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Regulation of the spermatogonial niche factor GDNF
by retinoic acid

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THE THESIS EXPLAINED

Spermatogenesis in mammals is a high efficient process which results in a daily production of millions of mature spermatozoa (Johnson *et al.*, 1980) and relies on a stem cell pool of spermatogonial stem cells (SSCs).

Sertoli cell, the unique somatic cell within the seminiferous tubules of testis, constitutes a specialized microenvironment called “SSCs niche” and directly controls SSCs behaviour. Sertoli cells secrete the niche factor GDNF, which acts on stem cells and is known to be crucial for their survival and self-renewal, *in vitro* and *in vivo*. On the other side, the key to commitment for SSCs is represented by retinoic acid (RA), the active metabolite of vitamin A, which is released within the testicular microenvironment. GDNF and RA are both essential for spermatogenesis, in fact in the absence both of GDNF or RA, the spermatogenic process is blocked (Meng *et al.*, 2000; Wolbach & Howe, 1925).

In this work we demonstrated that RA negatively regulates GDNF in immature murine Sertoli cells, affecting both mRNA and secreted protein, confirming previous evidence in literature (Hasegawa *et al.*, 2013). RAR α is the nuclear receptor involved in this regulation acting through a novel-identified Retinoic Acid Responsive Element (RARE) on *Gdnf* murine promoter. Data obtained with HDACi suggested that the underlying molecular mechanism involved an epigenetic repression mediated by HDAC enzymes. These results were confirmed by ChIP-qPCR experiments, that indicated that *Gdnf* promoter undergoes a de-acetylation after RA treatment. We then focused our attention on adult mouse, where *gdnf* regulation is conserved. Finally, we investigated this regulation in human testis, and we found that in our culture conditions testis tissue positively responds to RA stimulation while RA effect on *gdnf* expression is heterogeneous.

INTRODUCTION

1. Overview on Spermatogenesis

In mammals, spermatogenesis is a complex, efficient and highly regulated process that starts from puberty and guarantees a continuous formation of spermatozoa, the male gametes responsible for the transmission of the genetic makeup to subsequent generations.

Spermatogenesis takes place in the testis. Testes are two oval-shaped organs covered by a fibrous multi-layered tunica, properly named *vasculosa*, *albuginea* and *vaginalis*. Within the testis, septa of connective tissue create lobules that hold highly convoluted seminiferous tubules. These last are tubular structures enclosed by a thick basal lamina and smooth muscle cells, that hold germ cells and somatic Sertoli cells.

The continuous production of mature spermatozoa depends on the activity of an adult, rare, unipotent stem cell: the spermatogonial stem cell (SSC), that in mice constitutes only 0.03% of total germ cells (Tegelenbosch & de Rooij, 1993).

The complex process of spermatogenesis can be divided into three phases: proliferative phase, meiotic phase and spermiogenesis, that will be described below.

In the first phase, cells in the spermatogonial compartment begin a series of mitotic divisions aimed at the production of new spermatogonia and at the replacement of SSCs that periodically get lost. In the mouse, the spermatogonial compartment has been traditionally divided into two categories: the undifferentiated (which contains the SSCs) and the differentiating spermatogonia, both located on the basal membrane of the seminiferous tubule. The first category includes the A-single (A_s); A-pair (A_{pr}) which are two daughter cells remained connected by an intracellular bridge due to an incomplete cytokinesis; A-aligned (A_{al}) spermatogonia which are chains of 4, 8, 16 or occasionally 32 clone cells connected by intercellular bridges.

Differentiating spermatogonia comprise A1, A2, A3, A4, Intermediate and B spermatogonia. All these cells are characterized

by the expression of KIT, SOHLH1 and SOHLH2 (Barrios *et al.*, 2012; Suzuki *et al.*, 2012). The last mitotic division of the B spermatogonia generates the preleptotene spermatocytes, the cells entering in meiosis.

Human spermatogonial subpopulation is less characterized with respect to the murine counterpart. It can be divided into three categories: A dark (A_d), A pale (A_p) and B spermatogonia. A_d and A_p can be distinguished on the basis of nuclear morphology and intensity of staining with hematoxylin (Clermont & Leblond, 1959; Clermont & Antar, 1973; Clermont, 1966). A_d and A_p spermatogonia are considered as the counterpart for murine undifferentiated spermatogonia, while type B represents the differentiating cells, that divide to produce primary spermatocytes, secondary spermatocytes and spermatids (Boitani *et al.*, 2016). It was commonly accepted that A_d constitute a pool of quiescent spermatogonia which can give rise to A_p when needed, and were thought to be the human 'true' SSCs (Amann, 2008; Boitani *et al.*, 2016). Recently, a new classification of the human spermatogonial subsets based on spermatogonial kinetics and marker expression have questioned the classical view of A_d and A_p (Di Persio *et al.*, 2017). Clearly, the identification of the human SSCs deserve further studies.

Spermatogonia differentiate into primary spermatocytes and enter the second phase of spermatogenesis, the meiotic phase. The first meiotic division is characterized by a long prophase, including leptotene, zygotene, pachytene and diplotene spermatocytes. Diplotene spermatocytes undergo two rapid subsequent divisions producing four haploid round spermatids from each germ cell, which undergo the spermiogenesis phase. In this last phase haploid spermatids get mature and differentiate into spermatozoa, cells characterized by elongated shape, compact nucleus and flagellum. At the end, spermatozoa can be released into the lumen of the seminiferous tubule in a controlled process named spermiation.

During their maturation, germ cells gradually move in an organized and coordinated movement from the basement membrane towards the lumen of the tubule, where spermatozoa will be released. In the mouse, the time required to form a sperm from a A1 spermatogonia, hence the total duration of spermatogenesis, is 35 days, while in

human is approximately 74 days (Heller & Clermont, 1963) (Heller & Clermont, 1964).

In mammals, within the seminiferous tubule it is possible to recognize different cellular associations called stages of the seminiferous epithelium, that succeed each other in time in any given area (Leblond & Clermont, 1952). “A complete series of the successive cell associations found along a seminiferous tubule” was described by Clermont as “the wave of the seminiferous epithelium”, while “a complete series of the successive cellular associations appearing in any one given area of the seminiferous tubule” is known as the cycle of the seminiferous epithelium (Leblond & Clermont, 1952).

In mouse, the time required for a stage to reappear in the same area is 8,6 days (Oakberg, 1956 b) while in human the duration of one cycle of the seminiferous epithelium is 16 days (Clermont, 1963). This highly complex tissue organization guarantees a continuous release of mature spermatozoa into the epididymis, that occurs in each area once every 8,6 days in mouse (Oakberg, 1956 a) every 16 days in humans (Clermont, 1963).

Spermatogenesis in rodents has been widely characterized and its study represents a model for the comprehension of the human spermatogenesis. In mouse, twelve different cell associations or stages have been described, indicated by Roman numerals I-XII (Oakberg, 1956 a). In human, for a long time six stages have been defined. More recently, in order to better parallel human and murine cycle of the seminiferous epithelium (Amann, 2008), twelve stages have been characterized also in human, based on spermatids acrosome development (Muciaccia *et al.*, 2013). Spermatids, cells that undergo the most prominent cytological changes among cellular elements of the tubule (Clermont, 1960), make stages easily recognizable through the developmental step of their acrosome (Clermont & Leblond, 1955).

However, while in the mouse the twelve stages are distributed in a linear sequence as small segments of the seminiferous tubule, in human, a tubule cross-section typically contains 2-4 stages. So it was proposed that the human cellular associations are organized as “intertwined helical bands” along the seminiferous tubules (Schulze & Rehder, 1984), and this explains why the isolation of staged

seminiferous tubules based on transillumination is possible for rodents seminiferous epithelium but not for the human (Parvinen *et al.*, 1972).

Spermatogenesis is controlled by local molecules acting in autocrine or paracrine fashion, and by circulating hormones of the hypothalamic–pituitary–gonadal axis, through an endocrine level. Hypothalamus releases the *gonadotropin releasing hormone* GnRH, that in turn stimulates adenohypophysis to secrete *follicle stimulating hormone* FSH and *luteinizing hormone* LH. FSH hormone binds to its receptor FSHR on Sertoli cells, regulating the gene expression to support spermatogenesis (Walker & Cheng, 2005). The FSH signaling also promotes the production of Inhibin B by Sertoli cells, which acts as a negative feedback on the production of FSH (de Kretser *et al.*, 2001).

LH on the contrary stimulates Leydig cells to produce testosterone which in turn acts as a negative feedback on the pituitary gland to block LH production (Ewing, 1993).

1.1 Sertoli cells and the niche for the SSCs

Like other adult self-renewing tissues, the seminiferous epithelium function relies on stem cells, more precisely spermatogonial stem cells (SSCs), which are subject to various extrinsic signals provided by the "stem niche". The stem cell niche is the microenvironment in which stem cells reside, and which provides stimuli able to influence the fate decisions: self-renewal or differentiation. Stimuli include cellular, cell-cell and cell-matrix interactions, and signal molecules able to affect the transcriptional program (Voog & Jones, 2010). The SSCs niche is the space comprised by the basal lamina of seminiferous tubules and the somatic Sertoli cells, which establish a close contact with germ cells. Also peritubular myoid cells, Leydig cells, macrophages and nerves (Caires *et al.*, 2010) may be contributors of the niche. Interestingly, some Authors reported that the majority of A_{undiff} were clustered in regions of the tubules close to a blood vessel, suggesting that blood vessels could

provide an adequate support to SSCs (Chiarini-Garcia & Russell, 2001; Yoshida *et al.*, 2007).

However, it is well known that Sertoli cells are the main protagonists of the spermatogonial niche (de Rooij, 2017) (Figure 1). Sertoli appears as a large, columnar, arborized “nurse” cell (Griswold, 1995) which interacts with a well-defined number of germ cells, that vary among species (Russell & Peterson, 1984).

The most evident morphologic feature in Sertoli cell, used for cellular recognition, is its euchromatic nucleus and the large nucleolus inside it. Interestingly, the nuclear shape changes during the seminiferous epithelium cycle.

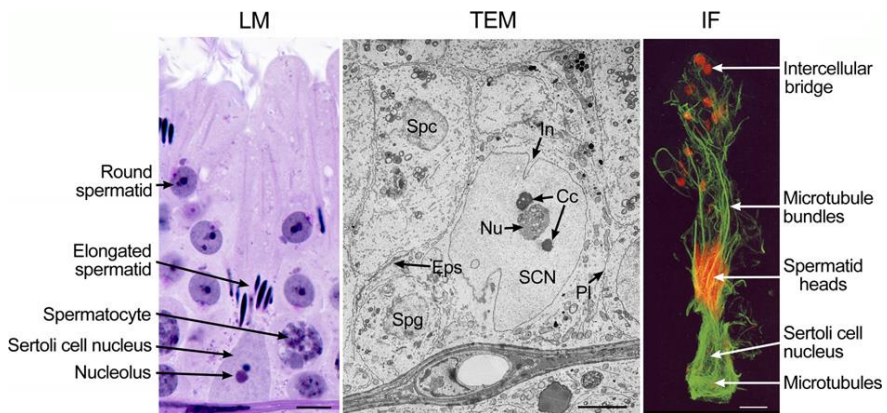


Figure 1 Sertoli cell observed using light microscopy (LM), transmission electron microscopy (TEM) and immunofluorescence microscopy (IF) (França *et al.*, 2016)

Most important markers for Sertoli cells are nuclear: SOX9 (SRY-box containing gene 9) (Frojdman *et al.*, 2000), androgen receptor (AR) (Sar *et al.*, 1990), GATA-binding protein 1 (GATA1) (Viger *et al.*, 1998); Wilms tumor protein-1 (WT1) (Sharpe *et al.*, 2003; Wang *et al.*, 2013); cyclin-dependent kinase inhibitor 1B (p27kip1) (Sharpe *et al.*, 2003).

Sertoli cells are the primary regulators of the sex determination: in the developing urogenital ridge, they establish the male sex of the gonad by expressing the Sex-determining Region Y (Sry) gene (Lovell-Badge, 1993) and by inhibiting the development of female

structures through the Anti-Müllerian Hormone (AMH) (Josso *et al.*, 1993). Sertoli cells actively proliferate during the fetal development and keep proliferating in the prepubertal period. In men two proliferation peaks occur during the first three months of life and then during puberty (Cortes *et al.*, 1987). Proliferation is regulated by many factors such as FSH and *Thyroid hormone* (TH), paracrine factors as TGF β , androgens and autocrine factors like IL-1 (Tarulli *et al.*, 2012). However, at puberty Sertoli cells stop proliferating, undergo terminal differentiation and the established Sertoli cell number determines the testicular size (França *et al.*, 2000).

Sertoli cells play an important structural role in the seminiferous tubule, dividing the basal compartment from the adluminal compartment of the seminiferous epithelium through the blood-testicular barrier (BTB), which is a structure formed by Sertoli cells tight junctions and composed by occludin, claudins and junctional adhesion molecules (Mruk & Cheng, 2010). During the cycle of the seminiferous epithelium, this structure, following retinoic acid pulse, faces dramatic restructuring (Hasegawa & Saga, 2012) allowing the transit of preleptotene spermatocytes from the basal to the adluminal compartment of the seminiferous epithelium.

In the basal compartment, Sertoli cells, with their cytoplasmic branches, embrace SSCs confining them in a highly isolated microenvironment enriched of factors secreted by Sertoli themselves, interstitial cells and molecules brought by the blood (Oatley & Brinster, 2012). The main factors controlling the balance between self-renewal and differentiation are: GDNF, FGF2, which increase the proliferation activity of SSCs and SCF, Activin A, CSF-1 and BMP4 that stimulate differentiation. Some of these factors are under hormonal control (Boitani *et al.*, 2016).

Another important function of Sertoli cell is its phagocytic activity, aimed at the clearance of residual body of leftover spermatid cytoplasm and apoptotic cells. The phagocytic activity relies on class B scavenger receptor type I (SR-BI) of Sertoli cells and phosphatidylserine exposed at the membranes of target cells (Nakagawa *et al.*, 2005).

Sertoli cells are also key regulators of androgen production, in fact they control Leydig cell population in the neonatal (Rebourcet *et*

al., 2014) and adult testis (Rebourcet *et al.*, 2014b). Since the Sertoli cells and the Leydig in the interstitial tissue do not establish a direct contact, it is possible that the action of the Sertoli on them is mediated by secreted factors, or indirectly through peritubular myoid cells (Rebourcet *et al.*, 2014b). Interestingly, in humans, Sertoli cell number, as well as Leydig cell number and function, decreases with aging (Johnson *et al.*, 1984). In this context, the reduction of Sertoli cell number in men could be at the base of testosterone decrease associated with aging (Rebourcet *et al.*, 2014 b).

