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Regulation of GDNF expression in Sertoli cells

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Abstract

Sertoli cells regulate male germ cell proliferation and differentiation and are a critical component of the spermatogonial stem cell (SSC) niche, where homeostasis is maintained by the interplay of several signaling pathways and growth factors. These factors are secreted by Sertoli cells located within the seminiferous epithelium, and by interstitial cells residing between the seminiferous tubules. Sertoli cells and peritubular myoid cells produce glial cell line-derived neurotrophic factor (GDNF), which binds to the RET/GFRA1 receptor complex at the surface of undifferentiated spermatogonia. GDNF is known for its ability to drive SSC self-renewal and proliferation of their direct cell progeny. Even though the effects of GDNF are well studied, our understanding of the regulation its expression is still limited. The purpose of this review is to discuss how GDNF expression in Sertoli cells is modulated within the niche, and how these mechanisms impact germ cell homeostasis.

Introduction:

Proper regulation of stem cell fate is critical to maintain adequate cell numbers in health and diseases. Evidence suggests that stem cell behavior is regulated by both extracellular signals from their microenvironment, or niche, and intrinsic signals within the cells (Li and Xie 2005). Much work has been recently done to understand how the niche controls stem cell self-renewal and differentiation and how, in turn, stem cells influence their environment (Chacon-Martinez, et al. 2018). The present review focuses on recent findings pertaining to glial cell-line derived neurotrophic factor (GDNF) as one of the major paracrine factors specifically responsible for self-renewal of spermatogonial stem cells (SSCs) within their niche, and proliferation of their direct progeny.

Mammalian sperm production occurs via a highly organized process called spermatogenesis, which is maintained throughout life by a small population of stem cells called spermatogonial stem cells (SSCs). Identifying SSCs and understanding their population dynamics has been a challenging task due to their low numbers (less than 0.03% of adult testicular cells)(Tegelenbosch and de Rooij 1993) and the lack of specific markers allowing the distinction between SSCs and subsets of undifferentiated progenitors (Grisanti, et al.

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Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

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2009, Chan, et al. 2014, Hermann, et al. 2015). Therefore, over the past decades, several models have been proposed that describe the dynamics of the mammalian SSC population. Leblond and Clermont were first to describe in the rat the existence of rarely dividing type A spermatogonia, that they considered reserve stem cells (A_0), coexisting with a population of renewing spermatogonia that they called A_1-A_4 (Clermont and Leblond 1953, Clermont and Bustos-Obregon 1968, Dym and Clermont 1970). The reserve stem cell would be able to repopulate the testis only after X-ray radiation or chemical injury (Dym and Clermont 1970). However, further investigations by Huckins and Oakberg demonstrated substantial radioactive thymidine incorporation in A_0 spermatogonia, indicating their active proliferation (Huckins 1971a, b, Oakberg 1971). Precise cell cycle length evaluation and whole mount preparations subsequently led to the identification of different subsets of A spermatogonia with widely different cell kinetics properties, and to the proposition of a now accepted rodent model where SSCs, also named A_{single} (or A_s) spermatogonia, either self-renew or differentiate to generate two A_{paired} (or A_{pr}) spermatogonia connected by an intercellular bridge (De Rooij 1973, Huckins 1978). These cells further divide to generate chains of 4 $A_{aligned}$ (or A_{al}) spermatogonia. Additional divisions amplify the germ cell population by generating chains of A_{al8} to A_{al16} cells. This step is considered an amplification step that increases the number of progenitors, and A_{single}, A_{paired} and $A_{aligned}$ are often referred to as undifferentiated spermatogonia (Huckins 1971a, Huckins and Oakberg 1978). Under the influence of retinoic acid, $A_{aligned}$ cells differentiate into A_1-A_4 cells, or differentiating spermatogonia, which further divide to become Intermediate spermatogonia, B spermatogonia, and primary spermatocytes. Spermatocytes will undergo meiosis and give rise to haploid spermatids that will progress through spermiogenesis to become spermatozoa (Haneji, et al. 1983, Russell, et al. 1990, van Pelt and de Rooij 1991, Chen, et al. 2016b, Griswold 2016). In human and non-human primates, the SSC population consists in A_{dark} and A_{pale} spermatogonia, distinguished by their size, nuclear morphology, and different intensity of hematoxylin staining (Clermont and Leblond 1959). Incorporation of radioactive thymidine indicated that A_{pale} spermatogonia were more active than A_{dark}, and the latter were also considered reserve stem cells (Clermont 1969). In humans, each A_{pale} divides into two type B spermatogonia, which in turn produce four spermatocytes (Clermont 1966). Recent investigations in the rhesus monkey, however, have shown that A_{dark} and A_{pale} shared similar molecular phenotypes and therefore might belong to the same population of A_{single} cells, albeit at different stages of the cell cycle (Hermann, et al. 2009).

While the A_{single} model of spermatogenesis in rodents and primates prevailed for decades, a novel “fragmentation” model was recently proposed in mice, whereby A_{paired} and $A_{aligned}$ spermatogonia can detach from the cellular doublets and chains and revert from a transit amplifying mode to a self-renewal mode (Nakagawa, et al. 2007, Klein, et al. 2010, Nakagawa, et al. 2010). This latter model, devised following lineage tracing and live imaging, indicates that A_{paired} and $A_{aligned}$ spermatogonia conserve some levels of plasticity, and are therefore not irreversibly committed to differentiation and meiosis, as previously thought. However, proliferation of undifferentiated spermatogonia after fragmentation is very slow (Hara, et al. 2014), and cannot produce the number of differentiating spermatogonia necessary to sustain the steady state of spermatogenesis. It might be because the *in vivo* imaging experiments used to observe chain fragmentation imposed stressful

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physiological conditions, or that only specific stages of the seminiferous epithelium cycle were visualized, where the proliferative activity of the cells is normally low (de Rooij 2017). It is now postulated that the A_{single} model prevails in the steady state of spermatogenesis, while the plasticity of A_{paired} or $A_{aligned}$ spermatogonia might allow them to restore spermatogenesis after external insult (Hara, et al. 2014, Lord and Oatley 2017). Kinetics of spermatogenesis regeneration after treatment with busulfan, combined with differential labeling of A_{single}, A_{paired} and $A_{aligned}$ spermatogonia confirmed that some A_{paired} and $A_{aligned}$ spermatogonia may contribute to the stem cell pool (Zhang, et al. 2016).

