer *et al.*, 2016; Schell *et al.*, 2008).

Other studies demonstrated an increase of TNF mRNA levels in human specimens with disturbed spermatogenesis and inflammatory lesions (Loveland *et al.*, 2017; Klein *et al.*, 2016). As results of a previous study showed an increase of TNF and MCP-1 mRNA in EAO mouse testis (Nicolas *et al.*, 2017a), the data in this study provide evidence for the importance of TNF and its interaction with SC and PTC in the initiation and maintenance of the inflammatory response in EAO mouse testis. Furthermore, these results suggest the possible existence of a similar mechanism between testicular inflammation in human and mouse.

Furthermore, results of this work provide evidence that TNF leads to elevated production of activin A in SC, which are commonly thought to be the main producers of activin A in mouse testis under physiological conditions. These results were the basis for investigations of the role of SC-derived activin A in development of chronic testicular inflammation. However, preliminary results from the study on *Inhba*SCKO mice showed no reduction of testicular activin A levels in the normal testis and moreover revealed that activin A level and EAO damage score still were positively correlated in TH-immunised *Inhba*SCKO mice, possibly indicating a minor contribution of SC to testicular activin A production during EAO development.

Characterisation of the *Inhba*SCKO mouse model

Phenotypic characterisation of the *Inhba*SCKO mouse model presented in this work resulted in conflicting evidence for the knock-down efficiency of *Inhba* in SC. Although testicular *Inhba* mRNA levels were reduced, and activin A immunohistochemistry staining showed changes in the compartmental distribution of activin A in SC, no reduction of testicular activin A protein levels was observed and limited resolution of the immunohistochemical method, as well as strong staining intensity in late germ cells and residual bodies, made it difficult to identify and quantify differences in activin A staining. Moreover, no differences in paired testis weights were observed in *Inhba*SCKO and *Inhba*FLOX mice.

Testicular weight reduction would have been expected, as activin A was shown to balance SC and germ cell proliferation in the foetal mouse testis, and research on testis development in the *Inhba* null mouse revealed a reduction of the testis weights, attributable to lower SC number (Mendis *et al.*, 2011). Additionally, despite SC-derived activin A was reported to be involved in the regulation of spermatogenesis (Hedger and Winnall, 2012; Mather *et al.*, 1990; Hakovirta *et al.*, 1993), no changes in gross testicular morphology or impairment of spermatogenesis were observed in the *Inhba*SCKO mouse.

However, there was also evidence for phenotypic differences of the *Inhba*SCKO mice compared to their *Inhba*FLOX controls. Overall, reduction of activin A in the SC affected lumen diameter in some areas, but not tubule diameter, indicating effects on the production of luminal fluid by the SC. Other differences of the *Inhba*SCKO mice and their *Inhba*FLOX controls include the emergence of leukocytic infiltrates in response to adjuvant treatment suggesting that the reduction of activin A in the SC has an effect on the susceptibility to inflammation, but studies using larger numbers of mice (which were not possible in the time-frame available) will be required to unravel the meaning of this finding.

There are two possible explanations for the lack of a clear phenotype:

One possibility is the low efficiency of the Cre-recombinase to knock-down *Inhba*. This has been reported in other studies (Turlo *et al.*, 2010; Heffner *et al.*, 2012). For example, Turlo *et al.* observed that Cre-mediated recombination in mice does not result in protein loss and lacks a phenotype when the Cre-recombination episomal product persisted in the cells (Turlo *et al.*, 2010). The retention of the product was demonstrated to be possible in cells with low proliferative capacity, such as the SC. However, both the *Amh-Cre* mouse and the *Inhba*-floxed mouse, used for generating the *Inhba*SCKO mouse in this work, have been used to knock-out genes before, including successful knock-outs in the SC (Tsuji-Hosokawa *et al.*, 2018; Barrionuevo *et al.*, 2009; Archambeault and Yao, 2010; Pangas *et al.*, 2007; Lecureuil *et al.*, 2002).

Another possibility is the compensation of activin A production by other cell types, especially the meiotic germ cells, Leydig cells and macrophages, despite activin A has been knocked down or deleted in the SC. In this thesis, activin A immunohistochemistry staining was observed within late germ cells and the cytoplasm of the SC as well as residual bodies and intracytoplasmic vesicles in the *Inhba*FLOX control. In *Inhba*SCKO mice, activin A expression was mostly restricted to residual bodies and intracytoplasmic vesicles and late germ cells. These changes in the compartmental distribution of activin A indicate that the activin A appears to be retained in the spermatids and their residual cytoplasm.

These data suggest that production of activin A by the spermatogenic cells is minimally affected in the *Inhba*SCKO mice, and that activin A in the SC of these mice may be derived from the spermatogenic cells rather than production by the SC themselves. These results, and the fact that knocking down activin A production in the SC does not reduce total activin A levels in the testis, would indicate that the SC are not the major source of activin A in the testis under normal conditions (healthy testis), as it was commonly assumed from previous results (Hedger and Winnall, 2012). The strong staining intensity highlights the possibility of spermatogenic cells being a major source of activin A in the mouse testis. However, the functional role of the germ cell-derived activin A is not known.

It is also not clear whether the spermatid activin A is secreted into the interstitium where it exerts a function, or whether it is simply metabolised by the SC when they digest the residual cytoplasm.

Taken together, further investigations will be required to explain the conflicting results of the *Inhba*SCKO phenotype. Once more animals will be available for the study, the efficiency of the SC-derived activin A knock-down needs to be assessed by measurement of endogenous *Inhba* mRNA levels by qRT-PCR and activin A concentration by ELISA in isolated SC from *Inhba*SCKO mice.

SC-derived activin A is not involved in inflammatory and fibrotic responses in EAO mouse testis

Due to time constraints placed by the Justus Liebig University candidature, the EAO study using *Inhba*SCKO was not completed. Hence, results presented in this thesis are preliminary. A greater number of animals will be available after thesis submission and included in the study. Moreover, untreated animals as well as *Inhba*WT controls, which were not available at the time of the thesis submission, will be included in the study and the effect of SC-derived activin A on the progression and severity of EAO further investigated.

Preliminary results of this work showed no differences in the induction rate of EAO, amount of leukocytic infiltration, severity of fibrosis and destruction of testicular architecture between *Inhba*SCKO and *Inhba*FLOX mice, indicating that activin A produced by SC has no influence on disease parameters. Moreover, positive correlation of activin A and the EAO damage score suggests that SC-derived activin A does not play a role in regulating progression to orchitis.

Interestingly, no differences in the paired testis weights were seen in TH-immunised *Inhba*FLOX mice compared to the adjuvant controls, and a significant decrease of paired testis weights was seen in TH-immunised *Inhba*SCKO mice compared to TH-immunised *Inhba*FLOX mice.

The reason for this is not clear. Previous studies would have suggested a decrease of testicular weights in EAO mouse testes (Nicolas *et al.*, 2017a; Nicolas *et al.*, 2017b). This relationship was seen in TH-immunised testes of *Inhba*SCKO mice which developed EAO, while reduction of testis weights was missing in EAO testes of *Inhba*FLOX mice. In fact, one TH-immunised *Inhba*FLOX mouse, which developed EAO with a damage score of 3, exhibited a weight increase of both testes. An explanation for this observation could be either an effect of the *Inhba* floxing or the mixed 129S6/SvEv/C57BL6J/SJL background of the mice.

Various studies demonstrated varying susceptibilities of mouse stains with different genetic backgrounds to EAO (Tokunaga *et al.*, 1993; Teuscher *et al.*, 1985). This possibly has an influence on the pathology and progression of the disease. Increase in the testis weights might be due to increased fluid accumulation, which might precede damage to the seminiferous epithelium. In fact, dilatation of testicular lymphatic spaces and increase in testis weight are well-established characteristics of mouse testes with passive EAO (Tung *et al.*, 1987; Tung *et al.*, 1989).

Notably, based on the similarity of the testis weights, development of EAO was assumed to occur bilaterally. However, by this method it is not possible to completely exclude the incidence of unilateral EAO. This might have resulted in incomplete evaluation of EAO parameters, because histology was linked with not accurate mRNA and protein levels, as one testis was used for histology assessment, and the other one for RNA and protein isolation.

Previous studies have shown that in severe EAO testis a strong immunofluorescence staining of activin A was detectable in SC as well as in cells within the inflammatory infiltrates. As knock-down of activin A derived from SC seems to have no effect on the development of fibrosis and inflammation, infiltrating immune cells, such as macrophages, are promising candidates which should be investigated for their involvement in inflammatory and fibrotic responses. Because severe fibrosis is a characteristic of late EAO, and germ cell-loss is another feature of this disease stage, the contribution of germ cell-derived activin A to fibrosis development in EAO is unlikely.

The involvement macrophage-derived activin A in tissue repair and fibrosis was shown in various studies (Aoki et al., 2005; Miron et al., 2013; Kozaki and Ouchi, 1998). In bleomycin-induced pulmonary fibrosis, not only the production of activin A by macrophages was observed but also amelioration of lung architecture destruction and attenuation of lung fibrosis following follistatin administration, suggesting an important role of activin A produced by macrophages in promoting acute inflammation and subsequent fibrosis (Aoki et al., 2005).

In addition, several studies have demonstrated the production of activin A following exposure to inflammatory stimuli in diverse cell types, most notably cells of the myeloid lineage such as monocytes, macrophages and dendritic cells, suggesting an important role of macrophage-derived activin A in inflammation (de Kretser et al., 2012).

However, the mechanism of how activin A regulates the development of fibrosis in different tissues and organs is not known yet.