Sertoli cells make testis an immune privileged organ. In fact, their immuno-regulatory properties create a microenvironment that suppresses immune reactions against spermatocytes and spermatids, that normally express proteins not recognized by the immunological self-tolerance (O’Rand & Romrell, 1977; Tung & Fritz, 1978). This action is exerted physically by the BTB that impinges the transit of leukocytes and molecules in the seminiferous epithelium and by the secretion of a variety of molecules such as apoptosis inhibitors, complement inhibitors and immunoregulatory factors like TGF β 1, CCL27,IDO and galectin-1 (Guazzone *et al.*, 2009; Meinhardt & Hedger, 2011; Doyle *et al.*, 2012).

2. Glial Cell Line-Derived Neurotrophic Factor (Gdnf)

The *glial cell line-derived neurotrophic factor* (Gdnf) was originally isolated in rat embryo midbrain cultures and identified as a crucial factor for midbrain dopaminergic neurons survival (Schubert, 1974). GDNF, is one of the four neurotrophic factors of the GDNF family (GFLs), together with *neurturin* (NRTN), *persephin* (PSPN), *artemin* (ARTN) (Takahashi, 2001). GFLs constitute a subgroup of the TGF- β superfamily, with whom they share conformational features, like the “cysteine knot” structure (Lin *et al.*, 1993).

The murine Gdnf gene is located on chromosome 15 and consists of about 27 kb. It consists of three exons interspersed with two introns of 4.1Kb and approximately 18.5Kb. In the mouse, the presence of

alternative intronic promoters that generate transcripts with different 5'-UTR ends was then described (Tanaka *et al.*, 2000a; Tanaka *et al.*, 2000b; Tanaka *et al.*, 2001). The organization of the human gene encoding GDNF is similar to the murine, and also in this case the transcripts undergo alternative splicing to the 5'UTR region, thus generating multiple isoforms in the 5'UTR region (Airavaara *et al.*, 2011).

GDNF is synthesized as 211 amino acid precursor protein, converted into the 134 amino acid active form and then secreted (Lin *et al.*, 1993). Differently from TGF- β family members, GFLs, bind to the glycosylphosphatidylinositol (GPI)-anchored GDNF family receptor α , GFR α -1, on the membrane surface (Jing *et al.*, 1996; Treanor *et al.*, 1996). This binding raises GDNF affinity for the co-receptor c-RET, a tyrosine kinase transmembrane receptor, so that the GDNF-RET-GFR α -1 complex triggers the intracellular cascade.

2.1 Gdnf expression in the testis

The comprehension of the *in vivo* function of GDNF in mammalian testis dates back to 2000 when Meng and colleagues created a murine model which overexpressed GDNF or carried one GDNF-null allele in the testis (Meng *et al.*, 2000). In the first case, transgenic mice exhibited an increased number of spermatogonial stem cells (SSCs) organized in clusters, that degenerated over time into non-metastatic tumours. On the contrary, mice holding a single functioning Gdnf allele showed a progressive depletion of stem cells reserve resulting in Sertoli-cell-only phenotype and long-term impaired spermatogenesis. These results suggested the requirement of an optimum range of GDNF concentration for SSCs self-renewal (Chen *et al.*, 2014). Later, GDNF was shown to be a crucial molecule for the expansion of SSCs *in vitro* (Kanatsu-Shinohara *et al.*, 2005; 2003; Kubota *et al.*, 2004).

Within the testis, the main source of GDNF is Sertoli cell (Trupp *et al.*, 1995, Golden *et al.*, 1999). More recently, peritubular myoid (PM) cells were found expressing GDNF in human and murine

testis (Spinnler *et al.*, 2010, Chen *et al.*, 2014). PM cells are smooth-muscle-like cells that surround the external basal lamina of seminiferous tubules and were traditionally thought related to structural support and contractility of seminiferous tubules. This finding raised the hypothesis that also PM cells could influence SSCs behaviour and could actively take part in the SSCs niche.

GDNF responsive cells are A_s and A_{pr} which mostly expressed the GFR α -1 receptor. GFR α -1 is expressed at lower levels also in A_{al} (Nakagawa *et al.*, 2010; Grasso *et al.*, 2012).

In human testis, the source of GDNF comprises Sertoli cells (Davidoff *et al.*, 2001) and myoid peritubular cells (Spinnler *et al.*, 2010, Chen *et al.*, 2014; Chen *et al.*, 2016).

Both Apale and Adark spermatogonia express the GDNF receptor GFR α -1 (Grisanti *et al.*, 2009), which in complex results expressed by $54 \pm 8\%$ of the cells in the spermatogonial compartment (Di Persio *et al.*, 2017).

The role of GDNF in human testis is still poorly understood, even if a similar function to that in rodents has been hypothesized. In 2012 the expression of GDNF mRNA and protein was analyzed in Sertoli cells from non obstructive azoospermia (NOA) patients, which are characterized by the loss of germ cells and impaired spermatogenesis. This study showed that NOA patients exhibited remarkably lower levels of GDNF mRNA and protein respect to the normal counterpart (Ma *et al.*, 2013). The same conclusions were drawn by Singh and colleagues which analysed GDNF expression in Sertoli cells from Sertoli cell-only (SCO) patients (Singh *et al.*, 2017). They showed a 3,4 fold less production of GDNF and a 10 fold reduction of GFR α -1 expression in SCO testes compared to the normal.

In target cells, GDNF downstream signalling leads to a wide gene expression modulation. In particular, by microarray analysis, 1124 genes were found differentially modulated after GDNF exposure. Among these, N-Myc and L-Myc, as well as cyclins D3 and B were found up-regulated. On the contrary, cell cycle inhibitors p21, p19 and p15 resulted down-regulated (Hofmann *et al.*, 2005).

GDNF also positively regulates a series of genes proved to be crucial to spermatogonial self-renewal, such as (Oatley *et al.*, 2006; Oatley *et al.*, 2007) Bcl6b (B cell CLL/lymphoma 6 member B),

Erm (Ets variant 5), Lhx1 (LIM homeobox 1). Moreover GDNF stimulates spermatogonia expression of its own receptor $GFR\alpha-1$ (Grasso *et al.*, 2012) and Fibroblast Growth Factor 2 receptor, $FGFR2$, increasing the responsiveness to this molecule that in turn stimulates SSCs self-renewal (Hofmann *et al.*, 2005).

Although *Gdnf* role as promoting SSCs self-renewal has been well established, a role in influencing SSCs differentiation was also hypothesized. In fact the activation of $ERK1/2$ in $GFR\alpha-1$ positive spermatogonia, under the control of GDNF, prevents them from differentiation (Hasegawa *et al.*, 2013). In the same direction are data from Ikami and colleagues, that discriminate spermatogonia in two subsets: $GFR\alpha-1^+$, that appeared to be resistant from the differentiating stimulus of retinoic acid, and the $NGN3^+$ that showed a rapid tendency to differentiate into KIT^+ differentiating spermatogonia (Ikami *et al.*, 2015). Concerning this last point, recently, Sharma and Braun revisited the commonly accepted role of GDNF. The Authors created a murine model in which the typical cyclical pattern of *gdnf* expression was disrupted so that it resulted constitutively expressed in all stages of spermatogenesis. An increased number of SSCs was detected, however, it did not match with a proportional increase in their Edu labelling index. This evidence suggested that GDNF could drive spermatogonial self-renewal by blocking the differentiation in particular of $LIN28^- A_s$ cells into $LIN28^+ A_s A_{pr} A_{al}$, rather than directly promoting proliferation (Sharma & Braun, 2018). On the contrary, Grasso and colleagues found that in murine seminiferous tubules *Kit* mRNA - a known marker of differentiating spermatogonia - was significantly up-regulated by GDNF at stages II-VIII, suggesting that GDNF could have a role in the differentiation of $GFR\alpha-1^+$ spermatogonia (Grasso *et al.*, 2012).

GDNF was also described as a chemoattractant molecule in enteric neural cells (Young *et al.*, 2001), in neuronal precursor cells in the rostral migratory stream (Paratcha *et al.*, 2006), in epithelial cells (Tang *et al.*, 1998), in transformed cells (Okada *et al.*, 1999) and in seminoma (Ferranti *et al.*, 2012).

It is known from literature the non-static nature of SSCs, as well as their not stochastic localization on the basal lamina (Chiarini Garcia *et al.*, 2001). Moreover, the entire SSCs pool undergoes continuous

turnover and a migratory activity of SSCs along the tubule has been considered functional to the replacement of SSCs that get lost, from neighbouring SSCs (Klein *et al.*, 2010). Dove and colleagues for the first time demonstrated the direct capacity of the GDNF to induce undifferentiated spermatogonia migration *in vitro*. Intriguingly, the more undifferentiated (PLZF-positive) spermatogonia were shown to highly express VASP, a member of the ENA/VASP protein family that interacts with the actin cytoskeleton. The expression of VASP is high in A_s and A_p and decreases to almost undetectable levels in A_{al} and differentiated spermatogonia. In intact seminiferous tubules and germline stem cell cultures, GDNF treatment up-regulates VASP in a dose-dependent fashion. These data suggest that GDNF may impinge on the stem/progenitor compartment, affecting the actin cytoskeleton and cell migration (Dove *et al.*, 2013).

2.2 Gdnf regulation

GDNF function is crucial for SSCs self-renewal and for whole spermatogenesis maintenance, thus it has to be finely regulated. GDNF levels are not constant during the seminiferous epithelium cycle. In the mouse, both GDNF mRNA and secreted protein swing during the 12 stages of seminiferous epithelium, reaching a peak around stages II-VI, a decrease at VII-VIII and a minimum at stages IX-XI (Grasso *et al.*, 2012). A similar trend was found in rat (Johnston *et al.*, 2011) and hamster (Sato *et al.*, 2011). Undifferentiated spermatogonia behaviour during the cycle of the seminiferous epithelium parallels GDNF fluctuations: from stage XI to stage IV they undergo proliferation and become arrested in phase G₀/G₁ at stages III-VI to prepare themselves for differentiation at stages VII-VIII. However, the number of A_s and A_{pr} do not significantly vary among the different stages, unlike A_{al} which increase from stage XI to III before going to differentiation at stage VII (Tegelenbosch & de Rooij, 1993).

As the entire spermatogenesis is under the hypothalamic- pituitary axis control, the niche factor GDNF is controlled by systemic

endocrine factors as well. In particular, GDNF is positively regulated by FSH hormone (Tadokoro *et al.*, 2002) on Sertoli cells, as they express FSH receptor, FSHR. Because FSHR is not present on peritubular cells, GDNF production in peritubular cells does not seem to be directly regulated by this hormone (Albrecht *et al.*, 2006). However, FSH pathway might be dispensable for GDNF production *in vivo*, given that FSHR- null mice are still fertile (Dierich *et al.*, 1998; Krishnamurthy *et al.*, 2000). Maximal Sertoli cell responsiveness to FSH occurs at stages II-VI (Parvinen *et al.*, 1980), indeed, *gdnf* mRNA results induced by FSH in stage II-VI and not in other stages (Lamberti & Vicini, 2014). Recently, Lamberti and Vicini characterized three cAMP responsive element (CRE) implicated in the FSH-induced response of *gdnf*. These regions were localized just upstream the TATA box and Transcriptional Start Site 1 (TSS1) and were found to be highly conserved among human, mouse and rat (Lamberti & Vicini, 2014) (Figure 2).

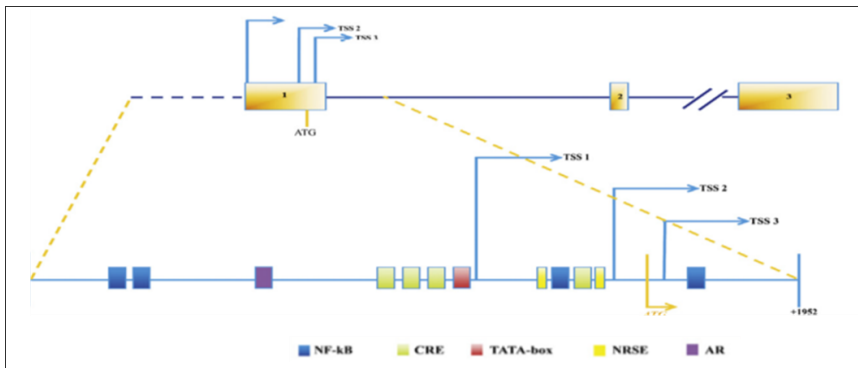


Figure 2 Schematic structure of the murine *Gdnf* gene showing the evolutionarily conserved binding sites in the flanking regions of the TSS1 (Lamberti & Vicini, 2014).

Recently, Hasegawa and colleagues still shed light on cyclical activation of MEK/ERK in Sertoli cells, that determines oscillating levels of *gdnf* and promotes the undifferentiated state by inducing the expression of GFR α -1/RET. Moreover they identified in a

transient pulse of retinoic acid from stage VII an antagonist of *gdnf* expression levels (Hasegawa *et al.*, 2013).

Testosterone was also demonstrated to stimulate both GDNF mRNA and protein in PM cells *in vitro* (Chen *et al.*, 2014). Moreover the loss of *Gdnf* in PM cells caused a progressive depletion of undifferentiated spermatogonia over the long term in mice (Chen *et al.*, 2016). On the contrary, is not clear the effect of testosterone on *gdnf* expression in Sertoli cells, even if is well established that they respond to testosterone, due to the expression of androgen receptor (AR). An androgen-responsive element (ARE) highly conserved in mouse, rat and human was found on the promoter region of *gdnf* (Figure 2), suggesting a direct effect of testosterone on the expression level of *gdnf* (Lamberti & Vicini, 2014).

3. Retinoic acid

Retinoic acid (RA) is a small lipophilic molecule, the active metabolite of Vitamin A. It can rightly be considered a morphogen, according to the definition as “a secreted molecule that diffuses from local sources to form concentration gradients, which specifies multiple cell fates” (Schilling *et al.*, 2016). In fact RA signalling is a crucial regulator of the primary body axis of the embryo (di Masi *et al.*, 2015).