Over the past few years, molecular tracing experiments coupled with single cell mRNA sequencing and transplantation assays uncovered the fact that the mammalian A_{single} cell population is heterogeneous (Grisanti, et al. 2009, Chan, et al. 2014, Hermann, et al. 2015, Mutoji, et al. 2016, Guo, et al. 2017, Neuhaus, et al. 2017, Green, et al. 2018, Guo, et al. 2018, Hermann, et al. 2018), and that subsets of A_{single} cells have different capacity for self-renewal (Aloisio, et al. 2014, Helsel, et al. 2017). According to these data, a new model is now emerging whereby in mice a subpopulation of A_{single} cells, marked by high expression of both the transcription factor ID4 and the membrane receptor GFRA1, a co-receptor for glial cell line-derived neurotrophic factor (GDNF), is the purest functional SSC population described to date in immature and adult mice (Sun, et al. 2015, Lord and Oatley 2017, Hermann, et al. 2018, Lord and Oatley 2018). However, while ID4 expression is restricted to true SSCs ($ID4^{high}$) and cells transitioning into A_{paired} spermatogonia ($ID4^{low}$), expression of GFRA1 extends from SSCs to A_{paired} and some $A_{aligned}$ spermatogonia (Ebata, et al. 2005, Hofmann, et al. 2005, Grasso, et al. 2012). Other molecules such as PAX7, BMI1, and SHISA6 also mark A_{single} cell subsets, but their contribution to the “ultimate” stem cell pool is so far less well defined (Aloisio, et al. 2014, Komai, et al. 2014, Tokue, et al. 2017).

The process of spermatogenesis occurs within the seminiferous epithelium, which provides the specific microenvironment that will maintain SSC self-renewal and drive germ cell differentiation. The fate of the SSCs (i.e. the decision to self-renew or differentiate), depends on the complex interplay of various factors within their niche, which includes the neighboring somatic cells, the extracellular matrix and different soluble factors in their vicinity (Oatley and Brinster 2012).

Within the seminiferous epithelium, the somatic Sertoli cells are considered a critical component of the niche through their role in the maintenance of the stem cell pool and differentiation of the germ line. The factors produced by Sertoli cells that are critical for SSC self-renewal and maintenance include glial cell line-derived neurotrophic factor (GDNF)(Meng, et al. 2000), fibroblast growth factor (FGF2)(Kanatsu-Shinohara, et al. 2003, Kubota, et al. 2004), CSF1 (Oatley, et al. 2009), WNT family proteins (Yeh, et al. 2011, 2012), and leukemia inhibitory factor (LIF)(Kanatsu-Shinohara, et al. 2007). They all contribute to SSC expansion *in vitro*, as indicated by increased testes colonization after transplantation. But the factor that unequivocally regulates SSC self-renewal as well as proliferation of progenitors *in vitro* and *in vivo* is GDNF, a member of the transforming growth factor beta (TGF-b) superfamily that binds to the GFRA1/RET receptor complex (Meng, et al. 2000, Kubota, et al. 2004, Hofmann, et al. 2005, Naughton, et al. 2006, Oatley, et al. 2006, Jijiwa, et al. 2008, Chen, et al. 2016a). Understanding how GDNF expression is

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regulated within the niche is of paramount importance to understand the first steps of spermatogenesis.

GDNF Signaling

GDNF belongs to the GDNF family of ligands (GFLs), which consists of GDNF, Neurturin (NRTN), Artemin (ARTN) and Persephin (PSPN)(Airaksinen and Saarma 2002, Sariola and Saarma 2003). These proteins are members of the transforming growth beta (TGF-b) superfamily. GDNF was the first GFL discovered and was purified as a trophic factor from the supernatant of a rat glioma cell line (Lin, et al. 1993). GDNF protects and repairs dopaminergic neurons in animal models of Parkinson's disease, and also rescues motor neurons *in vivo* (Drinkut, et al. 2016, Ruven, et al. 2018). GDNF is a glycosylated homodimer that binds specific GDNF-family-alpha receptors (GFRA1–4), which are bound to the plasma membrane of the cell by a glycosyl phosphatidylinositol (GPI) anchor (Figure 1)(Jing, et al. 1996, Baloh, et al. 1997, Jing, et al. 1997, Masure, et al. 2000). GFRA1–4 are co-receptors that upon GDNF binding activate the RET receptor tyrosine kinase present at the surface of the target cell (Trupp, et al. 1996). Binding of GDNF-GFRA to the extracellular domain of RET activates its intracellular tyrosine kinase domain, which triggers the activity of multiple pathways in responding cells (Manie, et al. 2001, Kawamoto, et al. 2004, Ibanez 2013).

Global GDNF loss-of-function experiments in mice have demonstrated that its role is critical for the development of the kidneys, brain, and enteral nervous system. Mice lacking GDNF die at birth primarily from bilateral renal agenesis, but they also lack an enteric nervous system (Moore, et al. 1996, Pichel, et al. 1996, Sanchez, et al. 1996). These mice also have defects in the development of central and peripheral noradrenergic neurons (Granholm, et al. 1997). Similarly, ablations of the RET transmembrane receptor or its co-receptor GFRA1 lead to death shortly after birth, due to renal and neuronal abnormalities (Schuchardt, et al. 1994, Cacalano, et al. 1998). Studies using GDNF heterozygous (*Gdnf* +/−) mice have demonstrated that a partial reduction of GDNF leads to an age-related accelerated decline in the nigrostriatal dopaminergic system function and motor deficits (Littrell, et al. 2013). Therefore, GDNF is currently tested in clinical trials on Parkinson disease patients, but results are so far inconclusive. In the kidneys, the loss of one allele for *Gdnf* results in approximately 30% fewer but normal sized glomeruli in young mice (Cullen-McEwen, et al. 2003), and only mild reductions in enteric neuron size and neuronal fiber counts (Gianino, et al. 2003). All mice heterozygous for *Gdnf*, *Gfra1* and *Ret* have problems with intestinal contractility and neurotransmitter release, demonstrating that this signaling pathway is critical for enteric nervous system structure and function (Gianino, et al. 2003). Disruption of GDNF/RET signaling in humans causes several distinct diseases. Notably, loss of RET is associated with thyroid C-cell reductions, and mutations leading to its constitutive activation leads to medullary thyroid cancer (Lindahl, et al. 2000, Lindfors, et al. 2006, Cote, et al. 2015). Alterations in the pattern of RET phosphorylation is associated with amyotrophic lateral sclerosis (Luesma, et al. 2014). Since GDNF/RET signaling also maintains the enteric nervous system, loss of its function in these neurons causes Hirschsprung's disease, a condition characterized by the absence of enteric ganglia in the distal colon resulting in functional obstruction (Edery, et al. 1994).