We speculate that due to persistence of inflammatory conditions in the EAO testis, the constantly high production of macrophage-derived activin A might contribute to fibrosis development. Hence, elucidating the effect of activin A derived by macrophages on the development of testicular inflammation and fibrosis in EAO could considerably contribute to our understanding of the disease mechanisms.

Hypothetic model of fibrosis development in EAO testis

Based on data presented in this thesis, the following model is proposed **(Fig. 43)**: During the course of EAO, inflammation, including production of TNF by infiltrating immune cells, such as macrophages and T cells, leads to an increased production of activin A by cells within the testis, which possible include macrophages and/or SC.

Activin A secretion by those cells in turn upregulates the production of ECM components in PTC, resident fibroblasts, macrophages and possibly other cell types. The persistence of TNF production due to chronic inflammation ultimately results in the development of fibrosis.

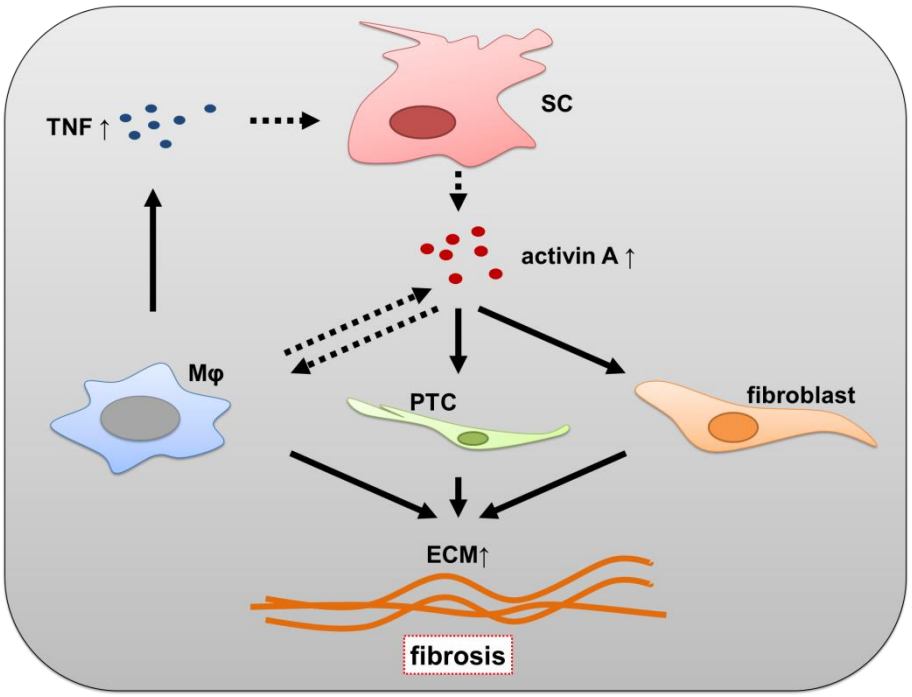

Figure 43: Schematic and simplified scheme summarising the hypothetic model of fibrosis development in EAO testis. Immune cells such as macrophages (Mφ) infiltrate EAO testis and produce TNF in response to inflammatory stimuli. This stimulates the synthesis of activin A by Sertoli cells (SC) and/or by Mφ themselves. Activin A increases the production of ECM components in PTC, resident fibroblasts and possibly Mφ. Chronic inflammation ultimately results in the excessive deposition of ECM resulting in development of fibrosis.

Activin A antagonists as potential therapeutic agents

The effect of activin A on the expression of collagen type I and fibronectin in NIH 3T3 cells and PTC was blocked by the addition of the activin A antagonist, follistatin. The suitability of activin antagonists as therapeutic agents is supported by recent research on the use of follistatin as inhibitor of activin A action in different pathologies (Hardy *et al.*, 2015; Maeshima *et al.*, 2014; Nicolas *et al.*, 2017b). Treatment of new born transgenic mice carrying an overexpression of β-subunit of the epithelial sodium channel in the airway epithelium resulting in cystic fibrosis (CF)-like lung pathology, mimicking human CF, with follistatin, decreased the airway levels of activin A. Follistatin also reduced key features of CF lung disease, causing respiratory failure and premature death, including mucus hypersecretion and airway neutrophilia, while increasing survival of the mice (Hardy *et al.*, 2015).

Moreover, follistatin administration reduced the fibrotic area in kidneys in a rat model of UUO that commonly features significant increased activin A levels in UUO kidneys compared to levels in contralateral kidneys (Maeshima *et al.*, 2014). Follistatin also decreased the expression of αSMA and production of collagen types I and III, and fibronectin in the follistatin-treated kidneys, whose elevation is a marker for the severity of the disease. These data not only demonstrate the strong pro-fibrotic role of activin A produced by interstitial fibroblasts in renal fibrosis, but also indicate that inhibition of activin A action may be an effective approach for the prevention of renal fibrosis (Maeshima *et al.*, 2014).

Moreover, in testes from mice with elevated levels of the circulating form of follistatin, induction of EAO was reduced, while the EAO damage score and extent of testicular inflammation were directly proportional to the levels of activin A (Nicolas *et al.*, 2017b). Although these data implicate an importance of activin A in EAO progression and show its potential as therapeutic target, EAO development in mouse testis was not sufficiently prevented by elevation of circulating levels of follistatin (Nicolas *et al.*, 2017b).

This may be due to the insufficiency of elevated circulating follistatin levels to raise the concentration of follistatin in the testis, but might also indicate the involvement of additional pro-fibrotic pathways.

Combining these data with the findings of the present study, which indicate a significant role for activin A in the development of testicular fibrosis following chronic inflammation, we propose that exploring the use of activin A antagonists as therapeutic agent may be beneficial in limiting inflammation, fibrosis and fertility impairment in patients with testicular disease. The efficiency of follistatin in ameliorating the severity of EAO and fibrosis development could be investigated by local elevation of testicular follistatin levels, using a lentiviral vector with a promotor specifically expressed in the testis.

Conclusion and future directions

Taken together, results of this thesis provide evidence for the involvement of different cell types, such as PTC, fibroblasts and immune cells in the development of testicular fibrosis associated with chronic inflammation, and pointing out a key role of activin A in regulating fibrotic responses in mouse and human testes.

Further studies are required to fully understand the involvement of activin A as well as the highly orchestrated and intricate web of cellular and chemical interactions regulating inflammatory and fibrotic responses in the EAO testis and contributing to severity of the disease.

These should include the investigation of the involvement of immune cells in the induction of fibrosis. A first experimental step could be the measurement of fibrotic marker expression following stimulation of isolated macrophages with activin A.

In addition, further investigations will be required to explain the conflicting results of the *Inhba*SCKO phenotype. The efficiency of the SC-derived activin A knock-down needs to be assessed by measurement of activin A concentration by ELISA in isolated SC from *Inhba*SCKO mice.

As preliminary results of this thesis indicate that SC are not responsible for the production of activin A contributing to EAO development, future experiments should include the investigation of the effect of activin A derived by macrophages on the development of testicular inflammation and fibrosis in EAO.

Furthermore, these preliminary observations showing the lack of effect of SC-derived activin A downregulation in *Inhba*SCKO mice on disease parameters of EAO should be confirmed using a larger number of mice, and by including an *Inhba*WT control group.

Moreover, the use of follistatin as a potential therapeutic target together with other anti-inflammatory drugs should be explored in order to reduce the disease severity and to ultimately restore fertility. E.g., the local elevation of testicular follistatin levels could be investigated using a lentiviral vector with a promotor specifically expressed in the testis.

5. SUMMARY

Infection and inflammation of the male reproductive tract have been estimated to be responsible for ~15% of all cases of infertility. Experimental autoimmune orchitis (EAO) in mice is a model of immunologic male infertility, pathologically characterised by lymphocytic inflammation causing breakdown of the testicular immune privilege, spermatogenesis impairment and fibrosis. EAO in mice was induced by immunisation with testicular homogenate (TH) + adjuvant + *Bordetella pertussis* toxin.

Activin A, a member of the transforming growth factor-β superfamily of immunoregulatory cytokines, is a regulator of fibrosis and inflammation in various tissues, highlighting an emerging potential as a target for novel diagnostic and therapeutic strategies. It was found to be increased in EAO mouse testes. However, it is not known whether activin A is directly involved in testicular inflammation and fibrosis observed in EAO.

Results of this study show that activin A subunit (*INHBA*) gene expression levels and the key fibrotic proteins, collagen and fibronectin, are elevated in human testicular biopsies with impaired spermatogenesis and leukocytic infiltrates, and that activin A is positively correlated with collagen and the extent of leukocytic infiltration in these biopsies. Collagen and fibronectin were also found significantly increased in murine testes with EAO, whereby fibronectin expression positively correlated with the severity of the disease. Moreover, pre-treatment of mice with a vector carrying a gene cassette of the activin antagonist follistatin 315 (rAAV-FST315) prior to immunisation with TH reduces testicular fibronectin expression, and hence, possibly positively influences EAO. Treatment of mouse primary peritubular cells (PTC) and NIH 3T3 cells with activin A resulted in increased levels of fibronectin mRNA and elevated production of collagen type I and fibronectin. Activin A treatment also increased collagen type IV mRNA levels in PTC, while in NIH 3T3 cells αSMA mRNA and protein expression were elevated by activin A. Moreover, the existence of cells co-expressing collagen type I and CD45 or F4/80 suggests the involvement of macrophages in the fibrotic response in the EAO testis. These data indicate that multiple cell types may contribute to fibrosis development in EAO testis including PTC, resident fibroblasts and macrophages, and implicate activin A as a key mediator of this process, at least in fibroblasts and PTC. Results of this work also showed that TNF stimulated activin A production in Sertoli cells (SC). The role of SC-derived activin A was investigated in mice lacking activin A expression in SC (*Inhba*SCKO). Preliminary results showed no differences in the EAO induction rate, amount of leukocytic infiltration and fibrosis severity between TH-immunised *Inhba*SCKO and *Inhba*FLOX mice, while activin A and the EAO damage score still were positively correlated indicating that SC-derived activin A does not play a role in regulating progression to EAO.