RA is formed from circulating retinol (ROL) or from local storage of retinyl esters by two sequential oxidative steps: first ROL is converted into retinaldehyde by alcohol dehydrogenases (ADH); then retinaldehyde dehydrogenases (RALDH) convert retinaldehyde in RA. RA controls the gene expression through the binding of a heterodimer constituted of the nuclear receptors RAR/RXR (in the form of three different isoforms RAR α,β,γ and RXR α,β,γ), which are located on conserved regions named Retinoic Acid Responsive Element (RARE) on the promoter of target genes. RARE elements are composed of two hexameric DR (direct repeats) - (A / G) G (T / G) TCA - spaced by 5pb (DR5) or 2pb (DR2). For both DR5 and DR2, the 5' of the sequence is bound by RXR and the 3' by RAR

(Cunningham & Duester, 2015). RARs receptors act as a molecular platform to recruit numerous factors that regulate gene transcription. In the classical model, the RA-dependent transcriptional activation requires that the RAR / RXR heterodimer in the absence of retinoic acid binds to the RARE sequence and represses the transcription of the target genes also through epigenetic modifiers. RAR / RXR in the repressive state recruits co-repressors such as NCOR-1 (Kumar *et al.*, 2016), the histone deacetylases HDACs and the repressive chromatin-remodeling complex PRC2 (Polycomb repressive complex 2) (Figure 3 A).

This leads to an increase in trimethylation of the lysine 27 of histone H3 (H3K27me3) which induces chromatin condensation and gene silencing. When retinoic acid is present, it binds to RAR and induces a conformational change of the RAR/RXR heterodimer that promotes the release of repressors and the recruitment of factors that activate transcription such as NCOA1 or SRC1, NCOA2 and NCOA3. In turn, the co-activators recruits the HATs histoneacetylases and the Tritorax complex that remove the negative epigenetic modifications (H3K27me3) and add histone activating transcription modifications: histone H4 acetylations and the histone H3 lysine 4 trimethylation. These modifications induce relaxation of the chromatin, allowing access on the promoter of the transcription factor target gene and the RNA polymerase.

The effect of RA on transcription is not always activatory. There are examples, in fact, where the RA leads to the repression of the transcription, recalling the Polycomb complex and the deposition of repressive changes. Some retinoic acid-regulated genes known in the literature are fibroblast growth factor (Fgf8), homeobox gene B1 (Hoxb1), pou5f3 in zebrafish and Wnt8a in mouse (Cunningham & Duester, 2015).

The retinoic acid-dependent pathways also include a non-transcriptional signalling pathway that activates a kinase cascade that will be integrated into the nucleus. Retinoic acid regulates the pathway of MAP kinases and ERK kinases by activating p42 / p44 and p38MAPK (Chung *et al.*, 2010; Dey *et al.*, 2007; Gianni *et al.*, 2002). In this case the RA is bound by the cytoplasmic portion of the RARs that will transmit the signal through the GTPase Rho and PI3K. The cascade of phosphorylations converges in the nucleus,

with the translocation of the MAPKs, which will activate further targets. In the nucleus the protein kinase MSK1 is activated, which in turn phosphorylates numerous factors involved in the transcription such as histones, RAR receptors and their cofactors (Masi *et al.*, 2015)

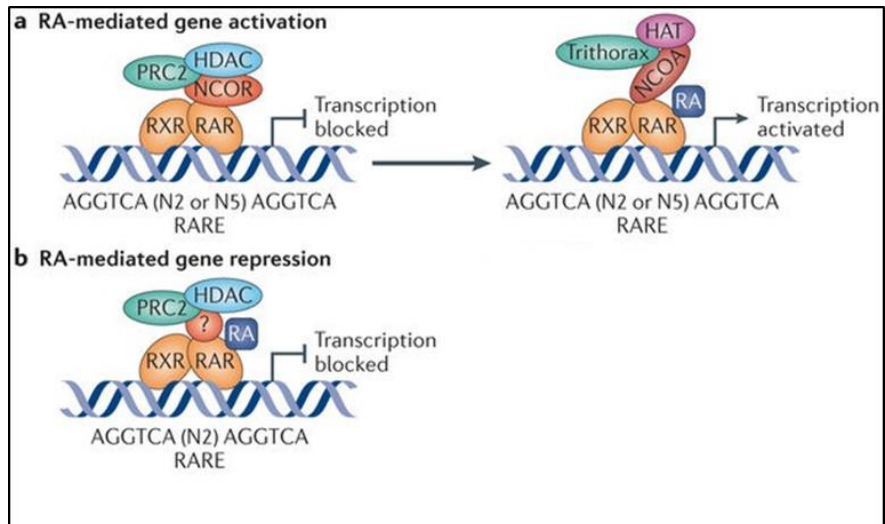


Figure 3 A) RA-mediated control mechanism of gene expression. RA mediated transcriptional activation B) RA mediated transcriptional repression (Cunningham & Duester, 2015).

3.1 Retinoic acid and spermatogenesis

In the testis, nuclear receptors and the enzymes involved in RA metabolism are widely expressed, so that the synthesis of RA seems to be a synergic process mediated by many cell types. ADH (alcohol dehydrogenase) 1 and 3 isoforms in Sertoli cells convert ROL into retinaldehyde; ADH4 in late spermatids catalyzes the same reaction (Deltour *et al.*, 1997; Molotkov *et al.*, 2002). Retinaldehyde is converted in retinoic acid by ALDH1A (aldehyde dehydrogenase 1A) in Sertoli cells and by ALDH1A2 in germ cells

(Arnold *et al.*, 2015). However, the main sources of RA seem to be Sertoli cells and pachytene spermatocytes (Endo *et al.*, 2017).

RA degradation is also important and is mainly controlled by peritubular cells which express three cytochrome P450 hydrolase CYP26A1, CYP26B1, CYP26C1 (Vernet *et al.*, 2006 B), but also within the seminiferous tubules by Sertoli cells and germ cells which also express CYP26 enzymes (Hogarth *et al.*, 2015 B).

The critical role of retinoids in the spermatogenic process was strikingly demonstrated by the rodent model fed with Vitamin- A-deficient diet (VAD) (Wolbach & Howe, 1925). In VAD animals only Sertoli cells, undifferentiated spermatogonia and some preleptotene were left in the seminiferous epithelium. Then, when animals were injected with RA, a massive differentiation of A_{al} into A1 spermatogonia was restored and the blocked spermatocytes could initiate meiosis (van Pelt & de Rooij, 1991).

RA pleiotropic functions could be summarized as follows: promotion of Sertoli cell maturation at puberty by suppressing proliferation; spermatogonial differentiation (van Pelt & de Rooij, 1991); induction of meiosis (Koubova *et al.*, 2006); controlling blood-testis barrier (BTB) integrity (Morales & Cavicchia 2002); spermiation (Vernet *et al.*, 2008); coordinating the adult spermatogenic cycle (Sugimoto *et al.*, 2012; Endo *et al.*, 2015).

In particular, RAR γ is the RAR isoform involved in spermatogonia differentiation and it is typically expressed in A_{al} spermatogonia. RAR γ ablation has demonstrated to not affect meiosis or spermiogenesis but to impair A_{al} transition into A1 (Gely-Pernot *et al.*, 2012).

In germ cells, RA can promote meiotic progression by directly stimulating *Stra8* transcription, which is a critical gene for meiosis, even if its function is still not clarified (Raverdeau *et al.*, 2012).

Because all the events triggered by RA occur in a synchronized fashion around stages VII-VIII, RA was thought to reach a peak in that stages. This hypothesis was demonstrated by Hogarth and colleagues that for the first time quantified RA local concentration in postnatal seminiferous epithelium of the testis (Figure 3 C). The Authors used a murine model in which spermatogenesis was disrupted through the administration of bisdichloroacetyldiamine (WIN18,446), a strong inhibitor of RALDH enzymes, and then

spermatogenesis was synchronized with a RA injection (Hogarth *et al.*, 2015 A).

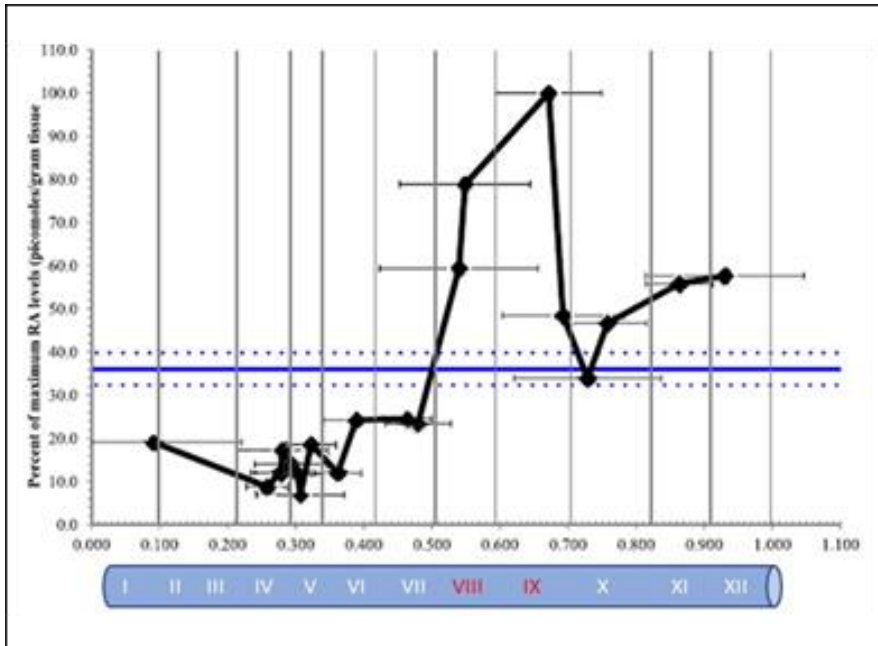


Figure 3 C) Schematic representation of RA levels in relation to the stages of the seminiferous epithelium (modified by Hogarth *et al.*, 2015 A)

A cyclical metabolism of RA during the cycle of the seminiferous epithelium is the basis for oscillating RA levels: in fact, in the first part of the cycle, storage of RA seems to be predominant; RA synthesis, catalyzed by RALDH enzymes, occurs at stages VII-XII ; RA degradation by CYP26 cytochromes takes place after spermatogonia differentiation, at stages VIII-XI (Vernet *et al.*, 2006 B; Sugimoto *et al.*, 2012). In particular, Hogarth and colleagues in 2015 underlined the stage-specific expression of two enzymes responsible for the reversible conversion of retinol into retinaldehyde, properly RDH10 (retinol dehydrogenase 10) and DHRS4 (dehydrogenase/reductase SDR family member 4) (Hogarth *et al.*, 2015 A).

In Sertoli cells, the most abundant isoform of retinoids nuclear receptors is RAR α (Vernet *et al.*, 2006 B). The importance of this receptor was attested by KO mice models, which are sterile (Lufkin *et al.*, 1993). Moreover, RAR α deficient germ cells hardly colonize a recipient testis (Doyle *et al.*, 2007). On the contrary, RAR β , RAR γ and RXR γ null mutants show a normal spermatogenesis (Lohnes *et al.*, 1993; Luo *et al.*, 1995; Krezel *et al.*, 1996), RXR β KO mice displayed defects in spermatids maturation and spermiation (Kastner *et al.*, 1996), while the effect of RXR α absence has not been investigated because RXR α -/-animals die *in utero* (Sukov *et al.*, 1994; Kastner *et al.*, 1994).

While the role of Vitamin A in rodents has been largely investigated, the role in human testis is still unknown. For this reason, a VAD study in nonhuman primates would be helpful to determine the effect of RA depletion in primate testis (Hogarth *et al.*, 2011).

Since the role of RA is crucial in rodents spermatogenesis, inhibition of the endogenous biosynthesis of this molecule has also been proposed as a contraceptive strategy in men, through the inhibition of ALDH1a2 enzymes by the pharmacological compound WIN 18, 446 (Heller *et al.*, 1961). However, nowadays, an ideal molecule has still to be optimized to reversibly block spermatogenesis, without interfering with the metabolism of RA in other organs and without generating a disulfiram reaction (Hogarth *et al.*, 2011), which is a toxic reaction that causes a reddening of the face when consuming alcohol, due to an incomplete metabolism of this molecule.

AIM

This study arises in the context of the research activity carried out in my laboratory, aimed for years at the study of SSC and its niche, both in mouse and in human.

GDNF is a crucial factor for the self-renewal of spermatogonial stem cells (SSCs) which, starting from puberty in the mammal, support the continued production of mature germ cells, spermatozoa (Meng *et al.*, 2000). In both mouse and human, GDNF is produced and secreted by somatic Sertoli cells and peritubular myoid cells. Sertoli cells are the main contributors to the formation of SSCs niche, a highly specialized micro-environment in which SSC takes the so-called "fate decision" i.e. to undertake the differentiation process ("commitment") or to maintain the "stemness" (de Rooij, 2017). In this context, GDNF represents a molecule-paradigm for the understanding of the spermatogonial niche. In particular, we have shown that the production of GDNF in the seminiferous tubules is stage-specific and therefore in a given area of the seminiferous tubule its concentration varies cyclically (Grasso *et al.*, 2012). Later, we demonstrated that GDNF acts *in vitro* as a chemoattractant for undifferentiated spermatogonia (Dovere *et al.*, 2013). Since GDNF promotes SSC self-renewal, these data suggest that GDNF could facilitate the homing and maintenance of SSCs in their niche. The niche is influenced by paracrine factors released by different cell types in the testis and endocrine factors such as hormones of the hypothalamic-pituitary axis, which therefore control the process of spermatogenesis at several levels. We then cloned and characterized the murine gene promoter region for active GDNF in Sertoli cells. The TSS has been identified, several evolutionarily conserved transcription factor binding sites and three CRE sites (cAMP-responsive elements) demonstrating the direct involvement in FSH-dependent endocrine regulation (Lamberti & Vicini, 2014). In recent years, further mechanisms of regulation of GDNF expression have been highlighted that make it a finely regulated molecule (Garcia *et al.*, 2017). An inhibition of RA-mediated GDNF levels was described by Saga and colleagues in 2013. Retinoic acid is an active metabolite of vitamin A with an

almost ubiquitous role in cell differentiation and embryonic development, as well as in the testis. Retinoic acid is fundamental for the differentiation of spermatogonia and, as GDNF, it is produced cyclically and in a stage-specific manner in the seminiferous tubules. GDNF levels are low in those stages of the seminiferous epithelium where retinoic acid levels are high and vice versa. This evidence suggests that *in vivo* GDNF and retinoic acid are antagonists in the regulation of the spermatogonial stem compartment. Because the molecular mechanism underlying this negative regulation is not described, in this thesis we have focused our attention on the molecular mechanism by which RA negatively affects the expression of GDNF. Primary cultures of Sertoli cells from immature mice (Vicini & Conti, 1997) and cultures of seminiferous tubules from adult mice were used as experimental models (Kotaja *et al.*, 2004).