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Role of GDNF family ligands in the testis

The importance of GDNF for germ cell development was uncovered by the seminal work of Meng and colleagues (Meng, et al. 2000) who demonstrated that mice heterozygous for *Gdnf*, though fertile, exhibit increased numbers of seminiferous tubules lacking spermatogonia as the animals aged. Conversely, transgenic animals overexpressing *Gdnf* display an accumulation of undifferentiated spermatogonia. Thereafter, it was demonstrated that GFRA1 and RET proteins and mRNA are expressed in these cells (Viglietto, et al. 2000, Dettin, et al. 2003, Hofmann, et al. 2005), confirming that they are able to respond to GDNF influence. During development, *Gdnf*, *Ret*, and *Gfra1* mRNAs are expressed at high levels in the male embryonic gonad as early as embryonic day 12.5 (E12.5), but their expression in the developing ovary is low (Nef, et al. 2005, Miles, et al. 2012). *Gdnf* is mainly detected in SF1-expressing somatic cells (Beverdam and Koopman 2006). In *Ret*−/− embryos, germ cells undergo apoptosis starting at E14.5 (Miles, et al. 2012), however ablation of *Gdnf* has little impact at this stage of germ cell development. Absence of the GDNF protein could be compensated by another GFL, Persephin (PSPN), which is also highly expressed between E12.5 and E15.5 (Milbrandt, et al. 1998, Miles, et al. 2012). Since prospermatogonia enter a period of mitotic arrest at E15.5, the role of GDNF from this time point until birth might be restricted to germ cell survival.

Shortly after birth, prospermatogonia migrate toward the basement membrane and become established SSCs (McGuinness and Orth 1992). GDNF then binds to the RET/GFRA1 receptor complex and induces their self-renewal (Meng, et al. 2000, Naughton, et al. 2006, Kanatsu-Shinohara and Shinohara 2013). As GFRA1 and RET are concomitantly expressed in A_{paired} and some $A_{aligned}$ spermatogonia, it is likely that GDNF also induces and maintains the proliferation of these cells as SSCs embark on the path of differentiation (Viglietto, et al. 2000, Hofmann, et al. 2005, Sharma and Braun 2018). However, deciphering the functional importance of GDNF and its receptor complex in spermatogenesis has been difficult since global knockout mice die around birth, mainly from renal agenesis (Sanchez, et al. 1996). Further, GDNF is expressed not only by Sertoli cells but also by peritubular myoid cells (Chen, et al. 2014, Chen, et al. 2016a), which makes interpretation of knockout experiments challenging. By using xenograft transplantations of neonatal knockout testes, Naughton et al. demonstrated that the absence of GDNF or its receptors RET and GFRA1 after birth led to a lack of SSCs and failure of spermatogenesis (Naughton, et al. 2006). Similarly, inactivating the RET Y1062 phosphotyrosine docking site led to progressive loss of germ cells and their absence by day 21 after birth (Jain, et al. 2004, Jijiwa, et al. 2008). However, these mice were heterozygous, and although the penetrance of the mutation was high and recapitulated Hirschprung's disease, some remaining GFRA1-positive cells with self-renewal ability were detected after more careful analysis (Takashima, et al. 2015). Therefore, at present, there is no satisfying model of a testis-specific *Gdnf* knockout. Additionally, it can be argued that GDNF is not the sole growth factor triggering SSC self-renewal and progenitor proliferation, as mentioned above. In particular, FGF2 can expand SSCs in cultures, maintain their stem cell activity and restore spermatogenesis in busulfan-treated mice as efficiently as GDNF (Takashima, et al. 2015). Therefore compensation by other self-renewal factors needs to be accounted for.

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As previously mentioned, the GDNF family of ligands contains four related molecules that can putatively bind to the GFRA1/RET complex in the testis, albeit with different affinities (Viglietto, et al. 2000). Neurturin (NRTN) is expressed by Sertoli cells preferentially after puberty, and its expression overlaps with that of GFRA2 in spermatocytes and spermatids (Viglietto, et al. 2000, Meng, et al. 2001). Indeed, in other tissues, GFRA2 functions as a specific NRTN receptor, and mice deficient in *Nrtn* or *Gfra2* display similar phenotypes (Heuckeroth, et al. 1999, Rossi, et al. 1999, Wanigasekara, et al. 2004). According to Meng and colleagues, GFRA2 expression is not detectable in spermatogonia, and *Nrtn* overexpression in transgenic mice leads only to transient depletion of spermatocytes and spermatids (Meng, et al. 2001). *Nrtn* knockout testes are normal and mice are fertile (Heuckeroth, et al. 1999). Therefore, while NRTN and GDNF are both expressed by Sertoli cells (Viglietto, et al. 2000, Widenfalk, et al. 2000, Meng, et al. 2001, Johnston, et al. 2011), and RET is expressed by all undifferentiated spermatogonia (Yoshida, et al. 2004, Naughton, et al. 2006, Yoshida, et al. 2006), the phenotypes of *Gdnf*- and *Nrtn*-overexpressing testes are comparatively different, as are the respective knockouts. This demonstrates that the cellular distribution of the GFRA co-receptors, and not that of RET, determines the target cell population for GDNF and NRTN. Altogether, these data also indicate that a significant crosstalk between NRTN and GRFA1, or between GDNF and GFRA2, is unlikely in the testis. Further, recent data indicate that GFRA3 is expressed at low level by rodent spermatogonia (Green, et al. 2018), which explain why its ligand Artemin (ARTN) triggers the formation of short chains of $A_{aligned}$ spermatogonia from cultured rat SSCs (Hamra, et al. 2007). An ARTN-like ligand has also been proposed as a homolog of GDNF in fish testes (Lucini, et al. 2004, Gautier, et al. 2014). However, ARTN does not appear to be expressed in the mammalian seminiferous epithelium, and its global ablation does not alter fertility (Honma, et al. 2002). Consequently, *in vitro* experiments using rodents and human SSCs cultured with rARTN may not reflect the *in vivo* situation. Finally, GFRA4, the receptor for Persephin (PSPN), is expressed by rat and mouse germ cells, in particular at the transition from spermatogonia to early spermatocytes (Masure, et al. 2000, Green, et al. 2018). The *Gfra4* transcript appears to be truncated and produces a soluble protein in this organ (Lindahl, et al. 2000), while *Pspn* global knockout mice are fertile (Tomac, et al. 2002). Therefore, according to these studies, PSPN does not appear to exert a significant influence on mammalian spermatogenesis. PSPN, through GFRA4/RET, mainly participates in the development of thyroid parafollicular cells (C cells) and chromaffin cells of the adrenal medulla, which are destined to produce calcitonin and adrenaline/noradrenaline respectively in the adult (Lindahl, et al. 2000). The mining of recently published single-cell RNA-seq data confirmed that in mice and humans, *Gfra1* is mostly found in undifferentiated spermatogonia. It is co-expressed with *Id4*, *Nanos2/3* and *Etv5*, which are markers of SSCs (Green, et al. 2018, Guo, et al. 2018, Hermann, et al. 2018). Transcripts for *Gfra2* are found in human undifferentiated spermatogonia, but are rare in the mouse equivalent germ cell population. Further, *Gfra2*, *Gfra3* and *Gfra4* are detected in spermatocytes and spermatids in both species. Additional studies will be required to understand the functional importance of GFRA2–4, in particular around meiosis. Altogether, these data demonstrate that in the testis, GDNF is the most critical GFL and that it exerts its influence on SSCs and progenitors probably exclusively through the GFRA1/RET receptor complex.