6. ZUSAMMENFASSUNG

Infektionen und Entzündungen der männlichen Geschlechtsorgane tragen zu schätzungsweise 15% aller Fälle von Unfruchtbarkeit bei. Die experimentelle Autoimmun - Orchitis (EAO) der Maus dient als Model zur Untersuchung immunologisch bedingter Infertilität beim Mann und ist pathologisch durch die leukozytäre Infiltration charakterisiert, welche zur Störung des testikulären Immunprivilegs führt, sowie zur Beeinträchtigung der Spermatogenese und Entstehung von Fibrose. Die EAO wurde in Mäusen durch Immunisierung mit Hodenhomogenat (HH) + Adjuvant + *Bordetella pertussis* Toxin ausgelöst.

Aktivin A, ein immunregulatorisches Zytokin aus der Transforming Growth Factor-β Superfamilie, ist ein Regulator von Fibrose und Inflammation in einer Vielzahl von Geweben, was sein wachsendes Potenzial als neuer diagnostischer und therapeutischer Marker zeigt. Obwohl in EAO Maushoden eine Erhöhung der Aktivin A-Expression nachgewiesen werden konnte, ist bisher nicht bekannt, ob Aktivin A direkt an der Entstehung der Entzündung und Fibrose im EAO Hoden beteiligt ist.

Ergebnisse dieser Arbeit zeigen eine erhöhte mRNA Expression der Aktivin A Untereinheit (*INHBA*), sowie der Fibroseproteine, Kollagen und Fibronektin, in humanen Hodenbiopsien mit beeinträchtigter Spermatogenese und leukozytären Infiltraten. Des Weiteren konnte in diesen Biopsien eine positive Korrelation von Aktivin A, Kollagen Expression und dem Ausmaß der leukozytären Infiltration gezeigt werden. Kollagen und Fibronektin waren in Maushoden mit EAO ebenfalls signifikant erhöht, wobei die Expression von Fibronektin positiv mit der Schwere der Erkrankung korrelierte. Zudem führte eine Vorbehandlung von Mäusen mit einem Vektor, der eine Genkassette des Aktivin Antagonisten Follistatin 315 beinhaltete (rAAV-FST315), vor der Immunisierung mit HH, zu einer Reduktion der Expression von Fibronektin im Hoden, was einen positiven Einfluss auf die Schwere der EAO hatte. Die Behandlung primärer Peritubularzellen (PTC) und NIH 3T3 Zellen der Maus mit Aktivin A resultierte in erhöhter Fibronektin mRNA-Expression und verstärkter Produktion von Kollagen Typ I und Fibronektin. Darüber hinaus, führte die Stimulation mit Aktivin A zu einem Anstieg des Kollagen Typ IV mRNA in PTC, während es in NIH 3T3 Zellen die αSMA mRNA- und Protein-Expression erhöhte.

Zudem weisen testikuläre Zellen, die Kollagen Typ I und CD45 bzw. F4/80 ko-exprimieren, auf eine Beteiligung von Makrophagen an der fibrotischen Antwort im EAO Hoden hin. Diese Daten suggerieren, dass vermutlich eine Vielzahl von Zelltypen, einschließlich der PTC, residenten Fibroblasten und Makrophagen, an der Entwicklung von Fibrose im EAO Hoden

beteiligt ist und implizieren, dass, zumindest in Fibroblasten und PTC, Aktivin A ein Hauptmediator dieses Prozesses ist.

Ergebnisse dieser Arbeit zeigten auch, dass TNF die Aktivin A Produktion in primären Sertoli Zellen (SC) stimulierte. Die Rolle des von SC gebildeten Aktivin A wurde in Mäusen untersucht, in denen die Expression von Aktivin A in SC fehlte (*Inhba*SCKO). Vorläufige Ergebnisse zeigten keine Unterschiede in der EAO Induktionsrate, dem Ausmaß der leukozytären Infiltraten und Schwere der Fibrose zwischen HH-immunisierten *Inhba*SCKO und Kontrollmäusen, wobei Aktivin A noch immer mit der Schwere der Erkrankung korrelierte, was ein Hinweis dafür ist, dass das von SC gebildete Aktivin A wahrscheinlich keine entscheidende Rolle bei der Regulation der EAO Entwicklung spielt.

7. ABBREVIATIONS

18S	18S rRNA
Actb	β-actin
ActR1	Activin A receptor, type I
ActR2	Activin A receptor, type II
αSMA	Alpha smooth muscle actin
Azan	Azocarmine and aniline blue
B2m	β2-microglobulin
BAMBI	Bone morphogenetic protein and activin membrane-bound inhibitor
BSA	Bovine serum albumin
BTB	Blood-testis barrier
$CaCl_2$	Calcium chloride
Ccl2	Chemokine (C-C motif) ligand 2
CCR2	CC-chemokine receptor-2
cDNA	Complementary deoxyribonucleic acid
CF	Cystic fibrosis
CFA	Complete Freund's adjuvant
Col1a2	Collagen type I, alpha 2
Ct	Threshold cycle
CV	Coefficient of variation
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cells
ddH_2O	Double-distilled water
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate

EAE	Experimental autoimmune encephalomyelitis
EAO	Experimental autoimmune orchitis
ECM	Extracellular matrix
EDTA	Ethylene diaminetetraacetic acid disodium salt
ELISA	Enzyme-linked immunoabsorbent assay
F12	Nutrient mixture F-12
FCS	Foetal calf serum
FSH	Follicle-stimulating hormone
FST	Follistatin
g	G-force
Gapdh	Glyceraldehyde 3-phospate dehydrogenase
gDNA	Genomic Deoxyribonucleic acid
GnRH	Gonadotropin releasing hormone
h	Hour
HCl	Hydrogen Chloride
H&E	Haematoxylin and eosin staining
Hprt	Hypoxanthine guanine phosphoribosyl transferase
HRP	Horseradish peroxidase
IDO	Indolamine 2,3-dioxygenase
IF	Immunofluorescence
IFA	Incomplete Freund’s adjuvant
IHC	Immunohistochemistry
IL	Interleukin
Inhba	Inhibin beta A subunit
IFN-γ	Interferon-gamma
JNK	c-Jun N-terminal protein kinases
LH	Luteinizing hormone
LPS	Lipopolysaccharide

ABBREVIATIONS

MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein 1
$MgCl_2$	Magnesium chloride
min	Minutes
mRNA	Messenger RNA
NaCl	Sodium chloride
OD	Optical density
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PKA	Protein kinase A
PTC	Peritubular cells
PTPRC	Protein tyrosine phosphatase, receptor type, C
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RE	Relative expression
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
s	Seconds
SC	Sertoli cells
s.c.	Subcutaneous
SCO	Sertoli cell only syndrome
SMAD	Fusion of *Caenorhabditis elegans* SMA protein and the *Drosophila* Mad protein (Mothers against decapentaplegic)
SOX9	SRY-box 9
SRY	Sex determining region Y
TBST	Tris buffered saline-Tween
TGF	Transforming growth factor

TH	Testicular homogenate
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TRAF6	Tumour necrosis factor receptor associated factor 6
Treg cells	Regulatory T cells
UUO	Unilateral ureteral obstruction
UV	Ultraviolet

8. LIST OF FIGURES AND TABLES

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10. APPENDIX

10.1. Materials

10.1.1. Chemicals and reagents

2-Propanol	Sigma-Aldrich, Steinheim, Germany
Acetic acid (glacial) 100%	Merck, Darmstadt, Germany
Acid fuchsine	Merck, Darmstadt, Germany
Agarose	Invitrogen, Carlsbad, USA
Alcoholic Eosin Y solution	Sigma-Aldrich, Saint Louis, USA
Ammonia solution 32%	Honeywell Riedel de Haën, Seelze, Germany
Ammonium persulfate	Carl Roth, Karlsruhe, Germany
Aniline blue	Merck, Darmstadt, Germany
Aniline oil	VWR, Fontenay-sous-Bois, France
Azocarmine G	Chroma-Gesellschaft, Stuttgart, Germany
Benzoic acid methyl ester, pure (>99%)	Carl Roth, Karlsruhe, Germany
β-mercaptoethanol	Sigma-Aldrich, Saint Louis, USA
Biebrich scarlette	Merck, Darmstadt, Germany
Bordetella pertussis Toxin (#516562)	Merck, Darmstadt, Germany
Bouin's Fixative Solution (#BOUN-1L)	Amber Scientific, Midvale, WA, Australia
Bovine serum albumin (BSA) (A3294)	Sigma-Aldrich, Saint Louis, USA
Celestin blue	Merck, Poole, UK
Citric acid (244)	Merck, Darmstadt, Germany
DPX Mountant	Sigma-Aldrich, Steinheim, Germany
Entallan new mounting medium (#107961)	Merck, Darmstadt, Germany
Ethanol	Sigma-Aldrich, Steinheim, Germany
Ethidium bromide	Carl Roth, Karlsruhe, Germany
Ethylene diaminetetraacetic acid disodium salt (EDTA)	Merck, Darmstadt, Germany