RESULTS

4. Retinoic acid down-regulates *gdnf* in immature testis

In order to confirm the evidence reported in the literature by Hasegawa (Hasegawa *et al.*, 2013), which describes the down-regulation of *gdnf* mediated by retinoic acid in the mouse, we set up primary cultures of Sertoli cells, obtained from CD1 mice of 17 days, according to the protocol described by Vicini & Conti (Vicini & Conti, 1997).

Sertoli cells were treated for 24 hours in the presence of increasing concentrations of retinoic acid (RA) from 0,01 μM to 10 μM . Subsequently *gdnf* mRNA levels were evaluated by Real-Time PCR.

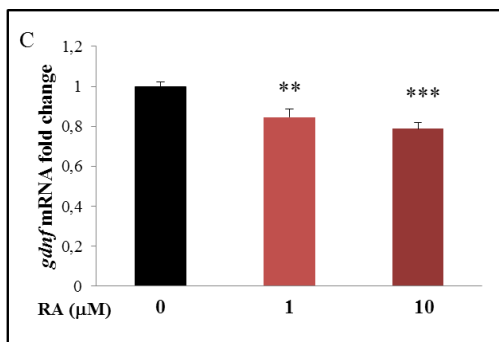
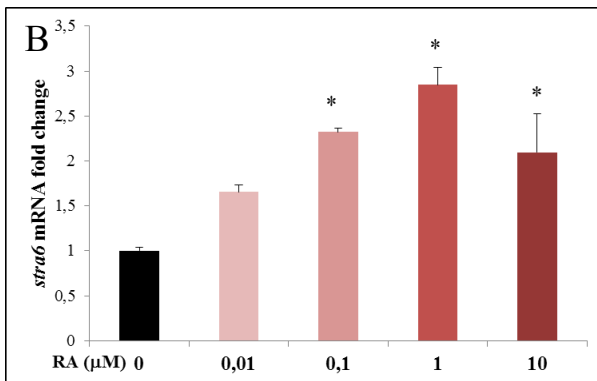
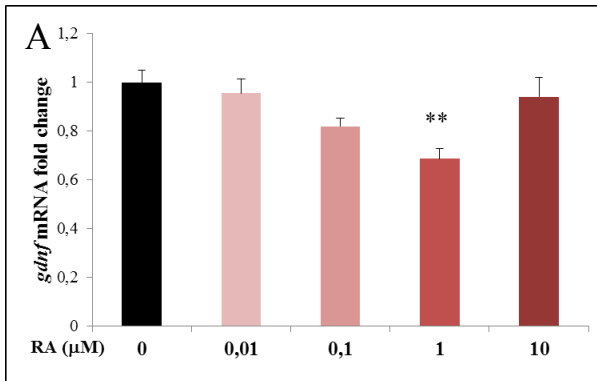
The results showed a negative modulation of the transcript in a dose-dependent manner with a significant effect at the concentration of 1 μM RA (Figure 4 A).

Moreover, as an internal control of treatment efficacy, we in parallel evaluated by Real-Time PCR the induction of *stra6* transcript, a well-known responsive gene of RA (Bouillet *et al.*, 1997). We observed a dose-response increase of *stra6* with maximum induction with 1 μM RA, confirming the efficacy of retinoic acid treatment (Figure 4 B).

To evaluate if *gdnf* RA-dependent regulation was recapitulated in testis organ culture, 1 mm³ fragments of testicular tissue obtained from 17 day-old CD1 mice were cultured for 8 hours in presence of 1 or 10 μM RA. Subsequently *gdnf* and *stra6* mRNA levels were evaluated by Real-Time PCR. *Gdnf* resulted significantly down-modulated at both concentrations respect to untreated fragments (Figure 4 C) as well as *stra6* mRNA that was induced with both treatments (Figure 4 D). Data suggest that the RA-mediated regulation of *gdnf* in Sertoli cells is still detectable in the immature organ culture.

Because GDNF is a soluble molecule, we then asked if also secreted levels were affected by RA. Therefore, GDNF protein levels in the conditioned media of Sertoli cells were quantified **after 24 hours**

exposure with 1 μM RA or left untreated. The ELISA assay performed on three cultures, showed that in the presence of RA GDNF levels were lower compared to the control (Figure 4 E).



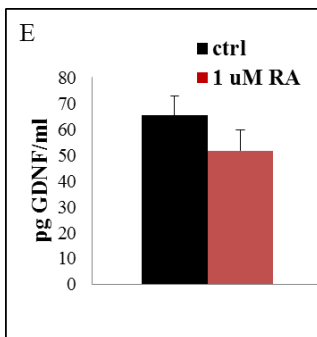
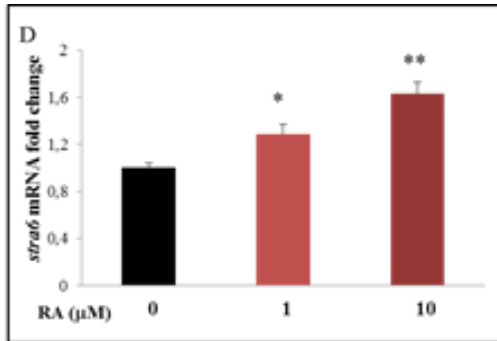


Figure 4 **A)** Histograms show the regulation of *gdnf* transcript after 24 hours treatment with increasing concentrations of retinoic acid (RA). mRNA levels were measured by Real-Time PCR, normalized on β -actin and expressed in fold change with respect to the control. Data are shown as mean \pm SEM of 3 cultures. ** $p < 0.01$ with Student-Newman-Keuls Method **B)** Histograms showing the regulation of *stra6* transcript in the same conditions. Data are shown as mean \pm SEM of 2 cultures. * $p < 0.05$ ANOVA with Dunn's Method. **C)** Histograms show the regulation in murine testicular organ culture of *gdnf* transcript after 8 hours treatment with increasing concentrations of retinoic acid (RA). mRNA levels were measured by Real-Time PCR, normalized on β -actin and expressed in fold change with respect to the control. Data are shown as mean \pm SEM of 3 cultures. * $p < 0,05$ *** $p < 0,001$ with Student-Newman-Keuls Method. **D)** Histograms show the regulation in murine testicular organ culture of *stra6* transcript in the same conditions. Data are shown as mean \pm SEM of 2 cultures. * $p < 0,05$ ** $p < 0,01$ with Student-Newman-Keuls Method. **E)** The histogram shows the effect of 24 hours RA treatment on secreted GDNF protein in Sertoli cells conditioned medium. Data are presented as the mean \pm standard error of the mean (SEM) from $n = 3$ cultures.

5. RA-dependent *gdnf* inhibition is quite rapid and does not involve neo-synthesis of a repressor

In order to deepen the kinetic of the RA action on *gdnf* mRNA, time-course analysis on RA-mediated regulation was performed on Sertoli cells.

Gdnf mRNA levels after RA treatment were evaluated by Real-Time PCR at different time points: 20 minutes, 1 hour, 2, 4, 8, 16, 24 hours. A significant reduction of *gdnf* mRNA (~40%) was appreciable not earlier than 2 hours and was maintained for 24 hours (Figure 5 A).

To evaluate whether the inhibition of *gdnf* depended on transcriptional stability or required protein synthesis, Sertoli cells were treated for 2 hours in the presence of actinomycin D, impinging ongoing transcription (actD, 5 µg / ml), or cycloheximide, a powerful inhibitor of protein synthesis (CHX, 10 µg / ml), with or without RA.

Interestingly, actD treatment alone induced a reduction of about 80% of *gdnf* mRNA levels compared to the control, indicating an extremely low stability of the mRNA and suggesting that active transcription is essential for *gdnf* mRNA half-life. On the contrary, RA and actD co-treatment did not give rise to further appreciable lowering of *gdnf* mRNA compared to actD per se.

In contrast, treatment with CHX increased mRNA levels, making plausible that the ongoing synthesis of a repressor controls basal *gdnf* levels. However, in cells treated with CHX, RA was still able to significantly inhibiting mRNA levels. This data suggests that RA mechanism of action on *gdnf* transcript does not require protein synthesis (Figure 5 B).

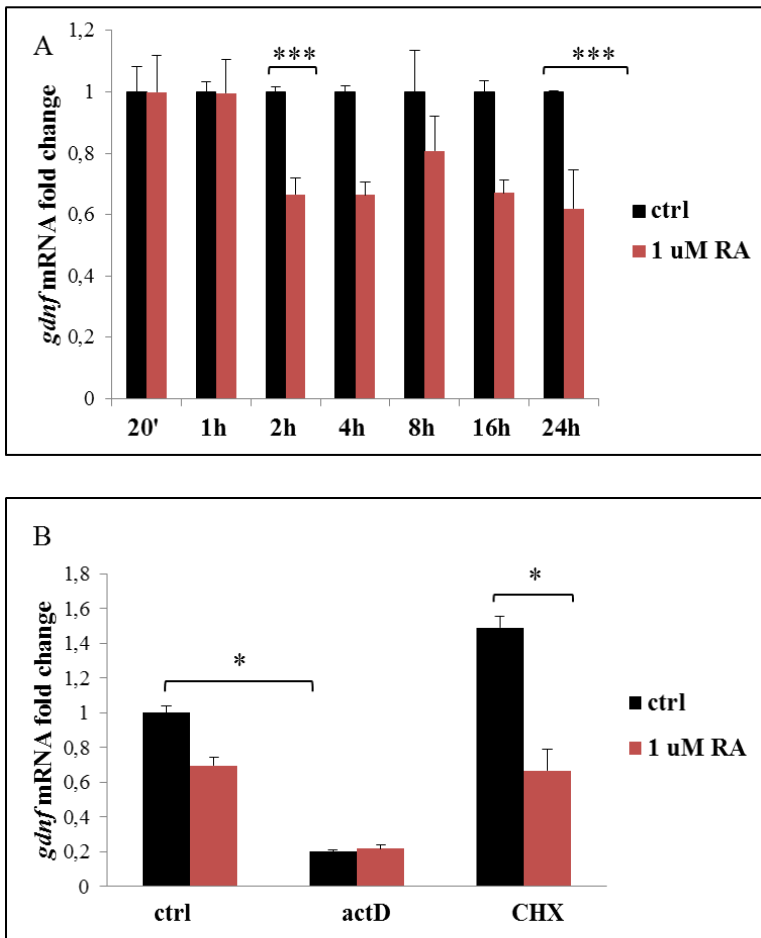


Figure 5 A) The histogram shows the effect of different time treatment with 1 uM RA on *gdnf* mRNA in primary cultures of murine Sertoli cells. The mRNA levels were quantified by Real-Time PCR, normalized on β -actin and expressed as mean \pm SEM from 2 cultures and expressed in fold change compared to the untreated control. *** $p < 0,001$ with t-test **5 B)** Histogram shows the regulation in Sertoli cells of *gdnf* transcript after 2 hours treatment with actinomycinD (actD) or cycloheximide (CHX) in the presence or absence of RA. The mRNA levels were quantified by Real-Time PCR, normalized on β -actin and expressed as mean \pm SEM from 2 cultures and expressed in fold change compared to the untreated control. * $p < 0.05$ with ANOVA (Dunn's Method) on Ranks.

6. RAR α inhibition impinges *gdnf* down-regulation

To determine whether the down-regulation of *gdnf* involved the nuclear retinoic acid receptors RARs, Sertoli cells were treated with BMS-204493, inverse agonist inhibitor of all three RARs isoforms (RAR α , RAR β and RAR γ) (Raverdeau *et al.*, 2012). Cells were pre-treated for 2 hours in the presence of 10 μ M BMS-204493, and thereafter, 1 μ M RA was added to the culture medium for 24 hours. At the end, *gdnf* mRNA levels were measured. Real-Time PCR results show the statistically significant reduction of *gdnf* levels after retinoic acid treatment compared to controls. Treatment with BMS-204493 alone did not modulate *gdnf* baseline levels but when retinoic acid was added, a significant down-regulation was no longer appreciable (Figure 6 A).

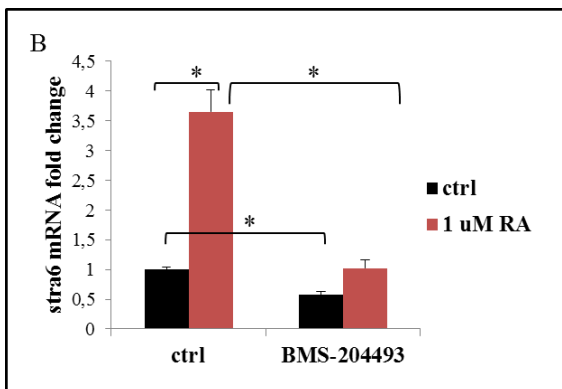
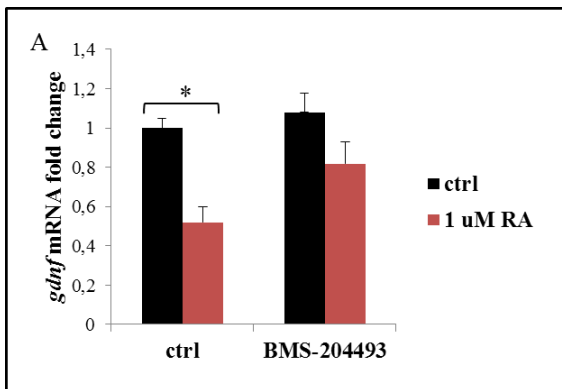
As an internal control of the effectiveness of the pharmacological inhibitor, the levels of *stra6* mRNA were measured. The treatment with BMS-204493 negatively affects basal levels of *stra6* and significantly blocks the induction of *stra6* by RA (Figure 6 B)

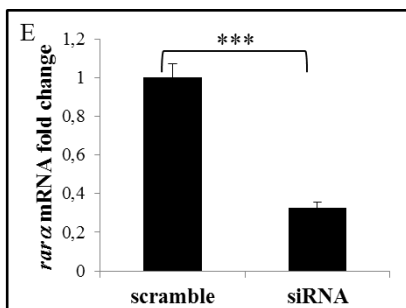
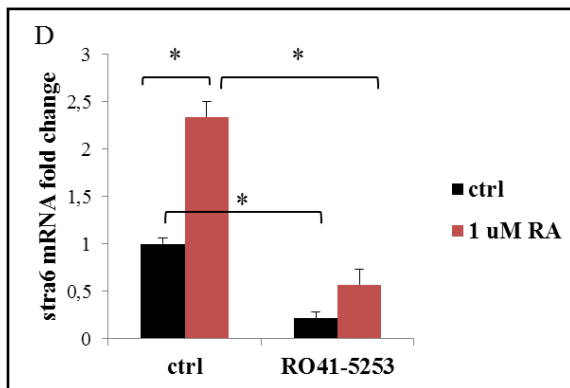
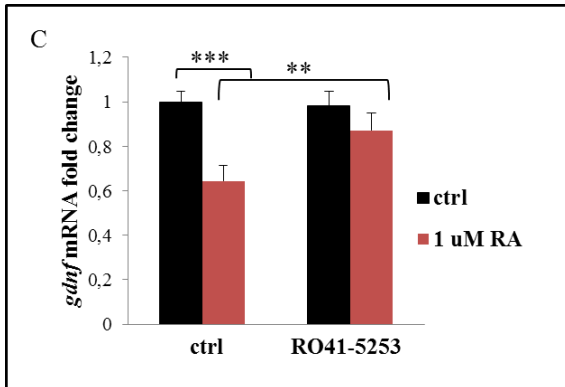
Since RAR α is the most expressed isoform in Sertoli cells (Vernet *et al.*, 2006 B), we investigated the involvement of this specific isoform, treating Sertoli cells in the presence of RO41-5253, a selective pharmacological antagonist of RAR α (Ertesvag *et al.*, 2007). Cells were pre-treated for 2 hours with 3,5 μ M RO41-5253, and then 1 μ M RA was added to the culture medium for the next 24 hours, then the levels of *gdnf* and *stra6* were analyzed by Real-Time PCR. In the presence of RO41-5253, the inhibitory effect of RA on *gdnf* mRNA was reversed, while the basal levels were not affected (Figure 6 C). Regarding *stra6* mRNA, both basal and induced levels were strongly reduced (Figure 6 D). These data suggest that the effect of RA on *gdnf* expression depends on the RAR α nuclear receptor activity.