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Two main signaling pathways triggered by the binding of GDNF to its receptor complex have been so far identified in undifferentiated spermatogonia *in vitro* and *in vivo*. In one of the pathways, RET phosphorylation induces SRC-family kinases (SFKs) and PI3K/AKT activation to allow spermatogonial proliferation (Braydich-Stolle, et al. 2007, Oatley, et al. 2007). Further analysis indicated that FYN kinase is the major SFK used by these cells, at least *in vitro* (Braydich-Stolle, et al. 2010). Recent studies demonstrated that only AKT3 is phosphorylated in response to GDNF in undifferentiated spermatogonia (Sharma and Braun 2018) and that the mTORC1 pathway is activated downstream of AKT, as spermatogonia differentiate from $A_{aligned}$ to A_{diff} (Busada, et al. 2015). The other pathway activated by the binding of GDNF to the RET/GFRA1 complex is the canonical RAS/ERK1/2 (MAPK) pathway (He, et al. 2008, Lee, et al. 2009). RAS signaling in these cells has been demonstrated *in vitro* and *in vivo*, and ultimately upregulates the expression of the transcription factor c-FOS, a known inducer of different cyclins (Sunters, et al. 2004, He, et al. 2008, Wolgemuth, et al. 2013). Despite these advances, it is still not clear which one of these pathways, or both, specifically triggers self-renewal or differentiation. For example, *Mycn* is upregulated downstream of the SRC/PI3K/AKT/mTORC1 pathway, which might induce spermatogonial proliferation as they differentiate (Braydich-Stolle, et al. 2007, Lucas, et al. 2012). However, a study using transplantation of SSCs treated with *Mycn* shRNA demonstrated that MYC family transcription factors are in fact crucial for SSC self-renewal (Kanatsu-Shinohara, et al. 2014, Kanatsu-Shinohara, et al. 2016). Nonetheless, experiments using SSCs cultured with GDNF prior to testis transplantations have shown without doubt that GDNF increases the number of SSCs, and that downstream targets of the GDNF/RET signaling pathway such as ID4, BCL6, ETV5, and LHX1 are critical for SSC self-renewal (Kubota, et al. 2004, Oatley, et al. 2006, Wu, et al. 2011). Interestingly, a study by the group of Sada and colleagues demonstrated that GDNF signaling is essential to maintain expression of the RNA-binding protein NANOS2 in SSCs (Sada, et al. 2012). The authors propose that NANOS2 downstream of GDNF prevents spermatogonial differentiation in the postnatal testis.

Cyclic expression of GDNF

In the postnatal testis, GDNF is mainly expressed by Sertoli cells within the seminiferous epithelium (Tadokoro, et al. 2002, Johnston, et al. 2011). Recent data have demonstrated that peritubular myoid cells (PM cells) surrounding the seminiferous tubules also express this growth factor and contribute to germ cell maintenance (Chen, et al. 2014, Chen, et al. 2016a). In PM cells, GDNF expression is clearly under the influence of testosterone, while this has not been demonstrated in Sertoli cells. The interplay and relationship between GDNF from different sources is beyond the scope of this review and we will discuss here expression and regulation of GDNF in Sertoli cells only.

In the adult testis, within given segments of the seminiferous tubules, spermatogenesis proceeds in stages (I-XII in the mouse, I-XIV in the rat) characterized by defined germ cell associations (Leblond and Clermont 1952, Oakberg 1956, Russell, et al. 1990, Griswold 2016). The cyclical production of soluble factors by rat Sertoli cells according to the stages of the seminiferous epithelium has been demonstrated in 2008 by Johnston and colleagues, who separated the stages by transillumination-assisted microdissection, and assessed Sertoli

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cell mRNA expression in each stage by microarray analysis (Johnston, et al. 2008). They subsequently demonstrated that GDNF expression is highest at stages XIII-I, and lowest at stage VII (Johnston, et al. 2011). Similarly, in the mouse, GDNF expression is highest at stages IX-I and lowest at stages V-VIII (Caires, et al. 2012, Garcia, et al. 2017, Sharma and Braun 2018). However, discrepancies between studies are noted, which might be due to specific culture conditions of testicular tubules and to the species investigated (Sato, et al. 2011, Grasso, et al. 2012). Further, patch-like distribution of GDNF was detected by immunohistochemistry in the basal region of Sertoli cells in the seminiferous epithelium of mice and hamsters (Sato, et al. 2011), and these signals closely co-localized with a subpopulation of GFRA1-positive spermatogonia along the basement membrane. More recently, GDNF was ectopically overexpressed in Stages V-VIII by Sertoli cells in transgenic mice (Sharma and Braun 2018). This aberrant expression of GDNF significantly increased the number of GFRA1+/LIN28$^-$ germ cells, which are believed to be a subtype of A_s spermatogonia with enhanced self-renewal capacity (Chakraborty, et al. 2014, Sharma and Braun 2018). Further, the same study also demonstrated that GDNF maintains SSC self-renewal by blocking their differentiation into more mature spermatogonia, rather than actively promoting proliferation, strengthening previous data (Sada, et al. 2012, Garcia and Hofmann 2013).