Ferric ammonium sulfate	Ajax Finechem, Seven Hills, NSW, Australia
Ferric chloride	Ajax Finechem, Seven Hills, NSW, Australia
Formalin	Trajan, Ringwood, Vic, Australia
Freund's adjuvant, complete (#F5881)	Sigma-Aldrich, Saint Louis, USA
Freund's adjuvant, incomplete (#F5506)	Sigma-Aldrich, Saint Louis, USA
Glycerol	Ajax Finechem, Seven Hills, NSW, Australia
Glycerol	Carl Roth, Karlsruhe, Germany
Harris's Haematoxylin Solutions (#HHS16)	Sigma-Aldrich, St Louis, USA
Histosol	Trajan, Ringwood, Vic, Australia
Hydrochloric acid, concentrated	Merck, Munich, Germany
Hydrochloric acid 37%	Carl Roth, Karlsruhe, Germany
Hydrogen peroxide 30% (H_2O_2) (#8070.1)	Carl Roth, Karlsruhe, Germany
Liquid DAB+ Substrate Chromogen System (#K3468)	Dako, Carpinteria, CA, USA
Methanol	Sigma-Aldrich, Steinheim, Germany
Non-fat milk, powdered	Carl Roth, Karlsruhe, Germany
Orange G	Merck, Darmstadt, Germany
Paraffin wax	Sigma-Aldrich, Steinheim, Germany
Paraformaldehyde	Merck, Darmstadt, Germany
Phosphatase inhibitor cocktail (100X)	Cell Signaling Technology, Danvers, MA, USA
Phosphotungstic acid	VWR, Darmstadt, Germany
Phosphotungstic acid, aqueous	Ajax Finechem, Seven Hills, NSW, Australia
Picric acid	Merck, Darmstadt, Germany
ProLong Gold Mountant with DAPI	Invitrogen, Karlsruhe, Germany
Protease inhibitor cocktail (100X)	Cell Signaling Technology, Danvers, MA, USA
Scott's Tap Water Substitute Concentrate	Sigma-Aldrich, Saint Louis, USA
Sodium Dodecylsulfate (SDS pellets)	Carl Roth, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth, Karlsruhe, Germany
Sodium deoxycholate	Sigma-Aldrich, Steinheim, Germany

Tramadol (Zydol)	Grünenthal GmbH, Aachen, Germany
Tris (4855.2)	Carl Roth, Karlsruhe, Germany
Tris-hydrochloride (9090.3)	Carl Roth, Karlsruhe, Germany
Tris-hydrochloride – OmniPur	Merck, Darmstadt, Germany
Triton X-100	Sigma-Aldrich, Steinheim, Germany
Tween 20	Sigma-Aldrich, Steinheim, Germany
Vectashield mounting medium	Vector Laboratories, Peterborough, UK
Xylene	VWR, Darmstadt, Germany

10.1.2. Cell culture reagents

2-Mercaptoethanol (#31350-010)	Gibco, Grand Island, NY, USA
Bovine serum albumin (#7030)	Sigma-Aldrich, Steinheim, Germany
DMEM+GlutaMAX (# 61965-026)	Gibco, Grand Island, NY, USA
DMEM/F12+GlutaMAX (#10565-042)	Gibco, Grand Island, NY, USA
Dulbecco´s PBS (1X) w/o $CaCl_2$ & $MgCl_2$ (#14190-144)	Gibco, Grand Island, NY, USA
Fetal Bovine Serum (FBS) (#16000-044)	Gibco, Grand Island, NY, USA
Lipopolysaccharides from *E. coli* O127:B8 (#L3880)	Sigma-Aldrich, Steinheim, Germany
MEM Non-Essential Amino Acids (#M745)	Sigma-Aldrich, Steinheim, Germany
Penicillin/Streptomycin (#15140-122)	Gibco, Grand Island, NY, USA
Recombinant human/mouse/rat activin A (338-AC)	R&D systems, Minneapolis, USA
Recombinant mouse JE/CCL2/MCP-1 (D-64030)	PromoCell, Heidelberg, Germany
Recombinant mouse TNFα (D-63720)	PromoCell, Heidelberg, Germany
FST288	Purified from HEK-293 cells transfected with human follistatin 288
Trypan blue solution 0.4%	Sigma-Aldrich, Steinheim, Germany
Trizma hydrochloride solution, pH 7.5, 1 M (T2319)	Sigma-Aldrich, Steinheim, Germany

10.1.3. Enzymes

Collagenase A (#1013578001)	Roche Diagnostic, Mannheim, Germany
DNase I (#10104159001) (cell isolation)	Roche Diagnostic, Mannheim, Germany
RNase- free DNase (RNA extraction)	Qiagen, Hilden, Germany
Hyaluronidase, from bovine testes (#H3506)	Sigma-Aldrich, Steinheim, Germany
Trypsin, from porcine pancreas (#T5266)	Sigma-Aldrich, Steinheim, Germany

10.1.4. Kits

VECTASTAIN Elite ABC HRP Kit	Vector Laboratories, Burlingame, CA, USA
MaxFluor Mouse on Mouse Fluorescence Detection Kit (MaxFluor 488)	MaxVision Biosciences, Washington, USA
MaxFluor Mouse on Mouse Fluorescence Detection Kit (MaxFluor 550)	MaxVision Biosciences, Washington, USA
Mouse on Mouse (M.O.M.) Basic Kit	Vector Laboratories, Burlingame, CA, USA
Pierce BCA Protein Assay (#23227)	Thermo Scientific, Rockford, IL, USA
RNase-Free DNase Set (#79254)	Qiagen, Hilden, Germany
RNeasy FFPE kit	Qiagen, Hilden, Germany
RNeasy Mini kit	Qiagen, Hilden, Germany
Total collagen assay kit	Quickzyme, Leiden, Netherlands
Total protein assay kit	Quickzyme, Leiden, Netherlands

10.1.5. List of consumables, equipment and software

1.7 mL MaxyClear Microtubes	Axygen Inc., Union City, CA, USA
8 chamber polystyrene culture slides	Falcon, Corning Inc., Corning, NY, USA
100µm CELLSTAR EASYstrainer	Greiner Bio-One, Frickenhausen, Germany
Adobe Photoshop CS6	Adobe Systems, San Jose, CA, USA
AxioCam MRm Digital Camera	Carl Zeiss, Göttingen, Germany
AxioVision Rel 6.8	Carl Zeiss, Göttingen, Germany

Biofuge Fresco	Heraeus, Hanau, Germany
Borosilicate tubes (CT1075)	Pacific Laboratory Products, Blackburn, VIC, Australia
CO_2-incubator for cell culture	Binder, Tuttlingen, Germany
Cell scraper	Sarstedt, Nümbrecht, Germany
CFX96 touch Real-time PCR detection system	Biorad, Munich, Germany
CFX Manager Software 3.1	Biorad, Munich, Germany
Clean bench	BDK, Sonnenbühl-Genkingen, Germany
Complete Freud's Adjuvant (#F5881)	Sigma-Aldrich, Saint Louis, MO, USA
Corning 96 Well Assay Microplate (3361)	Sigma-Aldrich, Saint Louis, MO, USA
Cryostat CM30509	Leica, Wetzlar, Germany
Electronic balance SPB 53	Scaltec instruments, Göttingen, Germany
Fast Real-Time PCR 7900HT	Applied Biosystems, Foster City, CA, USA
Filter tips	Nerbe plus, Winsen/Luhe, Germany
FusionCapt Advance Solo 4 16.07	Vilber Lourmat, Eberhardzell, Germany
Fusion FX Western Blot & Chemi imaging	Vilber Lourmat, Eberhardzell, Germany
GelDoc System Universal Hood II	Bio-Rad, Hercules, CA, USA
Gel Jet Imager 2000 documentation system	Intas, Göttingen, Germany
Genesis Lite EIA software	Labsystems Helsinki, Finland
GraphPad Prism 6	GraphPad Software, San Diego, CA, USA
Haake shaking water bath SWB25	ThermoFisher Scientific, Waltham, MA, USA
Halt protease inhibitor cocktail	ThermoFisher Scientific, Waltham, MA, USA
Hard-shell 96-well PCR plates	Biorad, Munich, Germany
Hot plate magnetic stirrer Rct basic	IKA-Werke, Staufen im Breisgau, Germany
Incomplete Freud's Adjuvant (#F5506)	Sigma-Aldrich, Saint Louis, MO, USA
Inverted microscope CKX41	Olympus, Hamburg, Germany
Labofuge 400 R centrifuge	Heraeus, Hanau, Germany

Magnetic stirring hotplate MR 3001 K	Heidolph instruments, Schwabach, Germany
Microplate reader TriStar LB 941	Berthold technologies, Bad Wildbad, Germany
Microplate reader Multiskan RC	Labsystems, Helsinki, Finland
Microplate shaker MPS1	Raket Instruments, Boronia VIC, Australia
Microscope Axioplan 2 Imaging (IF)	Carl Zeiss, Göttingen, Germany
Microseal B adhesive seals (PCR plates)	Biorad, Munich, Germany
Microtome RM2125 RTS	Leica, Wetzlar, Germany
Microtome RM2255	Leica, Wetzlar, Germany
Microwave oven	Samsung, Schwalbach, Germany
Mini centrifuge MC 6	Sarstedt, Nümbrecht, Germany
Mini centrifuge PerfectSpin Mini	Peqlab, Erlangen, Germany
Consort Mini Electrophoresis Power Supply E143	Sigma-Aldrich, Steinheim, Germany
Mini ExM DNA gel system	Peqlab, Erlangen, Germany
NanoDrop ND 2000 Spectrophotometer	Peqlab, Erlangen, Germany
Needle G18, G26, G30	BD, Franklin Lakes, NJ, USA
Neubauer Counting Chamber	Boeco, Hamburg, Germany
OLYMPUS cellSens Dimension 1.18 software	Olympus, Notting Hill, VIC, Australia
PCR thermocycler	Peqlab, Erlangen, Germany
pH-meter 766	Knick, Berlin, Germany
Pico 21 microcentrifuge	Heraeus, Hanau, Germany
Pipette, graduated and sterile	Greiner Bio-One, Frickenhausen, Germany
Potter S homogeniser	B. Braun, Melsungen, Germany
Power supply Power Ease 500	Invitrogen, Karlsruhe, Germany
Power supply peqPOWER	Peqlab, Erlangen, Germany
Precellys Evolution homogeniser	Bertin Technologies, Montigny-le-Bretonneux, France
Precellys steel beads, 2.8mm	Bertin instruments, Montigny-le-Bretonneux, France