We then examined the effect of RAR α ablation performed by RNA interference (RNAi) using small interfering RNA (siRNA) against RAR α mRNA. Sertoli cells were transfected for 5 hours with 250 picomoles / well anti RAR α siRNA or with a scrambled siRNA, conveyed by 5 μ l / well Lipofectamine2000. At the end, cells were

treated for 24 hours in the presence or absence of 1 μ M RA and levels of *gdnf* were measured by Real-Time PCR.

The administration of anti RAR α siRNA lowered RAR α mRNA levels by around 70% respect to the scramble sequence (Figure 6 E). As expected, RA was able to down-regulate *gdnf* mRNA in Sertoli cells transfected with the scramble RNA. Conversely, in cells where RAR α was interfered, RA did not lower *gdnf* levels (Figure 6 F). This approach confirmed the RAR α involvement in *gdnf* regulation and strengthened previous conclusions obtained using the pharmacological inhibitors.





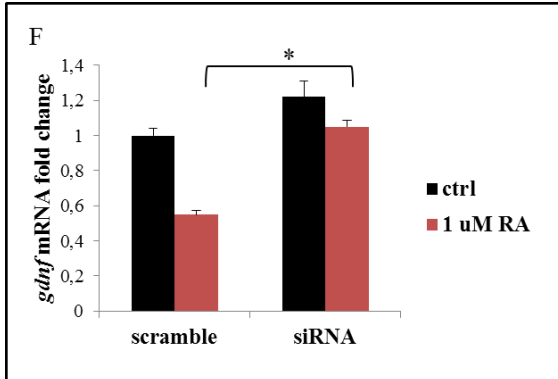


Figure 6 Effect of treatment with BMS-204493 on the levels of *gdnf* mRNA **A**) and *stra6* **B**) in the presence or absence of RA. **A**) Data are represented as mean \pm SEM from 4 cultures * $P < 0,05$ with Dunn's Method. **B**) Data are represented as mean \pm SEM from 3 cultures * $p < 0,05$ with Dunn's Method.

Effect of the treatment of Sertoli cells with 3,5 μ M RO41-5253 for 24 hours on the levels of *gdnf* **C**) and *stra6* **D**) in the presence or absence of RA, measured by Real- Time PCR. Data are represented as mean \pm SEM from 3 cultures. **C**) * $p < 0,05$ *** $p < 0,001$ with All Pairwise Multiple Comparison Procedures Tukey Test) **D**) * $p < 0,05$ with All Pairwise Multiple Comparison Procedures (Tukey Test) on Ranks.

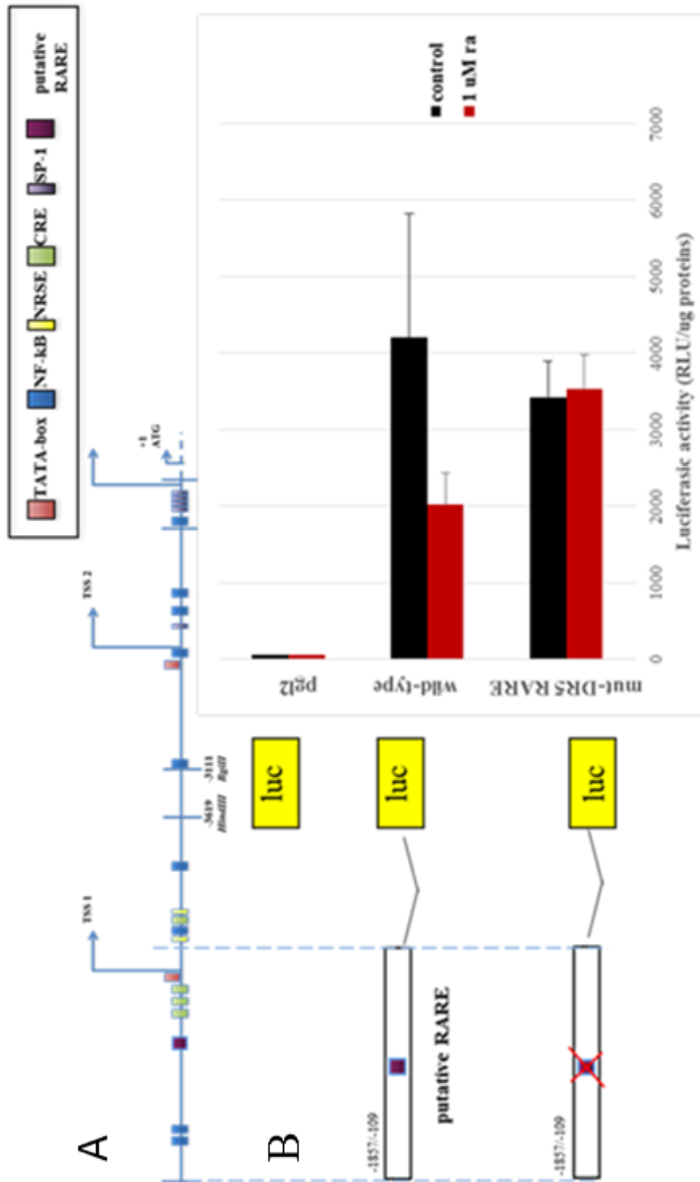
E) Effect of the anti-RAR α siRNA on *rara* transcript **F**) Effect of the treatment with scramble or anti-RAR α siRNA on *gdnf* levels in the presence or absence of RA, measured by Real- Time PCR. Data are represented as mean \pm SEM from 2 cultures * $P < 0,05$ with Dunn's Method.

7. Gdnf regulation depends on its promoter

We asked, therefore, whether the action of RA on *gdnf* mRNA levels involved the 5'-flanking regulatory region of the gene. To clarify the role of the promoter in the regulation of *gdnf*, we investigated the activity of the promoter by luciferase assays.

Sertoli cells were transiently transfected with a plasmid vector containing the 5'-flanking region of the gene Gdnf (-1857 / + 109) upstream of the luciferase gene (Lamberti & Vicini, 2014) and treated in the presence or absence of 1 μ M RA for 24 hours. At the end of the treatment, luciferase activity was measured in cell extracts. The activity is expressed as RLU (Relative Light Units) corrected for μ g of proteins. Retinoic acid reduces of about 40% of the luciferase activity compared to the control. These results indicate that the 5'-flanking region of the gene (-1857 / + 109) contains gene sequences implicated in the negative regulation of the levels of mRNA exerted by RA (Figure 7).

Since the presence of a Retinoic Acid Responsive Element on the 5'-flanking of the murine Gdnf gene is not known from the literature, we performed an in-silico analysis using the Softberry software, detecting the presence of a RARE DR5 5'-TGACCTCTCCCTGATCA-3' sequence on the murine Gdnf promoter (*mus musculus*), just upstream of the 3 CRE regions (Cyclic AMP Responsive Element) evolutionarily conserved in mouse, rat and man, previously identified by our group (Lamberti & Vicini, 2014). In order to demonstrate the involvement of this region, we performed a site-specific mutation of the DR5 in the genomic fragment (-1857 / + 109). With this mutated construct we transfected cells and measured luciferase activity, in the presence or absence of RA. The results show that the mutation of the putative DR5 binding site reverts the inhibition of RA treatment on luciferase activity (Figure 7 B). This suggests that the DR5 sequence identified in-silico is critical in RA mediated regulation confirming the involvement of RAR nuclear receptors (Figure 7 C) .



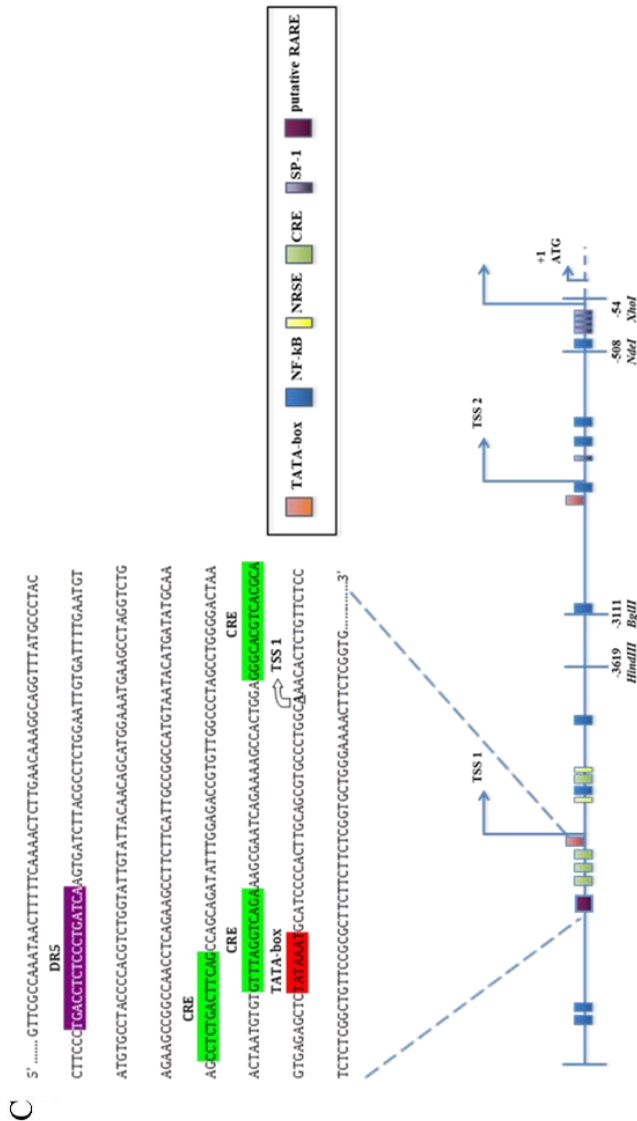


Figure 7 A) Schematic representation of the GDNF promoter with the RARE putative site (modified by Lamberti & Vicini., 2014) **B)** Effect of treatment with retinoic acid on the luciferase activity carried by wild-type and mut-DR5 RARE constructs transiently transfected in Sertoli murine cells. Luciferase activity was

expressed as Relative Light Units (RLU) and normalized for micrograms of cell lysates proteins. In the graph, the histograms show a representative experiment expressed as mean \pm SD. C) Schematic representation of the GDNF promoter with the RARE putative site (modified by Lamberti et al., 2014)

8. RAR α binds on Gdnf promoter

To directly demonstrate RAR α occupancy on the putative DR5 RARE, we performed ChIP-qPCR experiments.

Sertoli cells were cultured with or without RA for 2 hours, and the enrichment mediated by the RAR α antibody was measured to evaluate the recruitment at the DR5 RARE (Figure 8). The results demonstrate that both in basal and RA treatment conditions RAR α occupy the DR5 RARE.

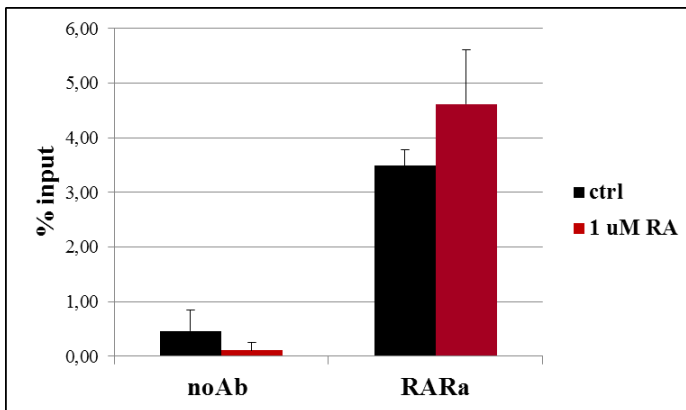


Figure 8) Real-time PCR amplification of the promoter region of Gdnf in the DNA derived from Sertoli cells untreated or treated with 1 μ M RA for 2 hours, immune-precipitated using the RAR α antibodies or with beads without antibody as negative control (noAb). Data are reported as increase relative to the percentage of input.

9. RA-dependent *gdnf* inhibition is prevented by HDACi

The RA classical genomic effect on target genes transcription is more often stimulatory. In the few documented cases of direct genomic repressive action mediated by RA, recruitment of HDACs enzymes seems to be involved (Cunningham & Duester, 2015).

We then tested the hypothesis of the involvement of this class of enzymes in RA mechanism of action on the *Gdnf* gene. To this purpose, we tested the effect of two different inhibitors of class I and II histone deacetylases (HDACs), SAHA and Valproic acid (VPA) (Dokmanovic *et al.*, 2007).

Sertoli cells were treated for 2 hour with 1,25 μ M SAHA or 1 mM VPA in the presence or absence of 1 μ M RA. The expression of the transcript of *gdnf* was analyzed by Real-Time PCR.

The treatment with SAHA significantly reversed the RA-mediated inhibitory effect on *gdnf* (Figure 9 A). The treatment with VPA also had the effect to inhibit *gdnf* basal levels and the inhibitory effect of RA was no longer appreciable (Figure 9 B). These data suggested that histone deacetylases could be involved in this regulation.