Regulation of GDNF expression

While the influence of GDNF on SSCs and its progenitors is well established, little attention has been paid to the regulation of its production at the molecular level. The mechanisms responsible for its cyclic expression are so far not well understood, however it is evident that GDNF production must be modulated by positive or negative stimuli. For example, follicle-stimulating hormone (FSH) and cytokines might be positive regulators of GDNF expression in Sertoli cells (Tadokoro, et al. 2002, Lamberti and Vicini 2014), while NOTCH signaling provides for a novel mechanism of downregulation (Garcia, et al. 2017).

Regulation of GDNF expression by FSH

Gonadotropin-releasing hormone (GnRH) secreted by neurons in the hypothalamus is the master regulator of spermatogenesis. Cyclic production of GnRH induces the release of the glycoproteins follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary, and these hormones will act on testicular somatic cells to regulate spermatogenesis. LH binds LH receptor (LHR) at the surface of Leydig cells, which ultimately induces the production of testosterone. FSH binds to its receptor (FSHR) at the surface of Sertoli cells and is responsible for the proliferation of these cells until around day 15 (P15) after birth in mice (Vergouwen, et al. 1991, Singh and Handelsman 1996, Baker and O'Shaughnessy 2001). Both FSH and LH (indirectly via testosterone and androgen receptor) exert their effects on spermatogenesis through the expression of Sertoli cell growth and differentiation factors. Upregulation of GDNF expression by FSH is supported by short-term *in vitro* studies using immature primary Sertoli cells cultured in presence of the hormone (Tadokoro, et al. 2002, Ding, et al. 2011, Lamberti and Vicini 2014), but the data obtained are not necessarily applicable to adult Sertoli cells. Tadokoro and colleagues also treated *Sl*/*Sl*^{d} mice, which lack A1-A4 differentiating spermatogonia, for 5 days with a GnRH antagonist

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(Nal-Glu) (Tadokoro, et al. 2002). They demonstrated a significant reduction of GDNF mRNA expression and a decreased rate of proliferation of undifferentiated spermatogonia in the testes of these mice, as measured with proliferating cell nuclear antigen (PCNA) expression. They attributed this decrease to a decrease in FSH, but were at the time unaware that the GnRH antagonist, which also suppresses LH and testosterone production, might also have reduced myoid cell-derived GDNF expression. In addition, the fact that *Fshb* knockout mice are fertile (Kumar, et al. 1997) indicates that this hormone is not an essential driver of GDNF production. Germ cell transplantation data recently revealed that SSC development in these mice is not significantly impaired (Tanaka, et al. 2016), although it is possible that GDNF provided by the peritubular myoid cells compensates for the loss of *Fshb* (Chen, et al. 2014, Chen, et al. 2016a).

Other mechanisms of GDNF upregulation

Other factors that induce GDNF expression in Sertoli cells include growth factors and chemokines/cytokines such as FGF2, TNF-α, and IL1-β (Simon, et al. 2007). Testicular macrophages located in the interstitial space between the tubules are thought to secrete some of these molecules (Bhushan and Meinhardt 2017). Sertoli cells express receptors for these factors, but because these studies have only been performed *in vitro* with a Sertoli cell line, TM4, which may not have conserved normal gene expression patterns, the results await confirmation from *in vivo* models. Constitutive activation of CTNNB1 (β-catenin) in Sertoli cells seems to induce high levels of GDNF expression in a transgenic mouse model (Tanwar, et al. 2010). This causes accumulation of undifferentiated spermatogonia and defective postnatal germ cell differentiation. Activation of CTNNB1 by treatment with LiCl causes a fivefold increase in *Gdnf* promoter activity, and the sequence of the proximal *Gdnf* promoter contains a TCF7 consensus-binding site. These results therefore suggest that *Gdnf* gene expression is directly regulated by CTNNB1 *in vivo*.

GDNF promoter analysis

Lamberti and Vicini recently performed an in-silico analysis of evolutionarily conserved regions (ECRs) between human, rat, and mouse, followed by the identification of conserved DNA binding sites for known transcription factors (Lamberti and Vicini 2014). The human, rat and mouse genes share high similarities in intron lengths and coding exons, in particular in exon 1. The genomic 5’-flanking region of exon 1 shows very high sequence similarity among the three species, suggesting the presence of a conserved promoter. Conserved DNA binding sites among the three different species include a canonical TATA-box, several NF-kB binding sites, an androgen receptor (AR) binding site, and several cAMP-response elements (CRE) within the −2000 to +1 bp proximal promoter (Figure 2). Deletion of the CRE sites in a luciferase reporter plasmid reduced the response to dibutyryl-cAMP in transfected primary Sertoli cells in comparison to control. The presence of NFKB1 binding sites agrees with the observation that GDNF expression increases upon treatment of Sertoli cells with TNF-α and IL1-β (Simon, et al. 2007). The GDNF proximal promoter region also contains sequences bound by putative transcriptional repressors. A negative regulatory region containing 2 NRSE-like binding sites can be found within the 5’ UTR, but specific ablation of this region did not significantly increase reporter gene activity in immature primary Sertoli cells, and treatment with REST siRNA did not increase GDNF expression.

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Further analysis of the proximal promoter demonstrated the presence of several E-Boxes and N-boxes to allow the binding of basic helix-loop-helix proteins with possible repressor activity as explained below (Garcia, et al. 2017) (Figure 2).