PTC-200 thermal cycler	Biozyme, Oldendor, Germany
Quantity One 1-D Analysis Software (4.6.3)	Bio-Rad, Hercules, CA, USA
Safe-Lock Tubes 2.0 ml	Eppendorf AG, Hamburg, Germany
Safety work bench BDK for cell culture	ThermoFisher Scientific, Waltham, USA
Semi dry blotting chamber Sedec	Peqlab, Erlangen, Germany
Shaker 3005 orbital	GFL, Burgwedel, Germany
Stainless steel beads, 5 mm	Qiagen, Hilden, Germany
SuperFrost Plus microscope slides	R.Langenbrinck, Emmendingen, Germany
Syringe, 1 ml Luer-Lok Tip	BD, Franklin Lakes, NJ, USA
Test tube shaker uniTEXER 1	LLG Labware, Meckenheim, Germany
Thermo Electron Corp Wellwash 4 Mk4 automated Plate Washer	ThermoFisher Scientific, San Diego, CA, USA
Thermo-shaker PCMT	Grant instruments, Shepreth, UK
Tips	Sarstedt, Nümbrecht, Germany
Tissue culture flasks (T75)	Sarstedt, Nümbrecht, Germany
Tissue culture plates	Sarstedt, Nümbrecht, Germany
Screw cap tubes, graduated and sterile	Greiner Bio-One, Frickenhausen, Germany
XCell SureLock mini-cell electrophoresis system	Invitrogen, Karlsruhe, Germany

10.1.6. SDS PAGE and Western blot reagents

Amersham Protran 0.2 µm NC membrane	GE Healthcare, Darmstadt, Germany
BlueEasy Prestained Protein Marker	Nippon Genetics Europe, Düren, Germany
NewBlot PVDF Stripping Buffer, 5X	LI-COR Biosciences, Lincoln, Nebraska, USA
SuperSignal West Pico Chemiluminescent Substrate	Thermo Fisher Scientific, Waltham, USA
Whatman 3MM Chr Cellulose Chromatography Paper	GE Healthcare, Darmstadt, Germany
Ponceau S (C.I. 27195)	Carl Roth, Karlsruhe, Germany
Rotiphorese gel 30	Carl Roth, Karlsruhe, Germany

10.1.7. Agarose gel electrophoresis and PCR reagents

DNA ladder 1 Kb plus (#10787-018)	Invitrogen, Carlsbad, USA
DNA Ladder H3 RTU (100 bp)	Nippon Genetics Europe, Düren, Germany
6 x Blue/orange DNA loading dye	Promega, Mannheim, Germany
Desoxyribonukleosidtriphosphate (dNTP) 10mM	Promega, Mannheim, Germany
Go Taq G2 Flexi DNA polymerase	Promega, Mannheim, Germany
iTaq Universal SYBR Green Supermix (#172-5124)	Biorad, Munich, Germany
Reverse Transcriptase (M-MLV RT)	Promega, Mannheim, Germany
Oligo-dT_{15} primer	Promega, Mannheim, Germany
Power SYBR green PCR master mix	Applied Biosystems, Warrington, UK
Recombinant RNasine Ribonuclease inhibitor	Promega, Mannheim, Germany
SuperScript III First-Strand Synthesis System	Invitrogen, Carlsbad, USA
SYBR Safe DNA gel stain	Invitrogen, Carlsbad, USA
Taq DNA Polymerase, recombinant	Invitrogen, Carlsbad, USA

10.1.8. ELISA reagents

BupH carbonate-bicarbonate buffer pack	Thermo Fisher Scientific, Waltham, USA
Casein Colloid, 5% (M2052)	Sanquin, Amsterdam, Netherlands
Phosphate buffered saline tablet (P4417)	Sigma-Aldrich, Saint Louis, MO, USA
Streptavidin Poly-HRP (1:4000) (M2051)	Sanquin, Amsterdam, Netherlands
TMB Single Solution (00-2023)	Thermo Fisher Scientific, Waltham, MA, USA

10.2. Buffers and solutions

10.2.1. Buffers for tissue lysis and agarose gel electrophoresis

Lysis buffer I	***1X TAE electrophoresis buffer (pH 8.0)***
0.0257 M NaOH	242 g/l Tris base

0.205 M EDTA

ddH_2O

18.61 g/l EDTA

1% (v/v) Glacial acetic acid

Neutralisation buffer II

6.8 g/l Tris-HCl

ddH_2O

10.2.2. Buffers and solutions for SDS PAGE and Western blot

RIPA buffer

150 mM NaCl

0,1% (v/v) Triton X-100

0,5% (w/v) Sodium deoxycholate

0,1% (w/v) SDS

50 mM Tris-HCl pH 8.0

2 mM EDTA

ddH_2O

→ add protease inhibitors: 1:100

→ add phosphatase inhibitor cocktail: 1:100

4X Laemmli sample buffer (pH 6.8)

4% SDS

20% (v/v) Glycerol

0.125 M Tris-HCl (pH 6.8)

0.004% (w/v) Bromophenol blue

ddH_2O

→ add 20% (v/v) β-mercaptoethanol just before use

10X Tris buffered saline (TBS) (pH 7.6)

24.2 g/l Tris base (Mol. weight: 121.14 g/l)

88 g/l NaCl

ddH_2O

0,1% (v/v) Tween-20

10X SDS running buffer (pH 8.3)

30.3 g/l Tris base

144.0 g/l glycine

10 g/l SDS

1X TBST washing buffer

10% of 10X TBST in ddH_2O

0,1% (v/v) Tween-20

1X SDS running buffer

10% of 10X SDS Running Buffer in ddH_2O

58.15 g/l Tris base

0.4% (v/v) SDS

30.3 g/l Tris base

10X Semi-dry Transfer Buffer

29.3 g/l Glycine

10X Wet transfer buffer

144.0 g/l Glycine

58.15 g/l Tris base

0.4% (v/v) SDS

30.3 g/l Tris base

1X Semi-dry transfer buffer

10% (v/v) 10X Semi-dry transfer buffer in ddH_2O

20% (v/v) Methanol

1X Wet transfer buffer

10% (v/v) 10X Wet transfer buffer in ddH_2O

20% (v/v) Methanol

Blocking solution

5% (w/v) Non-fat dry milk

1X TBST

Primary antibody dilution buffer

5% (w/v) BSA

1X TBST

Secondary antibody dilution buffer

5% (w/v) Non-fat dry milk

1X TBST

Ponceau staining solution

0.2% (w/v) Ponceau S

3% (v/v) Acetic acid

Stripping buffer (1 L)

2% (v/v) SDS

0.0625 M Tris (pH 6.8)

ddH_2O

→ add 700 µl β-mercaptoethanol to 100 ml stripping buffer right before use

Separating gel:

	7.5%*	***10%****	***12.5%****
ddH_2O	4.85 ml	4.01 ml	3.17 ml
1.5 mM Tris-HCl pH 8.8	2.5 ml	2.5 ml	2.5 ml
10% SDS	100 µl	100 µl	100 µl
Acrylamide	2.5 ml	3.34 ml	4.17 ml
10% (w/v) APS	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl
Total	10 ml	10 ml	10 ml

* Percentage of gel was used according to molecular weight of proteins (based on 37.5:1 ratio of acrylamide/bis-acrylamide) > 7.5% gel: 120 - 250 kDa; 10% gel: 40 - 120 kDa; 13% gel: 15 - 40 kDa

Stacking gel:

	4%
ddH_2O	3.00 ml
1.5 mM Tris-HCl pH 6.8	1.25 ml
10% SDS	50 µl
Acrylamide	0.65 ml
10% (w/v) APS	25 µl
TEMED	5 µl
Total	5 ml

10.2.3. Buffers and solutions for tissue fixation and staining

Bouin's fixative

25% (v/v) Formalin

75% (v/v) Saturated aqueous picric acid

5% (v/v) Glacial acetic acid

Acidic alcohol solution for H&E staining

0.5% (v/v) Hydrochloric acid

in 70% (v/v) Ethanol

Citrate buffer for antigen retrieval

1.92 g/l Citric acid (10mM)

in ddH_2O

pH 6.0

Blocking buffer for immunofluorescence

10% (v/v) Normal goat serum in TBST

10.2.3.1. Buffers and solutions for Masson's trichrome staining

Acidic alcohol solution for H&E staining

0.5% (v/v) Hydrochloric acid

in 70% (v/v) Ethanol

Celestin blue solution

5% (w/v) Ferric ammonium sulfate

0.5% (w/v) Celestin blue

14% (v/v) Glycerine

in ddH_2O

Weigert's iron haematoxylin solution

1% (v/v) Haematoxylin in ethanol

1.15% (w/v) Ferric chloride

Biebrich scarlette-acid fuchsine solution

0.9% (w/v) Biebrich scarlet

0.1% (w/v) Acid fuchsine

1% (v/v) Concentrated hydrochloric acid
in ddH_2O

1% (v/v) Glacial acetic acid
in ddH_2O

Aniline blue staining solution

2.5% (w/v) Aniline blue
2.5% (v/v) Glacial acetic acid
in ddH_2O

10.2.3.2. Buffers and solutions for azan staining

Aniline alcohol

0.1% (v/v) Aniline oil
in 90% (v/v) Ethanol

Azocarmine G solution

0.1% (w/v) Azo-carmine G in ddH_2O solution (filtered)
1% (v/v) Glacial acetic acid

