Mammalian gonocyte and spermatogonia differentiation: recent advances and remaining challenges

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Abstract

The production of spermatozoa relies on a pool of spermatogonial stem cells (SSCs), formed in infancy from the differentiation of their precursor cells, the gonocytes. Throughout adult life, SSCs will either self-renew or differentiate, in order to maintain a stem cell reserve while providing cells to the spermatogenic cycle. By contrast, gonocytes represent a transient and finite phase of development leading to the formation of SSCs or spermatogonia of the first spermatogenic wave. Gonocyte development involves phases of quiescence, cell proliferation, migration, and differentiation. Spermatogonia, on the other hand, remain located at the basement membrane of the seminiferous tubules throughout their successive phases of proliferation and differentiation. Apoptosis is an integral part of both developmental phases, allowing for the removal of defective cells and the maintenance of proper germ–Sertoli cell ratios. While gonocytes and spermatogonia mitosis are regulated by distinct factors, they both undergo differentiation in response to retinoic acid. In contrast to postpubertal spermatogenesis, the early steps of germ cell development have only recently attracted attention, unveiling genes and pathways regulating SSC self-renewal and proliferation. Yet, less is known on the mechanisms regulating differentiation. The processes leading from gonocytes to spermatogonia have been seldom investigated. While the formation of abnormal gonocytes or SSCs could lead to infertility, defective gonocyte differentiation might be at the origin of testicular germ cell tumors. Thus, it is important to better understand the molecular mechanisms regulating these processes. This review summarizes and compares the present knowledge on the mechanisms regulating mammalian gonocyte and spermatogonial differentiation.

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Introduction

Spermatogenesis is the process that allows for the continuous formation of spermatozoa throughout adult male life, and for the transfer of genetic material from one generation to the next. It includes two phases separated in humans by a few years of relative testicular quiescence, contrasting with the uninterrupted development of rodent spermatogenesis. The first phase takes place from embryonic ages to infancy. It consists of successive phases of primordial germ cells (PGCs) and gonocytes (also known as pre- or pro-spermatogonia) that can be distinguished by unique features, leading to the formation of the first spermatogonia, including spermatogonial stem cells (SSCs) (Culty 2009). The second phase, starting at prepuberty and extending throughout male adult life, comprises spermatogenic cycles that are initiated by the commitment of spermatogonia to differentiate, progressing through meiosis and spermiogenesis to end with the formation of immature spermatozoa (Hermo *et al.* 2010). Spermatogenesis occurs within the seminiferous tubules, a group of well

organized, long convoluted tubules connecting at both ends to the rete testis, which assure sperm transport to the efferent duct, the initial part of the epididymis (Hermo *et al.* 2010). The seminiferous tubules are made up of supporting Sertoli cells and germ cells (Bellve *et al.* 1977), and are surrounded by peritubular myoid cells. The other critical cells of the testis are the interstitial Leydig cells, responsible for androgen production, a key factor in the regulation of male reproductive tract development and spermatogenesis (Christensen 1975, Steinberger & Steinberger 1975). Although there are differences in the number of days it takes for a spermatogenic cycle to be completed between species, the process itself is similar between humans and rodents, and thus, rodents are commonly used animal models to study this process (Adler 1996).

Male germ cells represent a highly specialized type of cells that undergo considerable morphological, biochemical, and molecular changes throughout their development, starting from the most primitive type, the diploid fetal PGCs, and ending with the formation of haploid flagellated gametes (Setchell *et al.* 2003).

The rodent spermatogenic cycle includes three main phases: a mitotic phase that takes place in SSCs, undifferentiated (A_{single} , A_{pair} and A_{aligned}), differentiating (A_1 to A_4 , intermediate), and differentiated (type B) spermatogonia at various phases of maturation, followed by a lengthy meiotic phase including successive types of primary spermatocytes, secondary spermatocytes and the formation of haploid spermatids (round, elongated), which undergo spermiogenesis to become spermatozoa that will further achieve maturation in the epididymis (Hermo *et al.* 2010). The human cycle is very similar, despite presenting stages spread over much longer periods (days instead of hours) and with the exception of having only three types of spermatogonia, A_{dark} , A_{pale} , and B. This continuous and dynamic process results in the production, in humans, of millions of mature sperm per gram of testis weight each day (Amman & Howards 1980). Numerous studies have added to the ever-growing understanding of mechanisms underlying sperm formation. Over the last decade, an increasing number of studies have broadened our knowledge of the genes critical for SSC self-renewal and proliferation. In comparison, few studies have focused on the fetal to perinatal phases of germ cell development at the origin of SSCs.