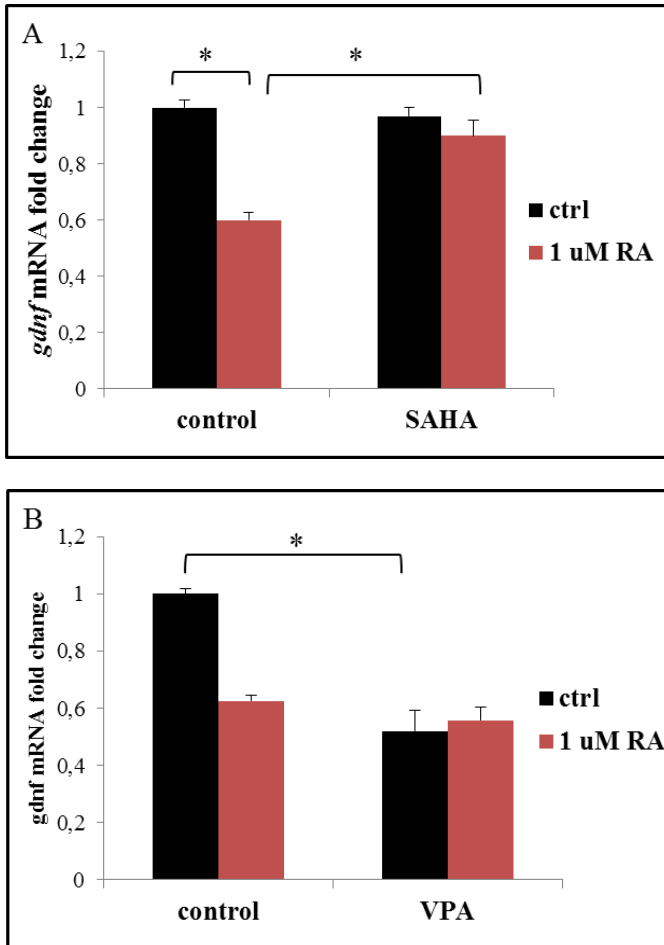


Figure 9) Effect of HDACi on the transcription levels of *gdnf*, in the presence or absence of RA, measured by Real-Time PCR. **A)** Effect of SAHA and **B)** Valproic Acid administration .

Values are expressed as mean \pm SEM of 3 independent experiments * $p < 0.05$ with Dunn's Method.

10. RA-dependent *gdnf* inhibition is based on promoter de-acetylation

The previously obtained results suggest the presence of an epigenetic mechanism at the base of the RA negative regulation on the *Gdnf* promoter.

To directly verify this hypothesis, we performed ChIP-qPCR experiments to evaluate the acetylation levels of histone H4 in a 105 bp region straddling the transcriptional start site 1 (TSS1) of the *Gdnf* promoter, in the presence or absence of RA.

Sertoli cells were cultured with or without RA for 2 hours, and at the end the enrichment mediated by the anti-H4 acetylated antibody (H4Ac) was measured to evaluate the acetylation status of a promoter fragment around the 105 bp long (Figure 10). The results demonstrate that RA treatment induces a significant reduction of histone H4 acetylation levels in the region around the TSS1, suggesting that the RA-mediated negative regulation is based on epigenetic mechanisms.

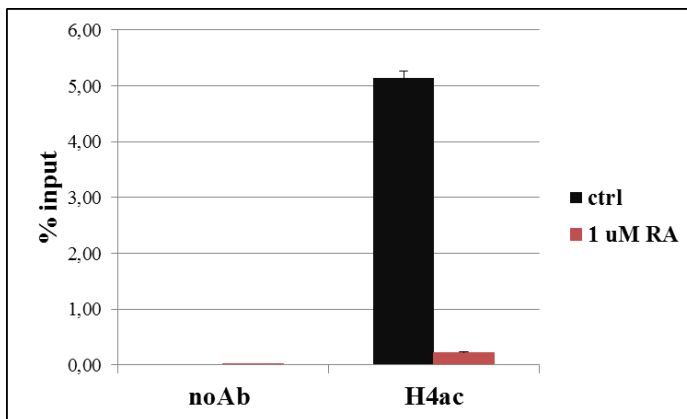
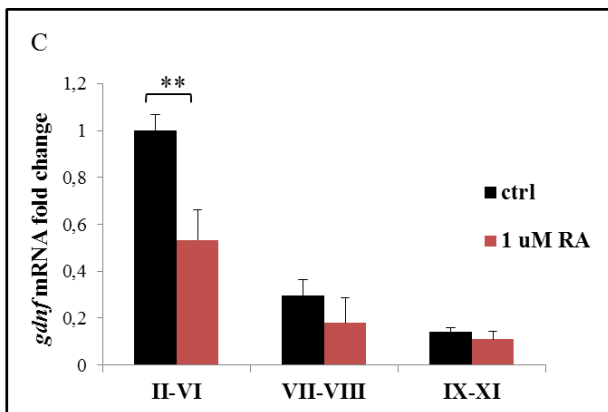
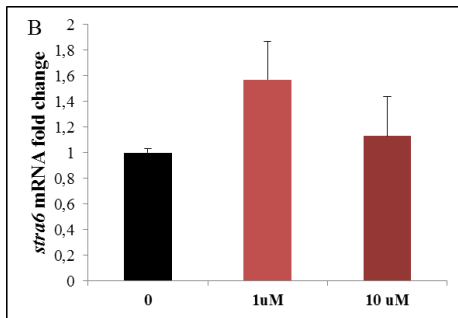
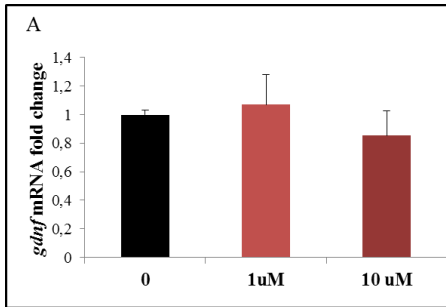


Figure 10 Real-time PCR amplification of the promoter region of *Gdnf* in the DNA derived from Sertoli cells untreated or treated with 1 μ M RA for 2 hours, immune-precipitated using the anti-H4-Ac antibodies or with beads without antibody as negative control (noAb). Data are reported as increase relative to the percentage of input.

11. *gdnf* RA-mediated regulation is conserved in adult mouse and is stage-dependent

To assess whether RA-mediated *gdnf* regulation was conserved in the sexually mature mouse, and since it is impossible to obtain pure Sertoli cultures from the adult testis, we cultured for 8 hours 1 mm³ fragments of testicular tissue obtained from 3 month old mice in presence of 1 or 10 μ M RA. Subsequently *gdnf* and *stra6* mRNA levels were evaluated by Real-Time PCR. However, in those culture conditions, neither of the two RA concentrations lowered *gdnf* mRNA levels (Figure 11 A) or significantly affected *stra6* mRNA (Figure 11 B).

Because in adult testis the concomitant presence of multiple germ cell associations and cellular subtypes could hide a subtle gene regulation, we decided to put in culture isolated and staged seminiferous tubules from 3 month old CD1 mice. Seminiferous tubules were divided into three groups of stages (stages II-VI, VII-VIII and IX-XI) and for each group 3 cm total length were isolated. Tubules were kept in culture for 4 hours, in the presence or absence of 1 μ M RA, and mRNA levels assessed by Real-Time PCR. In line with data already published by our laboratory, the *gdnf* baseline levels were found oscillating, high in stages II-VI, and lowered in stages VII-VIII and IX-XI (Grasso *et al.*, 2012). The negative regulation mediated by RA was significant detected only in stages II-VI (Figure 11 C). We measured *stra6* levels which were also showily stage-dependent, lower at stages II-VI and IX-XI and very high at stages VII-VIII. Again, RA stimulation induced *stra6* expression only in stages II-VI (Figure 11 D). These data suggest that the RA-mediated regulation of *gdnf* and *stra6* transcripts are detectable in isolated seminiferous tubules from adult testis.



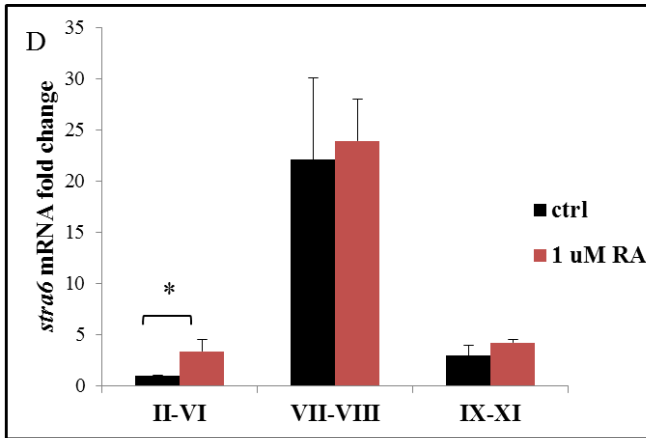


Figure 11) Histograms show the regulation in murine testicular organ culture of *gdnf* transcript **A**), and *strab6* transcript **B**) after 8 hours treatment with 1 or 10 RA. mRNA levels were measured by Real-Time PCR, normalized on β -actin and expressed in fold change with respect to the control.

Expression of *gdnf* **C**) *strab6* **D**) in staged seminiferous tubules cultured for 4 hours in the presence or absence of RA. The mRNA levels were evaluated by Real-Time PCR, normalized on β -actin levels and expressed as fold changes on the II-VI stage controls. Data are represented as means \pm SEM of three experiments. The statistical analysis used the T test to compare the RA treatment with respect to its control, for each group of stages. * $p < 0.05$ ** $p < 0.01$

12. RA could act as an indirect regulator of *gdnf*

We then asked if RA could exert an indirect effect on *gdnf* cyclical regulation in the seminiferous epithelium. Because the expression profile of *gdnf* during the cycle of the seminiferous epithelium parallels the profile of the FSH response in rat, which is high in stages XIII–V and decreases subsequently (Parvinen *et al.*, 1980; Parvinen, 1982), we asked if the variations in FSH response, which contributes to *gdnf* levels oscillations, were linked to RA. With this aim, we examined FSH receptor (*fshR*) mRNA expression in primary Sertoli cells (Figure 12 A) treated for 24 hours with RA, and we found lowered FshR mRNA levels. Then, we evaluated the same effect on staged seminiferous tubules, with or without 4 hours

1 μ M RA stimulation. We found that *fshR* basal levels higher at stages II-VI and lower at VII-VIII and IX-XI stages, confirming similar evidences in literature (Heckert & Griswold, 1993) and that the addition of 1 μ M RA significantly reduced *fshR* mRNA at stages II-VI but not in other stages.

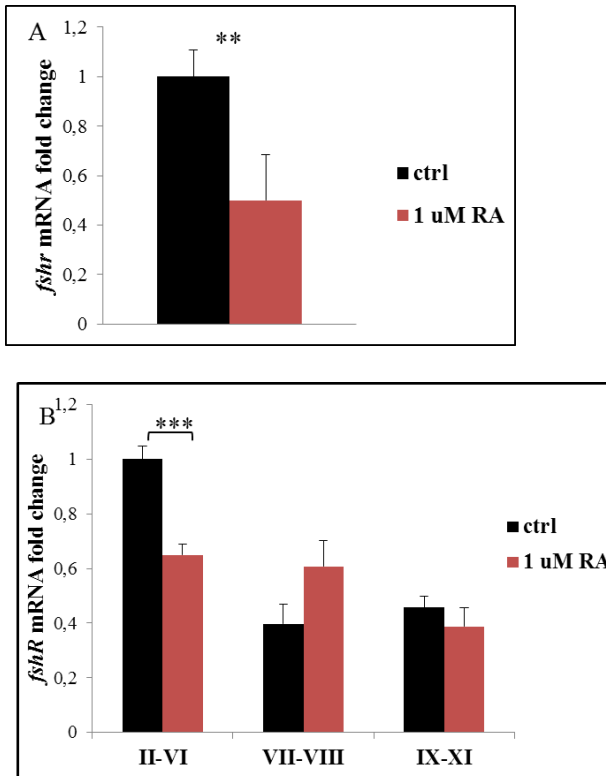


Figure 12 A) Effect of 24 hours treatment with 1 μ M RA on *fshR* mRNA in primary Sertoli cells. Data are expressed as mean \pm SEM of two cultures and The mRNA levels were evaluated by Real-Time PCR, normalized on β -actin levels and expressed as fold change on the untreated. The statistical analysis used the T test to compare the RA treatment with respect to its control $**p < 0.01$ B) Expression of *fshR* in staged seminiferous tubules cultured for 4 hours in the presence or absence of RA. The mRNA levels were evaluated by Real-Time PCR, normalized on β -actin levels and expressed as fold change on the II-VI stage controls. Data are represented as means \pm SEM of three experiments. The statistical analysis

used the *T* test to compare the RA treatment with respect to its control, for each group of stages. *** $p < 0.001$

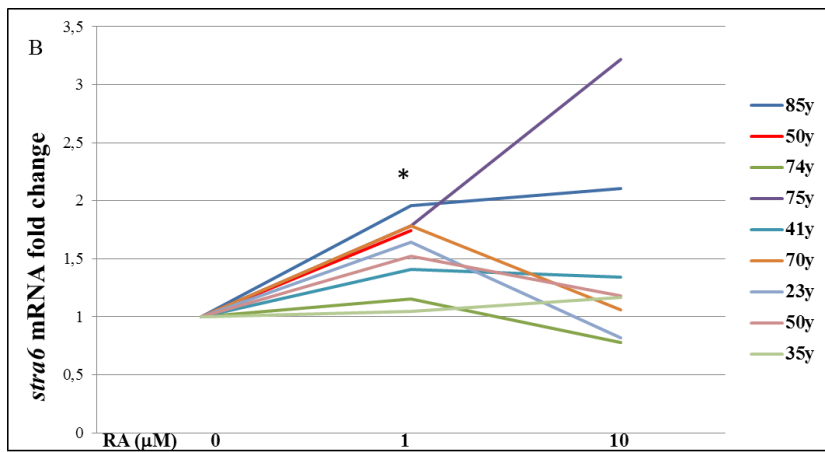
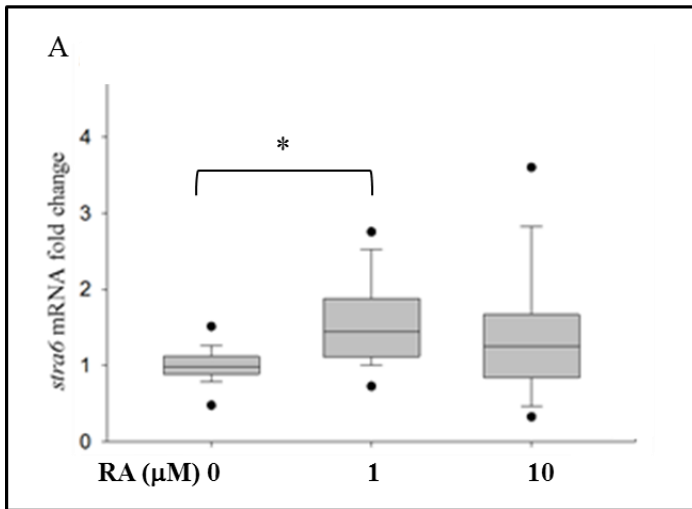
13. Gdnf RA-dependent regulation in human testis

We next asked whether RA could be a regulator of *gdnf* also in human testis.