Aniline blue-orange solution

0.5% (w/v) Aniline blue
2% (w/v) Orange G
8% (v/v) Glacial citric acid

10.2.4. Buffers and solutions for cell culture

1 mg/ml DNAse

1 mg/ml DNAse in D-PBS (+)

1 mg/ml collagenase

1 mg/ml collagenase in DMEM/F12
6 µg/ml DNAse

1 mg/ml trypsin

1 mg/ml Trypsin in DMEM/F12
20 µg/ml DNAse

1 mg/ml hyaluronidase

1 mg/ml hyaluronidase in DMEM/F12
6 µg/ml DNase

DMEM/F12+GlutaMAX

1% (v/v) Non-essential amino acids [100x]
1% (v/v) Penicillin / streptomycin (100 U/ml)
Store at 4ºC

DMEM+GlutaMAX

1% (v/v) Non-essential amino acids [100x]
1% (v/v) Penicillin / streptomycin (100 U/ml)
Store at 4ºC

Sertoli cell culture medium

0.1% (w/v) BSA in DMEM+GlutaMax

Filter sterile

Peritubular cell and NIH 3T3 fibroblast culture medium

10% (v/v) FCS in DMEM+GlutaMax

Peritubular and NIH 3T3 fibroblast starvation medium

1% (v/v) FCS in DMEM+GlutaMax

Hypotonic shock solution

20 mM Trizma hydrochloride

in ddH_2O

10.2.5. Buffer for EAO immunisation

Munõz buffer (pH 7.6)

25 mM Tris

0.5 M NaCl

0.017% (v/v) Triton X-100

in ddH2O

10.2.6. Buffers and solutions for ELISA

Carbonate buffer (pH 9.4)

0.2 M sodium carbonate-bicarbonate

ddH2O

Assay buffer (pH 7.4)

5% (w/v) BSA

0.01 M PBS

Blocking buffer (pH 7.4)

1% (w/v) BSA

50 mM Tris-HCl

ddH2O

20% BSA/Tris/Triton buffer (pH 7.5)

20% (w/v) BSA

0.1 M Tris base

0.9% (w/v) NaCl

5% (v/v) Triton X-100

ddH2O

Wash buffer

0.01 M PBS

0.05% (v/v) Tween 20

ddH2O

5% BSA/Tris/Triton buffer

20% BSA

0.1 M Tris

0.9% NaCl

	5% Triton X-100
	ddH2O
6% SDS solution	***1% Casein colloid/PBS***
6% SDS	1% Casein colloid
0.05 M PBS	0.05 M PBS

10.3. Animal sera for immunofluorescent staining

Goat serum	Biolegend, Dedham, MA, USA
Rabbit serum	Agilent Technologies, Santa Clara, CA, USA

10.4. Antibodies

Antibodies	**Dilution**	**Source**
Primary antibodies		
Mouse monoclonal anti-mouse β-actin (A5441)	1:5000 WB	Sigma-Aldrich, Saint Louis, MO, USA
Mouse monoclonal anti-mouse activin βA, E4	1:100 IF; 1:300 IHC; 650 ng/ml ELISA	Oxford Brooks University, Oxford, UK
Mouse monoclonal anti-mouse activin βA, E4, biotinylated	1:600 ELISA	Hudson Institute of Medical Research, Clayton, VIC, Australia
Rabbit monoclonal anti-mouse α smooth muscle actin	1:500 WB	Abcam, Cambridge, UK
Mouse monoclonal anti-mouse α smooth muscle actin FITC-conjugated IgG (F3777)	1:800 IF	Sigma-Aldrich, Saint Louis, USA
Rabbit monoclonal anti-mouse phospho-Smad2 (Ser465/467)(138D4)	1:1000 WB	Cell Signaling Technology, Danvers, MA, USA
Rabbit monoclonal anti-mouse phospho-Smad3 (Ser423/425)(C25A9)	1:1000 WB	Cell Signaling Technology, Danvers, MA, USA
Rabbit monoclonal anti-mouse Smad2 (D43B4) XP	1:1000 WB	Cell Signaling Technology, Danvers, MA, USA
Rabbit monoclonal anti-mouse Smad3 (C67H9)	1:1000 WB	Cell Signaling Technology, Danvers, MA, USA
Rabbit polyclonal anti-mouse collagen I	1:250 WB; 1:200 IF	Abcam, Cambridge, UK
Rabbit polyclonal anti-mouse collagen III	1:500 WB	Abcam, Cambridge, UK
Rabbit polyclonal anti-mouse fibronectin	1:500 WB; 1:300 IF	Abcam, Cambridge, UK
Rabbit polyclonal anti-mouse SOX9	1:200 IF	Abcam, Cambridge, UK
Rat monoclonal anti-mouse CD45 (30-F11)	1:200 IF	Thermo Fisher Scientific, Waltham, MA, USA
Rat monoclonal anti-mouse F4/80 (MCA497G)	1:200 IF	AbD Serotec, Kidlington, UK
Secondary antibodies		
AlexaFluor 488 goat anti-rabbit IgG (H+L)	1:1000 IF	Life Technologies Carlsbad, CA, USA
AlexaFluor 488 rabbit anti-rat IgG (H+L)	1:1000 IF	Life Technologies Carlsbad, CA, USA
AlexaFluor 594 goat anti-rabbit IgG (H+L)	1:1000 IF	Life Technologies Carlsbad, CA, USA
AlexaFluor 546 goat anti-rat IgG (H+L)	1:1000 IF	Life Technologies Carlsbad, CA, USA
HRP-conjugated goat anti-rabbit IgG (H+L) (#55689)	1:5000 WB	Cappel Laboratories, West Chester, PA, USA
HRP-conjugated goat anti-mouse IgG (H + L)	1:5000 WB	Thermo Fisher Scientific, Scoresby, VIC, Australia
HRP-linked goat anti-rabbit IgG (H + L)	1:1000 WB	Cell Signalling Technology, Danvers, MA, USA

10.5. Primers

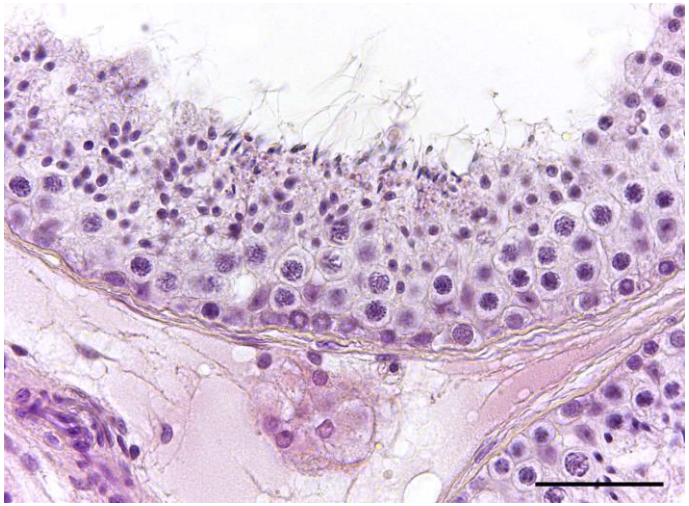
10.5.1. Primer sequences used in qRT-PCR