Regulation of GDNF via NOTCH signaling

In order to maintain germ cell homeostasis, pathways that negatively regulate growth factors essential for self-renewal or proliferation must exist. We recently demonstrated that NOTCH signaling in Sertoli cells modulates their expression of GDNF during embryonic development and after birth (Garcia, et al. 2013, Garcia, et al. 2014). NOTCH signaling is a highly conserved juxtacrine signaling pathway that mediates cell fate decisions during the development of multiple organs and tissues (Bray and Bernard 2010)(Figure 3). NOTCH1–4 are large cell-surface receptors that are activated by their ligands JAGGED (JAG1 and JAG2) and DELTA-like (DLL1, DLL3, and DLL4), which are transmembrane proteins displayed by neighboring cells (Wang 2011). Activation of the NOTCH receptor recruits ADAM10/TACE (Tumor necrosis factor (TNF)-α converting enzyme) and γ-secretase, which sequentially cleave and release the NOTCH intracellular domain (NICD) in the cytoplasm. NICD migrates to the nucleus where it forms a transcriptional complex with the DNA binding protein RBPJ (recombining binding protein suppressor of hairless) (Tanigaki and Honjo 2010), co-activators such as Mastermind (MAML) and histones acetyltransferases (Shao, et al. 2012)(Figure 3). The canonical targets of RBPJ include the HES and HEY families of transcriptional repressors, which are basic helix-loop-helix proteins (bHLH)(Iso, et al. 2003, Kageyama, et al. 2007, Bray and Bernard 2010). Transcriptional repressors of the HES family (HES1–7) bind to N-box promoter regions of their target genes, while repressors belonging to the HEY family (HEY1, HEY2, HEYL) bind to E-box promoter regions (Kageyama, et al. 2007). HES factors not only form homodimers, but they also form heterodimers with HEY1 or HEY2, which bind N-boxes with a higher affinity and repress transcription more efficiently (Iso, et al. 2003). HES/HEY proteins usually repress transcription by forming complexes with co-repressors of the Groucho/transducin-like Enhancer of split (Gro/TLE) family (Grbavec, et al. 1998). E- and N-boxes can also be bound by members of the MYC, MYOD, or NEUROG families of transcription factors, which drive cell proliferation and cell fate.

NOTCH pathway components are all expressed in germ cells and Sertoli cells in pre- and postnatal testes (Dirami, et al. 2001, Hahn, et al. 2009). However, the canonical pathway is not activated in male germ cells as demonstrated by the fact that fetal and post-natal germ cells are GFP negative in a transgenic NOTCH reporter mouse model called TNR-GFP. This model expresses GFP under the control of a promoter containing four RBPJ binding sites (Duncan, et al. 2005) (Garcia, et al. 2013). We recently established both a gain-of-function and loss-of-function mouse models of NOTCH signaling specifically in Sertoli cells. Constitutively activating NOTCH1 signaling in these cells led to infertility due to a complete lack of germ cells by P2 (Garcia, et al. 2013). In this model, E15.5 prospermatogonia, which normally do not proliferate at this stage of development, expressed the cyclins CCND1 and CCND3. They also expressed STRA8, KIT, and SYCP3 at the mRNA and protein levels, which are hallmarks of differentiating germ cells (Garcia, et al. 2013, Garcia and Hofmann 2013). Interestingly, expression of GDNF by Sertoli cells was significantly downregulated in

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the fetal testis and after birth, as well as in the adult mice. Overall these data indicated that GDNF expression was inversely correlated with NOTCH signaling activation, which drove early germ cell differentiation in the fetal testis. The loss of germ cells was attributed to a lack of niche molecules able to maintain differentiation at this stage of development (Garcia, et al. 2013, Garcia and Hofmann 2013).

Because the NOTCH receptors are redundant, functional studies relying on ablating expression of these proteins in mice, in particular NOTCH1, have been unsuccessful (unpublished data). Inhibiting gamma-secretase with a pharmacological inhibitor (DAPT) to prevent NICD cleavage and NOTCH activation was useful to demonstrate *in vitro* that NOTCH downregulation in isolated primary Sertoli cells promoted an increase of GDNF expression (Garcia, et al. 2013). However, treating cells or mice with this compound might lead to nonspecific results since the gamma-secretase complex is also critical for processing other integral membrane proteins, such as ERBB4, E- and N-cadherin (CDH1 and CDH2), ephrin-B2 (EFNB2) and CD44, a receptor for extracellular matrix proteins that functions as a transcription factor and also interacts with SRC family kinases within most cells (Ilangumaran, et al. 1999, Okamoto, et al. 2001). Therefore, since RBPJ is at the intersection of the NOTCH1–4 pathways, we disrupted the NICD- and DNA-binding exons of the *Rbpj* gene specifically in mouse Sertoli cells *in vivo*. Loss-of-function of *Rbpj* in these cells led to upregulation of *Gdnf* expression. We also observed an increase in the diameter of the seminiferous tubules, an increase in testis sizes and higher testis indexes. While the total number of Sertoli cells per testes remained the same, the number of GFRA1 and PLZF-positive cells (A_{undiff}) per Sertoli cell significantly increased and led to a 25–30% increase in the number of spermatocytes and elongated spermatids (hyperplasia)(Garcia, et al. 2014). The phenotype of the NOTCH signaling knockout was therefore the opposite to the phenotype of the NOTCH signaling overactivation mutant. Further, our data demonstrated that the canonical NOTCH pathway was active in Sertoli cells since expression of the transcriptional repressors *Hes1* and *Hey1* was upregulated in the overactivated NOTCH mutant, and downregulated in the knockout model. More recently, we demonstrated by ChIP-PCR that HES1 and HEY1 bind to the GDNF promoter, indicating that NOTCH signaling is a direct repressor of GDNF in Sertoli cells (Garcia, et al. 2017). Altogether, these results indicate that the canonical NOTCH signaling pathway is active in Sertoli cells and can be used —through the transcriptional repressors HES1 and HEY1— to inhibit GDNF production. Further, this mechanism may be used by Sertoli cells to dampen SSC self-renewal or progenitor proliferation when necessary.

Control of GDNF expression by germ cells

By producing specific growth factors, among them GDNF and FGF2, functional Sertoli cells create a unique physiological environment that directs SSC self-renewal and differentiation. In turn, different types of germ cells must influence Sertoli cells. These interactions between germ cells and Sertoli cells are facilitated by their direct contact within the seminiferous epithelium. To identify whether germ cells or Sertoli cells provide the ligand for NOTCH signaling in Sertoli cells, we specifically isolated populations of germ cells and Sertoli cells by FACS using transgenic mice expressing GFP specifically in Sertoli cells or germ cells. Germ cells highly expressed NOTCH ligands in comparison to Sertoli cells (Garcia, et al.