With this aim, we prepared human testicular organ cultures according to the “hanging drop” protocol (Jørgensen *et al.*, 2014). Fragments from testicular biopsies obtained from heart-beating organ donors (19y-85y) were cultured for 8 hours in α MEM medium containing 1 or 10 μ M RA.

Sertoli cell responsivity to RA was assessed measuring *stra6* induction by Real-Time PCR. *Stra6* was found up-regulated in a significant manner at 1 μ M RA (Figure 13 A and B).

Overall, *gdnf* expression was not affected by RA treatment at both 1 and 10 μ M (Figure 13 C). However, analysis of single donors revealed that a negative trend was appreciable in 6 out of 10 donors while 4 donors exhibited an opposite behavior with a strong up-regulation of *gdnf* (Figure 13 D). Taken together these results, indicate that RA-mediated *gdnf* regulation in human testis is heterogeneous.



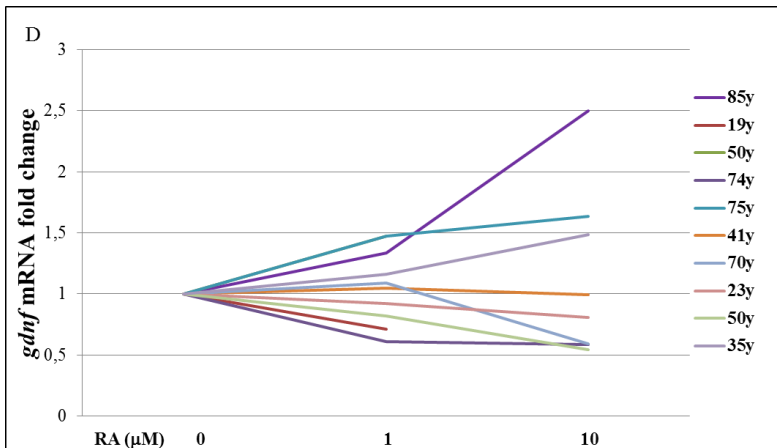
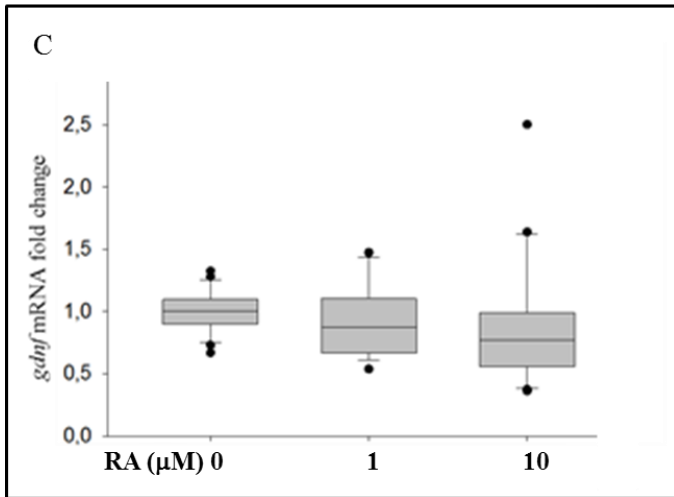


Figure 13 The graphs show boxplot (A, C) or single donors behavior (B, D) about the expression of *stra6* (A, B) and *gdnf* mRNA (C, D) in human testicular hanging drop cultures. The mRNA levels were evaluated by Real-Time PCR, normalized on *rps29* mRNA levels and expressed as fold change on the controls. Data are represented as medians \pm SEM. * $p < 0.05$ with Dunn's Method.

DISCUSSION

GDNF and RA are two crucial molecules for spermatogonial compartment. The first, secreted by somatic Sertoli cells, stimulates SSCs stemness, the second, released within the testicular microenvironment, stimulates spermatogonial progression. Both molecules are known to be crucial for spermatogenesis: ablation of GDNF and depletion of intra testicular RA led to spermatogenesis disruption (Meng *et al.*, 2000; Wolbach & Howe, 1925).

In murine testis, a negative regulation of GDNF by RA was recently described by Hasegawa and colleagues but the mechanisms behind were not clear (Hasegawa *et al.*, 2012). With the aim to understand the molecular mechanism underlying this regulation, we used murine primary Sertoli cell cultures as a model and firstly we confirmed the down-regulation of *gdnf* following 1 μ M RA, which is the concentration commonly used in literature for testicular treatments (Childs *et al.*, 2011; Jørgensen *et al.*, 2015). We demonstrate that RA-mediated *gdnf* regulation takes 2 hours and affects both mRNA and secreted protein levels. Protein synthesis was found dispensable for RA-mediated *gdnf* regulation but blocking ongoing transcription with ActD lowered *gdnf* mRNA by 80%. This suggests that *gdnf* transcripts have a short half-life, a feature that may contribute to *gdnf* transcripts oscillation during the 8,6 days of the cycle of the seminiferous epithelium (Grasso *et al.*, 2012; Sharma & Braun, 2018).

Immature and adult Sertoli cells express RAR α and RXR β , therefore we hypothesized an involvement of RAR α in the regulation of *gdnf* (Vernet *et al.*, 2006). Here we show that ablation of RAR α , performed both with a selective pharmacological antagonist and with a specific designed siRNA, was able to completely revert RA-mediated negative regulation. This data are in line with the observation that in vivo overexpression of a dominant negative form of RAR α reverts RA-negative *gdnf* regulation (Hasegawa *et al.*, 2013).

The murine *Gdnf* gene has three alternative promoters, among which, promoter 1 upstream of exon 1 is the only active in Sertoli cells. In promoter 1, the TSS1 is located approximately 30 bp

downstream from the canonical TATA-box sequence and approximately 900 bp upstream from the putative initiation of translation (Lamberti & Vicini, 2014). In Sertoli cells transiently transfected with the 1,7 kb 5' flanking-region of TSS1, a significant reduction of luciferase activity over basal conditions was measured in RA-treated cells. This was consistent with the presence of a retinoic responsive element (RARE) upstream of TSS1 and prompt us to search for RARE by in silico analysis. A novel DR5-RARE (5'-TGACCTCTCCCTGATCA-3') was indeed identified located 307 bp upstream of TSS1 (Lamberti & Vicini, 2014). Two line of evidence strongly suggest that DR5-RARE is involved in RA-modulation of *gdnf* expression level: i) deletion of DR5-RARE in the 1,7 kb 5' flanking-region of TSS1 completely reverted the RA-mediate inhibition of luciferase activity; ii) DR5-RARE is occupied by RAR α both in control and RA-treated cells as measured by Chip-qPCR analysis.

While the RAREs-mediated transcriptional activation is well established (Germain *et al.*, 2002), the mechanism by which RA represses transcription on the promoter of target genes is less known. The little information available on this type of regulation is based on few evidences, among whom studies on Fgf8, in which action of RA causes repressive changes around RARE on the promoter (Kumar & Duester, 2014). This prompted us to evaluate epigenetic mechanisms. Because a repressive action of RA with recruitment of HDAC complexes has been reported in literature, we investigated the effect on RA-mediated *gdnf* regulation of two pharmacological compounds, SAHA (Vorinostat) and Valproic Acid, which respectively inhibit class I and II, and class I and IIa HDAC enzymes (Dokmanovic *et al.*, 2007). In the presence of both drugs, RA effect on *gdnf* was no more evident. Reduced acetylation on *Gdnf* promoter was directly confirmed by ChIP-qPCR experiments showing that RA is able to modify the epigenetic profile of the TSS1 region, inducing de-acetylation of histone H4 with respect to the basal condition. This is in line with the model for RA-mediated gene repression proposed by Cunningham and Duester which predicts that, in the presence of RA, RARs recruits co-factors such as NCOR1 and NCOR2, PRC2 and HDAC complexes on the promoter of target genes (Cunningham &

Duester, 2015; Kumar *et al.*, 2016). Subsequent experiments of Chip-qPCR will be used to test this model for the recruitment of corepressors on the *Gdnf* promoter in the presence of RA.

To evaluate whether RA-mediated *gdnf* modulation is also conserved in the adult testis, and because it is not possible to obtain pure Sertoli cell culture from adult testis, we used in vitro testis organ cultures. In organ cultures, direct and indirect interactions between somatic testicular cells and germ cells are maintained, mimicking the in vivo situation. However, in these experimental conditions we did not find a significant *gdnf* modulation upon RA treatment probably due to a catabolic barrier preventing RA from entering the seminiferous tubules (Vernet *et al.*, 2006b). Therefore we employed a different in vitro model i.e., staged seminiferous tubules from adult mouse (Grasso *et al.*, 2012). In line with previous data, we here show that basal *gdnf* levels are cyclical, higher at stages II-VI and lower at VII-VIII and IX-XI (Grasso *et al.*, 2012). RA treatment significantly reduced *gdnf* transcription at stages II-VI, but not at VII-VIII and IX-XI. Interestingly, also RA-mediated *stra6* induction occurred only at stages II-VI. These results suggest that RA-mediated *gdnf* regulation is conserved in adult testis but is detected only in stages where the endogenous levels of RA are low (i.e II-VI) and not in stages VII-VIII where the endogenous levels of RA are maximal. However, RA did not mediate further gene regulation also at stages IX-XI. The absence of RA response may suggest that the RA refractory effect could continue until stage XI. This is in line with Hasegawa and Saga (Hasegawa & Saga, 2012), also supported by Page (Endo *et al.*, 2015), that assumes that RA levels grow at stages VII and keep high until stage XII.

Our data point to a direct regulation of RA of the *Gdnf* promoter through the DR5-RARE and epigenetic modification. We also asked if RA could indirectly control cyclical *gdnf* expression by antagonizing the *gdnf* “supporting” pathways in Sertoli cells. *Gdnf* expression in Sertoli cells is activated by MEK/ERK signaling, but MEK/ERK and RA pathways were shown to be independent from each other (Hasegawa & Saga, 2013). As FSH is an important *gdnf* stimulating factors both in vitro and in vivo, we asked if RA could antagonize FSH (Takodoro *et al.*, 2002; Grasso *et al.*, 2012; Lambert

& Vicini, 2014). We showed that in vitro, RA down-regulate FSH receptor mRNA both in immature and in adult testis. In particular, we found a cyclical expression of *fshR* mRNA, with a minimum at stages VII-VIII, confirming similar evidences in rat (Heckert & Griswold, 1993). These data indicate that RA may also indirectly affect *gdnf* levels by reducing FSH receptor levels.

Respect to the rodents model, the availability of human testicular biopsies for research purposes is extremely limited. For this reason, the understanding of spermatogenesis is fundamentally based on rodents and other primates studies. Even though the pivotal role for GDNF in rodent spermatogenesis is largely recognized, nothing is known about the role of the GDNF/GFRA1/Ret pathway in the human spermatogonial compartment. In human testis, the source of GDNF comprises Sertoli cells (Davidoff *et al.*, 2001) and myoid peritubular cells (Spinnler *et al.*, 2010, Chen *et al.*, 2014; Chen *et al.*, 2016). Moreover, a large subset of human undifferentiated spermatogonia, including the most primitive spermatogonia, express the GDNF co-receptor GFRA1, implying a possible physiological role of GDNF also in human spermatogenesis (Grisanti *et al.*, 2009; Di Persio *et al.*, 2017). Recent data from NOA and SCO patients suggest that GDNF in the human testis may a function similar to that of rodents (Ma *et al.*, 2013; Singh *et al.*, 2017). Regarding the role of Vitamin A in human testis, very little is known (Hogarth *et al.*, 2011). However, RA seems to play a critical role in human spermatogenesis, since impinging testicular RA biosynthesis by inhibiting ALDH1a2 enzyme, reversibly blocks spermatogenesis in men (Heller *et al.*, 1961). Here we investigated the *gdnf* regulation in human testis by in hanging drop cultures. RA treatment induces a significant up-regulation of *stra6* mRNA indicating that testicular cells are responsive to RA. On the contrary, there was no statistical significance of the effect of RA on *gdnf* expression. Interestingly, out of 10 samples, a negative trend was appreciable in six donors, while *gdnf* was up-regulated in four donors, indicating an heterogeneous response. The physiological function of GDNF and RA in human spermatogenesis certainly deserves further investigation.

To conclude, our results can ameliorate the comprehension of the SSCs niche functioning and its complex molecular network. Our

study is also the basis for further studies concerning the niche of SSCs in its physiological conditions but also in relation to the ever increasing number of cases of male infertility in which its balance could be compromised. Moreover can shed light on the RA-mediated gene repression of target genes.

MATERIALS AND METHODS

Primary culture of murine Sertoli cells

Primary cultures of Sertoli cells were obtained from 17-day CD1 mice, as previously reported (Vicini & Conti, 1997). Isolated testes were washed twice with Hank's solution (Sigma) and the tunica albuginea was removed. Subsequently testes were chopped, transferred to a Hank's solution containing 0.002% DNase I (Roche), 0.25% Trypsine (Difco) and agitated at 32 ° C for 13 minutes. The digestion was blocked by adding 10% fetal bovine serum. The seminiferous tubules were deposited for 4 minutes at unit gravity and washed twice with Hank's solution. The second digestion was performed with a solution of Hank's, 0.002% DNase I (Roche) and 0.2% of Collagenase A (Roche) at 32 ° C for 20 minutes, with agitation. At the end, the fragmented tubules were deposited at unit gravity, washed with Hank's solution, collected in a polystyrene tube and subjected to centrifugation at 18 g for 2 minutes. The pellet was then resuspended in MEM 1X (Minimum Essential Medium) (Gibco), containing 20 mM glutamine, HEPES 0.01 M, non-essential 0.1 mM amino acids and antibiotics.

The suspension with the fragments of seminiferous tubules is plated in 10 mm plates (9 ml of suspension) or in multiwell plates (6 wells) of 35 mm of diameter (1.5 ml of well suspension) and maintained at 34° C in a controlled atmosphere of 5% CO₂.