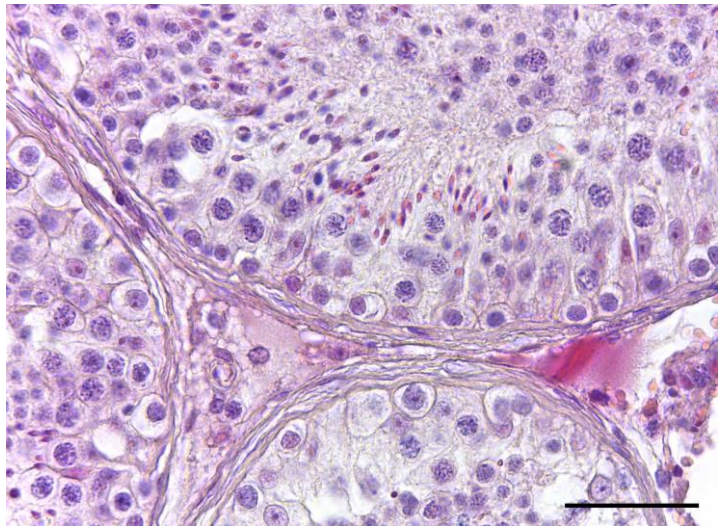
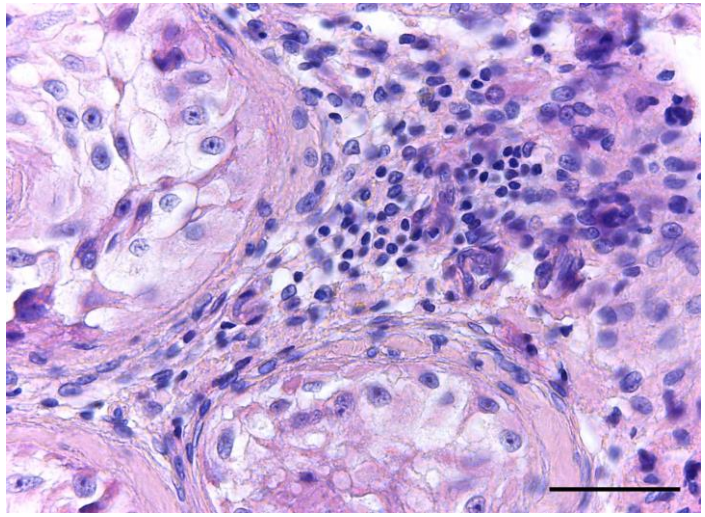
Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Entrez Gene ID	Amplicon size (bp)	Qiagen catalogue number
Mouse					
18S rRNA	TACCACATCCAAGGAAGGCAGCA	TGGAATTACCGCGGCTGCTGGCA	19791	180	-
Acta2 (αSMA)	CCTCCAGTTCCTTTCCAAAT	GCCAGGGCTACAAGTTAAG	11475	106	-
Actb (β-actin)	*TGACAGGATGCAGAAGGAGAT*	TACTCCTGCTTGCTGATCCAC	11461	156	-
B2m	CCGCCTCACATTGAA	TCGATCCCAGTAGACG	12010	198	-
Ccl2	QuantiTect Primer assay	Qiagen	20296	118	QT00167832
Col1a2	TTCACCTACTCTGTCCTAGTC	CAGGCGAGATGGCTTATGGCTTATTT	12843	88	-
Col3a1	CCA GCT GGG CCT TTG ATA CCT	TGC CCA CAG CCT TCT ACA CCT	12825	497	-
Col4 α1	TCAGGTCCACCTGGAATTA	GAAGTCCTTGAGAGCCTTTATC	12826	97	-
Fn1	AGAAGGCAGTAGCACAGA	TCTCCTCCACAGCATAGATAG	14268	110	-
Gapdh	TGACGTGCCGCCTGGAGAAA	AGTGTAGCCCAAGATGCCCTTCAG	14433	98	-
Hprt	*CTGGTAAAAGGACCTC*	CTGAAGTACTCATTATAGTCAAG	15452	110	-
Inhba	AGAACGGGTATGTGGAGATAGA	GACTCGGCAAAGGTGATGAT	16323	97	-
Il1a	CGAAGACTACAGTTCTGCCATT	GACGTTTCAGAGGTTCTCAGAG	16175	126	-
Il6	QuantiTect Primer assay	Qiagen	16193	128	QT00098875
Ptprc (Cd45)	ATGGTCCTCTGAATAAAGCCCA	TCAGCACTATTGGTAGGCTCC	19264	70	-
Tnf	QuantiTect Primer assay	Qiagen	21926	112	QT00104006
Human					
PTPRC (CD45)	*GACCCAGTTTCCCCATTGAC*	CTGAGGTGTTCGCTGTGATG	5788	102	-
GAPDH	*CCAGGTGGTCTCCTCTGACTTC*	GTGGTCGTTGAGGGCAATG	2597	81	-
INHBA	*GGTATGTGGAGATAGAGGATGAC*	TCCTGGCTGTTCCTGACTC	3624	105	-

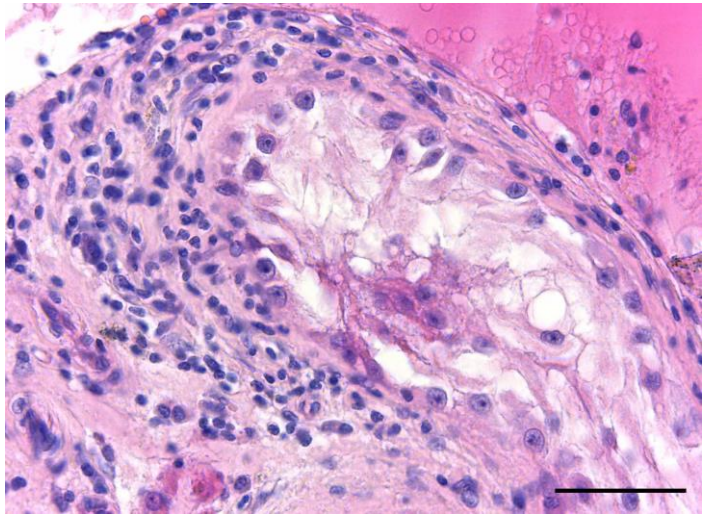
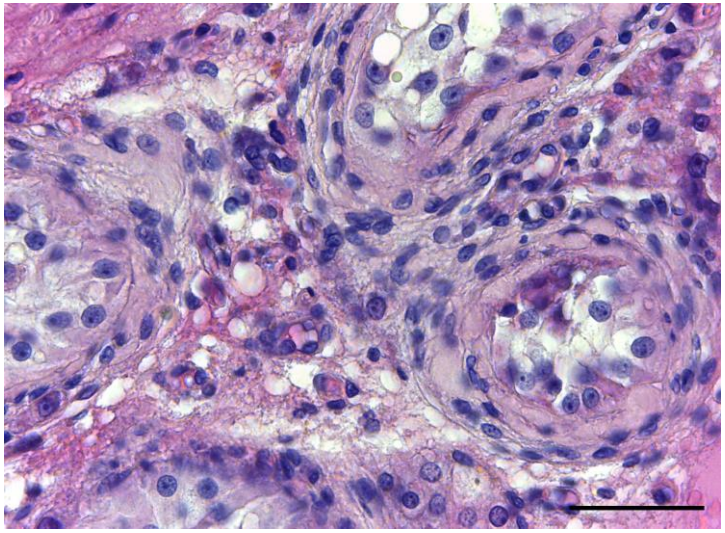
10.5.2. Primer sequences used in genotyping PCR

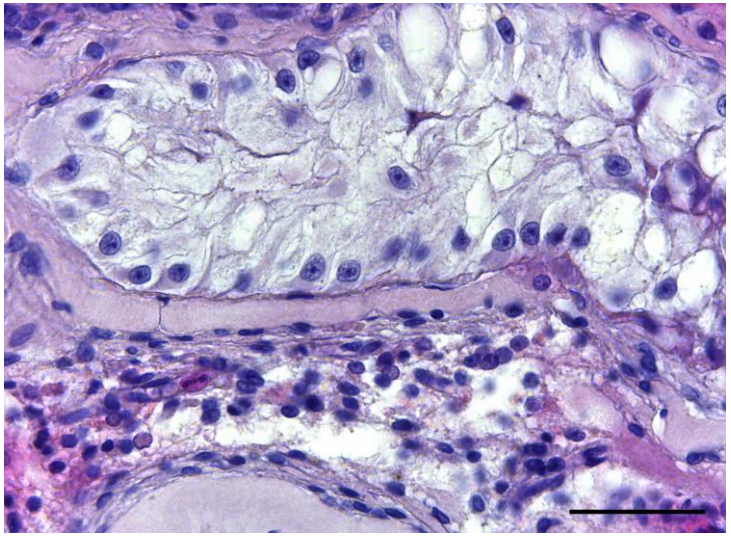
Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)
Inhba (WT)	AAGAGAGAATGGTGTACCTTCATT	TATAACCTGGGTAAGTGGGT	280
Inhba (Floxed)	AAGAGAGAATGGTGTACCTTCATT	AGACGTGCTACTTCCATTTG	400
Amh-Cre	*CCTGGAAAATGCTTCTGTCCG*	CAGGGTGTTATAAGCAATCCC	400

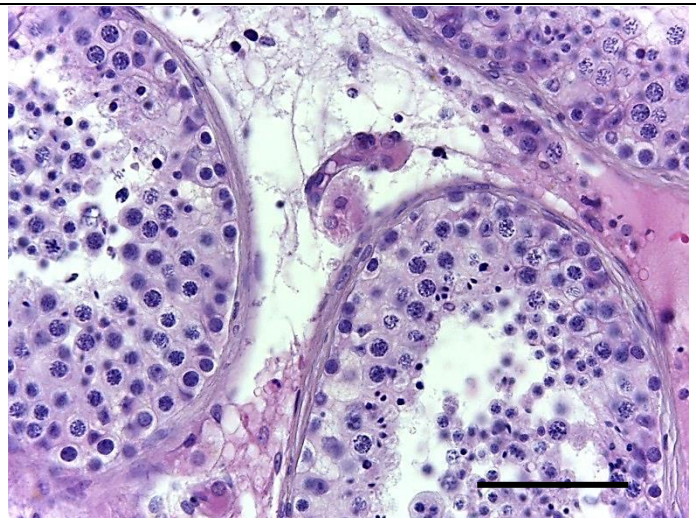
10.6. Table individual patient data

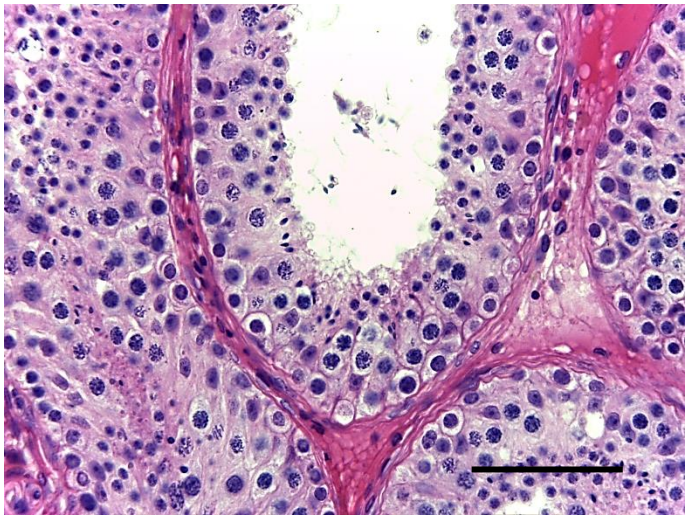
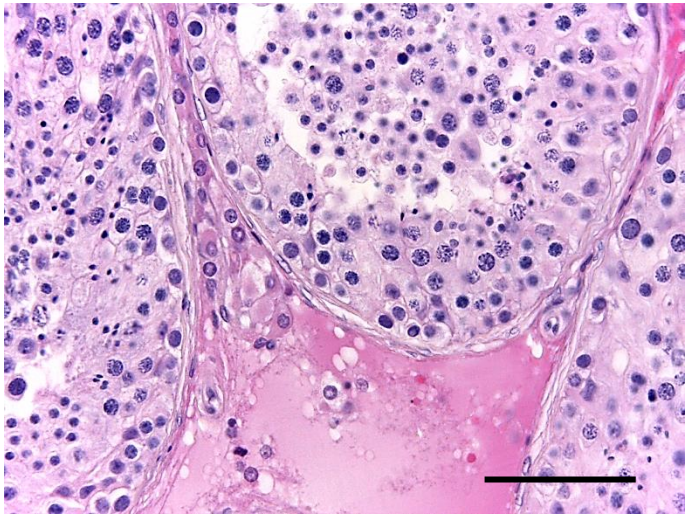
Testis category of paraffin embedded patient specimens	Histology (representative area)	Spermatogenesis score count*	Leukocytic infiltrates	Additional features (not depicted)	Relative *INHBA* mRNA levels	Relative *PTPRC* (CD45) mRNA levels	Total collagen (mg/mg total protein)
Intact Spermatogenesis		10	-	-	N/D	Not measured since no material available	0.0935