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2014). Alternatively, RFP-expressing germ cell populations were isolated by FACS and further fractionated using fluorescence-labeled antibodies against GFRA1 or KIT. Spermatocytes were isolated through their high expression of GFP from a DAZL-GFP transgenic model (Nicholas, et al. 2009). We demonstrated that the NOTCH ligand JAG1 was highly expressed in GFRA1-positive spermatogonia, although some expression was also noted in KIT-positive differentiating spermatogonia, and early spermatocytes (Garcia, et al. 2017). Because there are few GFRA1-positive spermatogonia in comparison to differentiating spermatogonia and early spermatocytes, it is reasonable to expect that the accumulation of A_{undiff} and A_{diff} spermatogonia around stage VII will increase NOTCH activity through JAG1 and trigger a decrease in GDNF expression by Sertoli cells, leading to the cyclic expression of this growth factor, a hypothesis that we are currently further testing. Therefore, the dosage of JAG1 presented by germ cells might be critical for NOTCH activation in Sertoli cells. Type A spermatogonia, when in sufficient numbers, might regulate their own homeostasis through downregulation of GDNF. These data are compatible with the fact that in normal mice, the absence of germ cells after busulfan treatment induces higher expression of GDNF (Tadokoro, et al. 2002, Ryu, et al. 2006, Garcia, et al. 2017).

Conclusion

GDNF is a critical component of the SSC niche, whose roles include stimulation of stem cell self-renewal, proliferation of progenitors and maintenance of the undifferentiated state. Despite its critical effects on germ cell homeostasis, surprisingly little is known about its regulation. Unraveling molecular mechanisms has been hampered by the difficulty of working with adult primary Sertoli cells, which do not proliferate and are unsuitable for long-term cultures. Another challenge is the fact that Sertoli cells often lose their characteristics after isolation, and that their normal pattern of gene expression varies across the stages of the seminiferous epithelium cycle, which is hardly reproducible in two-dimensional cultures. While additional mouse models will be necessary to assess overall gene function, they are insufficient to understand regulation of gene expression at the molecular level. Improving ex vivo organ culture systems might be useful to conserve adult Sertoli cell functions while elucidating complex signaling pathways interactions. Indeed, cross-talks between GDNF signaling and other pathways are still poorly understood as are RET-independent mechanisms driven by GDNF/GFRA1, or GDNF-independent mechanisms driving RET in germ cells. In addition, how germ cells communicate with Sertoli cells needs to be further explored. We have highlighted JAG1/NOTCH signaling as one possible mechanism that fulfills this role, but other modes of germ cell to Sertoli cell communication assuredly exist that need to be unraveled. Elucidating these signaling events in their proper architectural context is key to understanding human idiopathic infertility.

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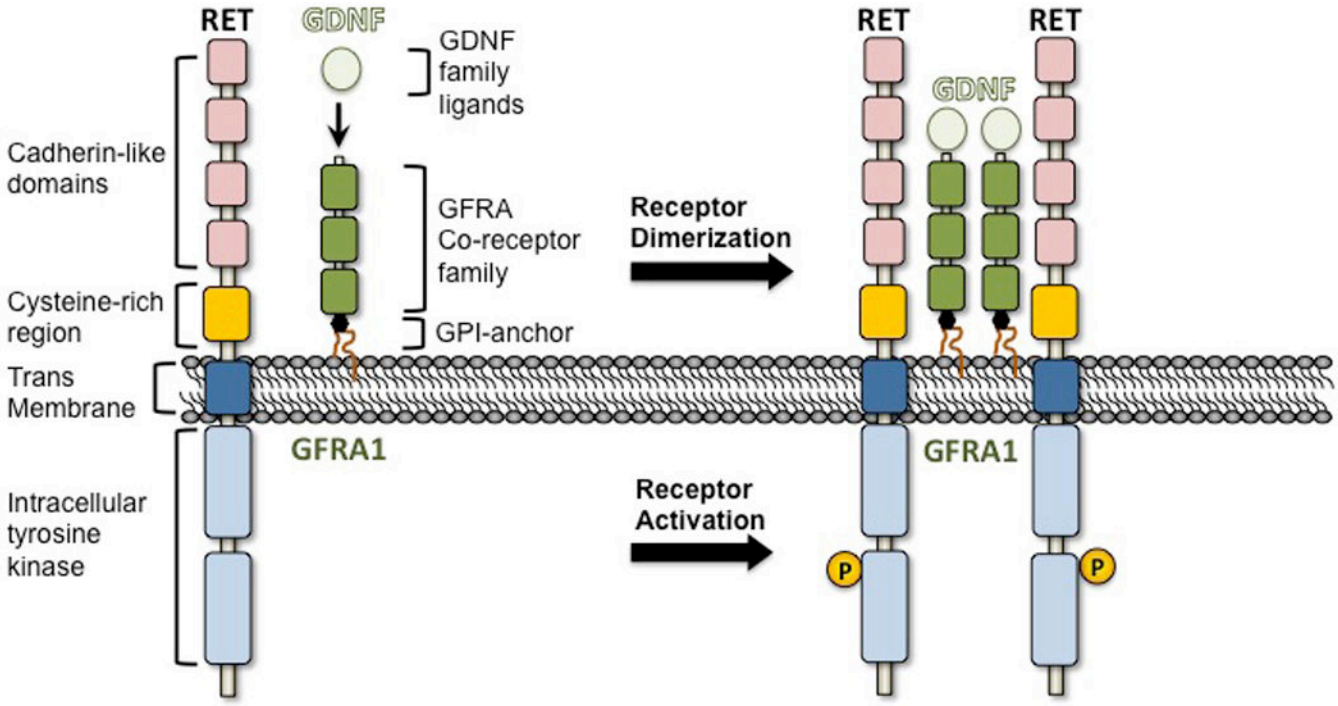

Figure 1: GDNF/RET signaling

Diagram illustrating activation of the RET receptor, which is triggered through interaction with GDNF bound to the GFRA1 co-receptor. GDNF binding leads to auto-phosphorylation of several tyrosine residues in the cytoplasmic region of RET.