With this procedure, a monolayer is formed consisting exclusively of Sertoli cells and germ cells. To remove the residual germ cells, after 48 hours a hypotonic solution (TrisHCL 20mM pH 7.4) was administered to selectively eliminate germ cells (Galdieri & Zani, 1981). Sertoli cells can then be used after 24 hours from hypotonic treatment.

Sertoli cells treatments

24 hours after hypotonic treatment, Sertoli cells are treated at 34° C 5% CO₂ with RA (Sigma-Aldrich, Milan, Italy); ActD (5 µg / ml) (Sigma-Aldrich, Milan, Italy); CHX (10 µg / ml) (Sigma-Aldrich, Milan, Italy); BMS-204493 (Sigma-Aldrich, Milan, Italy); RO41-

5253 (Santa Cruz Biotechnology); Valproic acid (Sigma-Aldrich, Milan, Italy); SAHA (Sigma-Aldrich, Milan, Italy).

Testicular organ culture

17-day CD1 mice or adult CD1 mice (3 months) were sacrificed, testes were taken, deprived of tunica albuginea and transferred to a plate with PBS. Testicular fragments of 1 mm³ were cut and placed on steel grids coated with 2% agar in organ culture dishes and maintained at 34 °C in a controlled atmosphere of 5% CO₂. PBS was added to the bottom of the dish to maintain fragments hydration.

Seminiferous tubules isolation

Adult CD1 mice (3 months) were sacrificed, testes were taken, deprived of tunica albuginea and transferred to a plate with PBS. At the stereomicroscope, with 25-50X enlargement, tubules were dissected and stages of the seminiferous epithelium identified through the different pattern of light absorption by transillumination (Kotaja *et al.*, 2004). The greatest differences in light absorption are due to the presence and disposition of spermatids. Three groups were isolated: low-density spermatids characterize a pale area which include the IX-XI stages; a greater number of spermatids located internally to Sertoli cells represents the dark spot area in stages II-VI; in the last stages of maturation, the spermatids form a continuous layer above the seminiferous epithelium which gives rise to a dark absorption pattern (dark area) in the VII-VIII stages (Figure 14).

For each group, 3 cm total length were measured, collected and cultured for 4 hours with MEM 1X (Gibco) medium with or without 1 µM RA, for subsequent mRNA extraction.

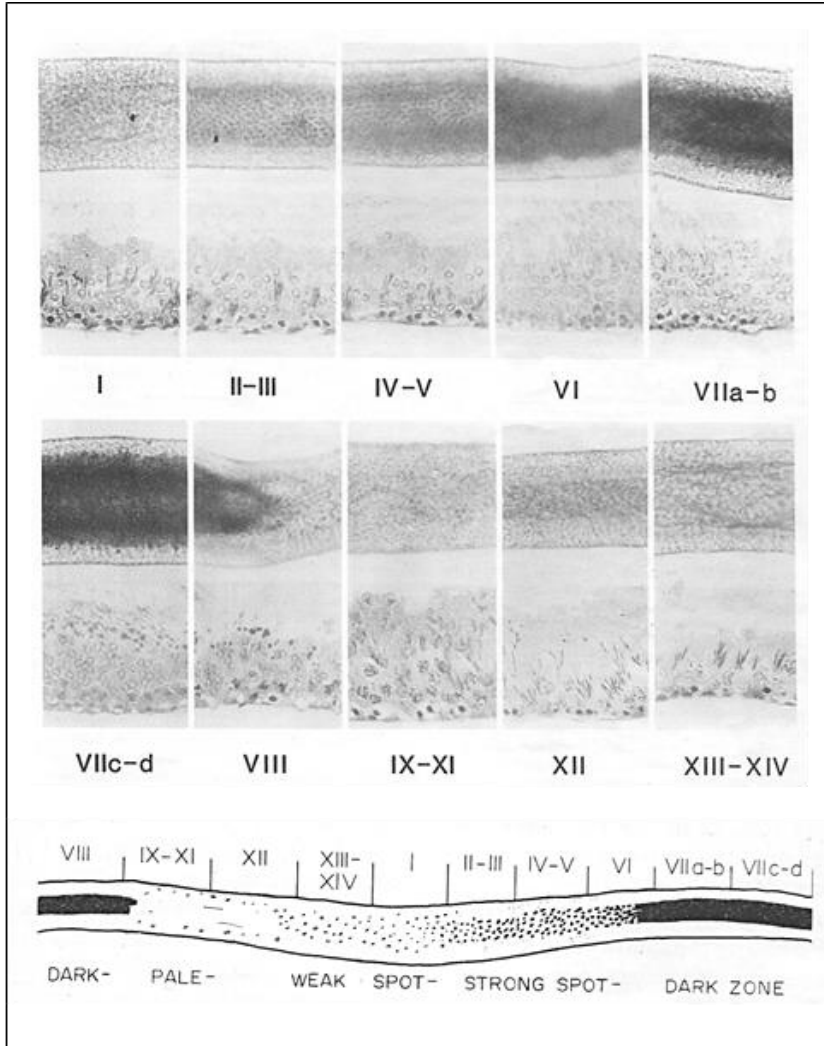


Figure 14 Absorbance pattern of stages II-VI, VII-VIII e IX-XI (Pelletier, 2011)

RNA extraction and Real-Time PCR

Total RNA extraction was performed using the TRIzol reagent (Invitrogen) following the manufacturer's instructions.

The obtained RNA was quantified by spectrophotometer (Nanodrop Microvolume Quantitation of Nucleic Acids).

The cDNA synthesis, from 1 µg of RNA for each sample, was performed using the High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific, Waltham, MA, 02451 USA) in a thermal cycler for 60 minutes at 37°C followed by 5 minutes at 95°C for the enzyme inactivation. A control sample, without the reverse transcriptase, was used to evaluate the presence of genomic DNA.

The cDNA was subjected to Real-Time PCR analysis performed in duplicate for each sample with FluoCycle™ II SYBR Green Mix (Euroclone) in 7500 Real-Time PCR System (Applied Biosystems, Life Technologies, Monza, Italy).

Data were analyzed with the comparative $2^{-\Delta\Delta CT}$ method using β -actin for mouse and *rps29* for human as reference genes and control conditions as calibrator.

Primers sequences, designed using Primer BLAST software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>), are listed in Table 1.

gene	primers
<i>gdnf</i> (<i>mus musculus</i>) Fw	TTGCATTCCTGCTACAGTGC
<i>gdnf</i> (<i>mus musculus</i>) Rev	GGTGGCTACCTTCCTCCTTC
<i>stra6</i> (<i>mus musculus</i>) Fw	ACACTCCACAGCCAGGATTC
<i>stra6</i> (<i>mus musculus</i>) Rev	ACATCTGTGTTGATCCCCGC
<i>rara</i> (<i>mus musculus</i>) Fw	GATCACAGACCTTCGGAGCACGA
<i>rara</i> (<i>mus musculus</i>) Rev	CGACTGTCCGCTTAGAGTGT
<i>fshR</i> (<i>mus musculus</i>) Fw	ACAGGGTCTTCCTCTGCCAA
<i>fshR</i> (<i>mus musculus</i>) Rev	TTTCTCCAGGTCCCCAAATCC
β -actin (<i>mus musculus</i>) Fw	TGACAGGATGCAGAAGGAGA
β -actin (<i>mus musculus</i>) Rev	GTACTTGCCTCAGGAGGAG
<i>gdnf</i> (human) Fw	GCAGACCCATCGCCTTTGAT
<i>gdnf</i> (human) Rev	CCACACCTTTTAGCGGAATGC

stra6 (human) Fw	GCTTCAGCCGTAGGACAGGA
stra6 (human) Rev	AGCTTCTTCCTGCAAAGGAGGT
rps29 (human) Fw	CGCTCTTGTCGTGTCTGTTC
rps29 (human) Rev	CCTTCGCGTACTGACGGAAA

Table 1 : list of the primers used for Real-Time PCR

Short interference RNA (siRNA)

To interfere the RAR α protein, a specific siRNA was designed with the INVIVOGEN software (5'-GGAAGCACUGAAAGUCUACGU-3' strand + 5'-ACGUAGACUUUCAGUGCUUCC-3' strand -)

Sertoli cells after the hypotonic treatment were grown in the absence of antibiotics and transfected for 5 hours with 5 μ l / well Lipofectamine 2000 (Invitrogen) and 250 picomoles of the anti RAR α siRNA or MISSION siRNA Universal Negative control (Sigma) in Opti-MEM medium (Invitrogen). At the end of the treatment, medium was removed and MEM 1X (Gibco) was added for the following 24 hours in the presence or absence of 1 μ M RA.

Plasmid mutation

To obtain internal deletion of the GDNF genomic promoter, the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Milano, Italy) was employed. Briefly, we performed a PCR associated technique to create deletion mutants of the construct using primers carrying the appropriate deletions of the region of interest, following the manufacturer's directions (Agilent Technologies, Milano, Italy). Primer couples (forward and reverse) were designed to keep the mutation in the middle of the primer. After PCR extension, the reaction was used to transform XL-10 gold bacteria. Several bacterial colonies were selected and sequenced to evaluate introduction of the desired mutation.

Primers used for mutagenesis on Gdnf promoter are listed in table 2.

Primers	
forward	GGCAGGTTTATGCCCTACCTTCCCAGTGATCTTACGCCTCTGG
reverse	CCAGAGGCGTAAGATCACTGGGAAGGTAGGGCATAAACCTGCC

Table 2: primers used for the plasmid mutation.

Luciferase assay and protein normalization

Sertoli cells after the hypotonic treatment were grown in MEM 1X medium (Gibco) in the absence of antibiotics for 24 hours. The next day cells were transfected using Lipofectamine 2000 (Invitrogen) and 1,5 µg / well of the control plasmids without a promoter (Pgl2-luciferase), wild type (-1857 / + 109-luciferase) or with the RARE DR5 site mutated (mut DR5: -1857 / + 109-luciferase) and cultured in Opti-MEM medium (Invitrogen). After 5 hours, the medium was removed and the cells grown with MEM 1X (Gibco) with or without 1µM RA for following 24 hours. At the end cells are washed with PBS and lysed in 1x Passive Lysis Buffer (Promega) and luciferase activity was measured with Luciferase Assay System (Promega) at the GloMax Discover luminometer (Promega). The activity was expressed in relative luminescence units (RLU) and normalized for µg of proteins, quantified with BCA assay (Thermo Scientific).

ChiP assay

Sertoli cell cultures were treated for 2 hours in the presence or absence of 1µM RA, then cross-linked with 1% formaldehyde (Sigma). After two lysis steps, the cell chromatin was sonicated to obtain fragments of about 800-500 bp. The size of the fragment was evaluated by electrophoretic run on 1% agarose gel. Subsequently the chromatin was diluted in a chromatin dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl). Sonicated chromatin was immunoprecipitated

with antibodies (4-5 µg of antibody in 40 µl of beads) anti-H4-Ac (Millipore 06-866) and (8 µg of antibody in 40 µl of beads) anti RARα (Santa Cruz sc-551), over night at 4° C. The immune complexes were collected by incubation with magnetic beads (Dynabeads™ M-280 Sheep anti-Rabbit IgG, Invitrogen) for 4 hours at 4° C and then subjected to 3 different washes: 4 times low salt concentration buffer (0.1% SDS, 1% TritonX-100, 2mM EDTA, 20mM TrisHCl, pH 8.1, 150mM NaCl); 3 times high salt concentration buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl); once Tris / EDTA buffer. and finally were eluted with elution buffer (1% SDS, 0.1 M NaHCO3) for 30 minutes at 37° C. DNA was reverse cross-linked with 0,2 M NaCl and recovered with the phenol / chloroform protocol followed by ethanol precipitation.

The extracted DNA was amplified by Real-Time PCR with LR1 primers, targeting 105 bp across the TSS1 GDNF; with Forward and Reverse RARE primers, which amplify 142 bp across DR5 RARE on the GDNF promoter; with LR3 primers which amplify 142 bp in the first GDNF intron, as a negative control. Real-time PCR was performed with FluoCycle™II SYBR Green Mix (Euroclone).

For data analysis the 'the percentage of input' method was used (<http://www.thermofisher.com/en/en/home/life-science/epigenetics-noncodingrna-research/chromatin-remodeling/chromatin-immunoprecipitation-chip/chip-analysis.html>).

Primers name	sequences
L1	GAAAAGCCACTGGAGGGCAC
R1	GCGGAACAGCCGAGAGAGGA
Forward RARE	GTCCTTGCTTAGGTTCCCA
Reverse RARE	CGTGGGGTAGGCACATACAT
L3	GAGGTGTAGCCTGGGTGTTC
R3	TTTGTTGCTAAACGGCTGC

Table 3: primers used for ChiP assay

Human organ cultures

All the experiments were performed on surgical testicular biopsies obtained from heart-beating organ donors, from the hospital Policlinico Umberto I of Rome. Donors were aged between 19 and 85 years and for each of them the free and informed consent of the family was obtained.

The Ethical Committee of the hospital approved the use of human material for our study, according to national guidelines for organ donation as issued by the Italian Ministry of Public Health.

Hanging drop culture was performed as described by Jørgensen (Jørgensen *et al.*, 2014). The testicular biopsies were cut into 2 mm³ fragments and then each fragment was placed into a drop of medium, positioned on the lid of the plate, containing DMEM (Sigma-Aldrich, Milan, Italy) with penicillin (100U/ml)/streptomycin (100 mg/ml) (Sigma-Aldrich, Milan, Italy), insulin (25µg/ml) (Sigma-Aldrich, Milan, Italy), apotransferrin (100µg/ml) (Sigma-Aldrich, Milan, Italy), sodium selenite (30nM) (Sigma-Aldrich, Milan, Italy) and 0.1% BSA. PBS was added to the bottom of the dish to maintain fragments hydration. Tissues were cultured at 34° C in 5% CO₂ with or without 1 or 10 µM RA for 8 hours. At the end of the culture fragments were washed in PBS and lysed with TRIzol (Sigma-Aldrich, Milan, Italy) for the RNA extraction. For all donors each condition was performed in duplicate and the RNA was isolated from ten fragments pulled together.

Statistical analysis

Statistical analysis was performed using the Sigma Plot 11.0 program. Quantitative data are shown as the mean ± standard error of the mean (SEM) or percentile plots for human data. To define the significance of the differences between two groups, data were analyzed using a t-test. To compare many groups, data were analyzed using a one-way analysis of variance (ANOVA) followed by a post hoc Dunn's method. The significance level was fixed at $\alpha = 0.05$.

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