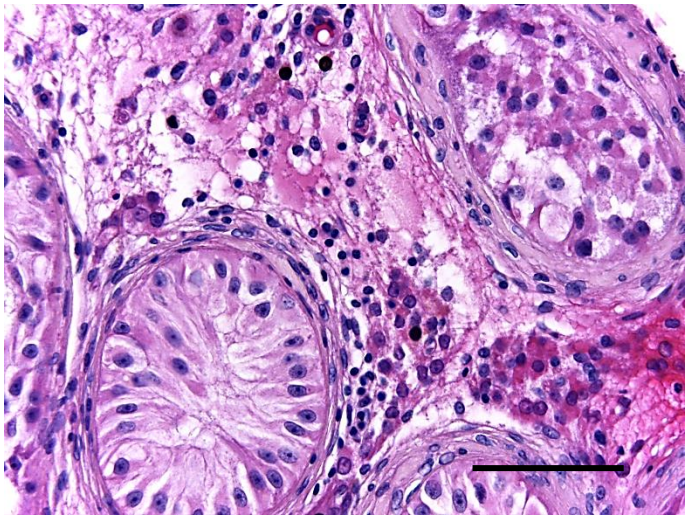
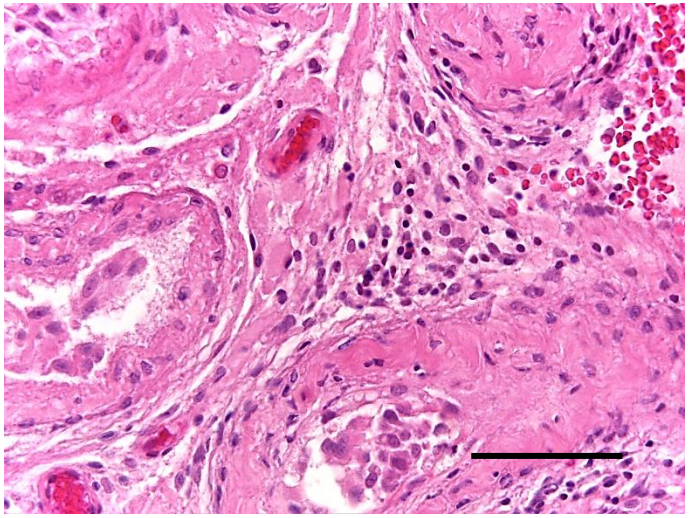
Intact Spermatogenesis		10	-	-	0.6319	N/D	0.1058
Impaired spermatogenesis (SCO)		0	+	-	2.5875	107.8930	0.1724

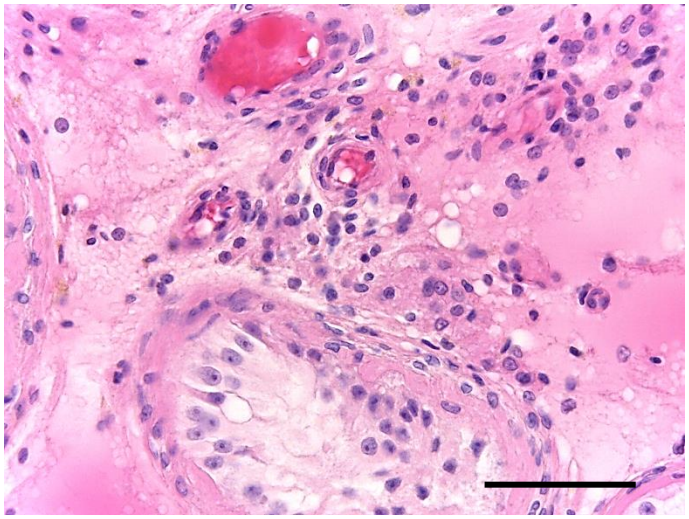
Impaired spermatogenesis (SCO)		0	+	-	6.1174	249.0285	0.2053
Impaired spermatogenesis (SCO)		0	+	Tubular atrophy ("shadows")	12.2022	380.5475	0.3265

Impaired spermatogenesis (SCO)		0	+	Tubular atrophy ("shadows") Intratubular spherical concrements (affected tubules with dense peritubular infiltrate)	15.8816	405.0274	0.2575

Testis category of snap frozen patient specimens	**Histology (representative area)**	**Spermatogenesis score count***	**Leukocytic infiltrates**	**Additional features (not depicted)**	**Relative *INHBA* mRNA levels**	**Relative *PTPRC* (CD45) mRNA levels**	**Total collagen (mg/mg total protein)**
Intact spermatogenesis		10	-	-	0.7143	0.4988	0.0602

Intact spermatogenesis		10	-	-	0.9072	1.9034	0.0369
Intact spermatogenesis		10	-	-	1.5503	1.0538	0.0514

Impaired spermatogenesis (SCO)		0	+	-	3.8298	3.0234	0.0832
Impaired spermatogenesis (SCO)		0	+	Tubular atrophy ("shadows")	2.9674	2.6668	0.1502

Impaired spermatogenesis (SCO)		0	+	Tubular atrophy ("shadows")	2.1407	3.2886	0.1296

*according to (Bergmann and Kliesch, 2010); see **Chapter 2.2.4.2.**

11. PUBLICATIONS

Publications originating from this dissertation to date

- **Kauerhof A.C.**, Nicolas N., Bhushan S., Wahle E., Loveland K. A., Fietz D., Bergmann M., Groome N. P., Kliesch S., Schuppe, H-C., Pilatz, A., Meinhardt A., Hedger M. P. and Fijak M. (2019) Investigation of activin A in inflammatory responses of the testis and its role in the development of testicular fibrosis. *Hum Reprod. Vol.34 (8), pp.1536-1550*.

Other publications

- Can, G., **Kauerhof, A.C.**, Macak, D. and Zegerman, P. (2018), "Helicase Subunit Cdc45 Targets the Checkpoint Kinase Rad53 to Both Replication Initiation and Elongation Complexes after Fork Stalling", *Molecular Cell*. doi.org/10.1016/j.molcel.2018.11.025

Conference abstracts

- **Monash Health Translation Precinct (MHTP) Research Symposium** (7 - 9 November 2018) Melbourne, Australia. Investigation of inflammatory responses in human and mouse testes implicates activin A as a potential inducer of testicular fibrosis. Poster presentation.
- **50. Annual Scientific Meeting of the Endocrine Society of Australia (ESA) and the Society of Reproductive Biology (SRB)** (19 - 22 August 2018) Adelaide, Australia. Investigation of intratesticular inflammatory responses in humans and mice implicates activin A as a potential inducer of fibrosis in the testis. Oral and poster presentation.
- **51st Annual Meeting of the Society for the Study of Reproduction** (10 - 13 July 2018) New Orleans, LA, USA. Potential involvement of activin A in development of fibrosis in chronic testicular inflammation by upregulating fibrotic genes in peritubular cells and fibroblasts. Poster presentation.
- **Victorian Infection and Immunity Network Conference** (14 - 16 February 2018) Lorne, Australia. Examination of the role of activin A and resident testicular cell types in fibrosis following experimental autoimmune orchitis (EAO) in mice. Poster presentation.
- **4th World Congress of Reproductive Biology (WCRB 2017)** (27 - 29 September 2017) Okinawa, Japan. Influence of activin A on the development of fibrosis and origin of collagen-producing cells in chronic testicular inflammation. Poster presentation.
- **10th Giessen Graduate Centre for the Life Sciences (GGL) Conference** (27 - 28 September 2017) Giessen, Germany. Investigating the role of activin A on the fibrotic response during chronic testicular inflammation. Poster presentation.
- **11th International Congress of Andrology - Lifestyle Factors and Male Reproductive Health** (6 - 9 May 2017) Copenhagen, Denmark. Investigating the role of activin A on the fibrotic response during chronic testicular inflammation. Poster presentation.
- **IRTG program renewal** (22 - 23 March 2017) Justus Liebig University, Giessen, Germany. Investigating the role of activin A on the fibrotic response during chronic testicular inflammation. Poster presentation.
- **9th Giessen Graduate Centre for the Life Sciences (GGL) Conference** (20 - 21 September 2016) Giessen, Germany. Investigating the role of activins on the fibrotic response and the generation of CD4+CD8+ T cells during chronic testicular inflammation. Poster presentation.

12. EHRENWÖRTLICHE ERKLÄRUNG

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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 from or are based on the content of published or unpublished work of others, and all information that relates to verbal communication. 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 29.03.2019

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Anastasia Christine Kauerhof

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