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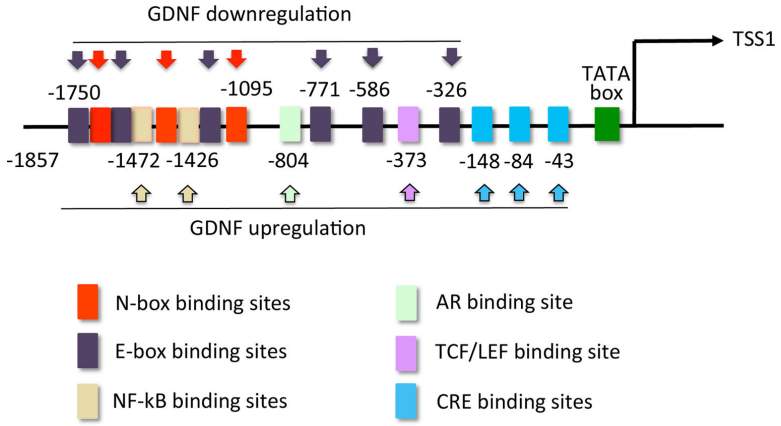

Figure 2: Mouse GDNF proximal promoter

Schematic representation of the mouse *Gdnf* proximal promoter, expanding on the data of Lamberti and Vicini, 2014. Potential regulatory elements are shown as boxes and are color-coded. HES1 and/or HEY1, together with co-repressors, will bind to N- or E-boxes within the *Gdnf* promoter and cause its downregulation. E- and N-boxes can also be occupied by bHLH transcriptional activators, which could putatively increase *Gdnf* expression. These activators, if any, are not known.

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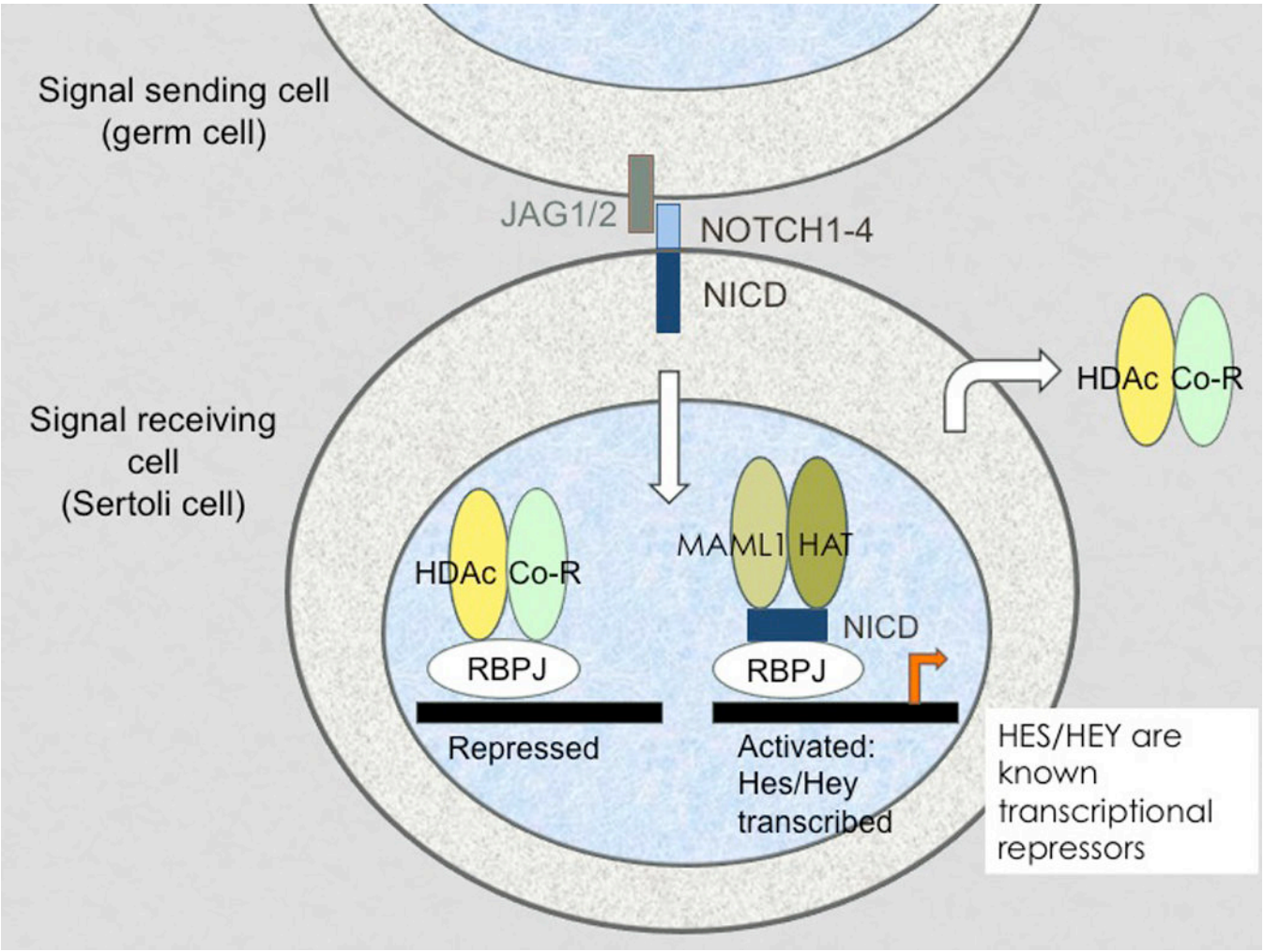

Figure 3: Schematic representation of the NOTCH signaling pathway

Ligands of the JAGGED (JAG) and DELTA-like families interact with NOTCH1–4 receptors on an adjacent cell. In the seminiferous epithelium, JAG1 at the surface of spermatogonia interacts with NOTCH1 at the surface of Sertoli cells. This releases the NOTCH intracellular domain (NICD), which translocates to the Sertoli cell nucleus. NICD forms a complex with RBPJ at the promoter of target genes. Binding of NICD releases co-repressors and allows the binding of co-activators. This leads to the transcriptional activation of the canonical NOTCH targets *Hes1* and *Hey1*, which are known bHLH transcriptional repressors.