The germ cell lineage is initiated by the specification of embryonic cells to adopt the germ cell fate under the control of bone morphogenic protein 4 (BMP4) and BMP8b, starting with the formation of PGC precursors expressing PRDM1 (BLIMP1) and PRDM14, detected around embryonic day (E) 6.25 in the proximal epiblast, which further evolves to alkaline phosphatase and Stella-expressing PGCs at E7.25 (Kurimoto *et al.* 2008; review in Saitou (2009)). At E7.5, these sexually undifferentiated PGCs start migrating toward the genital ridge, involving the interactions of the membrane receptors KIT (c-Kit/CD117) and CXCR4 with their respective ligands SCF and SDF1, where they will become resident and enclosed by Sertoli cells, forming testicular cords at E12.5dpc in rodents (reviewed in Jan *et al.* (2012)). During their migration, PGCs undergo genome-wide DNA demethylation, leading to the erasure of parental imprints (Reik *et al.* 2001, Seki *et al.* 2005). Sex determination is established at E12.5 in both germ and supporting somatic cells, based in part on the expression of the *Sry* gene in male somatic cells (Jameson *et al.* 2012). Once the testicular cords have formed, the germ cells present in the cord are referred to as gonocytes (Zhao & Garbers 2002, Rouillier-Fabre *et al.* 2003, Culty 2009, 2013).

Gonocytes are the sole source of a functional reservoir of SSCs necessary to ensure life-long production of sperm (Culty 2009, 2013). Gonocytes encompass a succession of cell types with distinct behaviors that are associated with different fetal and neonatal periods, including a period of fetal mitosis, a phase of quiescence starting around E17.5 in rats and ending at postnatal

day 3 (PND3), at which point gonocytes re-enter mitosis and start migrating toward the basement membrane of the seminiferous cords, where they undergo differentiation into SSCs or type A spermatogonia of the first spermatogenic wave (Culty 2009). These events, which occur 1–2 days earlier in mice, are not precisely synchronized, resulting in the co-existence of quiescent and proliferative gonocytes in neonatal testes (Culty 2013). One common characteristic of fetal and postnatal gonocytes is that they are the site of intense DNA remethylation, required for the acquisition of paternal imprints and transposon silencing (Trasler 2009). While platelet-derived growth factor-BB (PDGF-BB) and 17 β -estradiol were shown to induce neonatal gonocyte proliferation via MAPK activation (Li *et al.* 1997, Basciani *et al.* 2008, Thuillier *et al.* 2010), the factors inducing fetal gonocyte proliferation have not been fully identified. The difference between mitotic fetal and neonatal rat gonocytes was clearly illustrated by their different responses to all-trans retinoic acid (RA) in organ culture studies. In E14.5 gonocytes, RA was found to increase fetal gonocyte proliferation, while inducing apoptosis at a greater rate, leading to an overall loss of cells (Livera *et al.* 2000). However, in the same study, RA was reported to slightly increase PND3 gonocyte numbers and have no effect on apoptosis. Moreover, activin A and androgen were reported to be negative regulators of fetal gonocyte proliferation, whereas we did not find any effect of testosterone on PND3 gonocyte proliferation *in vitro* (Merlet *et al.* 2007, Thuillier *et al.* 2010, Mendis *et al.* 2011).

Studies have demonstrated that the timing of events is as critical as their amplitude to adequately regulate gonocyte development. For example, it has been recently shown that the constitutive activation of the Notch pathway in fetal Sertoli cells led to premature changes in the behavior of fetal gonocyte, including premature exit from quiescence, migration, and differentiation, resulting in the loss of germ cells before birth (Garcia & Hofmann 2013, Garcia *et al.* 2013). Interestingly, based on morphological and gene expression similarities, it has been suggested that improper gonocyte differentiation could lead to the formation of carcinoma *in situ*, the precursor pathology to testicular germ cell tumors (TGCTs; Skakkebaek *et al.* 1987, Sonne *et al.* 2009). Notably, the incidence of TGCTs has steadily been increasing over the past decades, for reasons that remain unknown (Huyghe *et al.* 2003). Although the increased exposure to endocrine-disrupting compounds is often blamed (Skakkebaek *et al.* 2001), the underlying mechanisms through which these tumors are developing are yet to be elucidated. Hence, a better understanding of normal gonocyte development could allow for a better comprehension of the origins of testicular tumors. Therefore, the goal of this review is to survey the available literature on gonocyte differentiation and to compare it with what

is known of SSC and spermatogonial differentiation in rodents, concluding with some information about these processes in human, primates, and other species. This will allow for a better understanding of the differences between these two critical but often over-looked phases of germ cell development.

Transitional gonocyte differentiation in rodents

Owing to the multiple changes occurring in the last phase of gonocyte development, it has been proposed to refer to them as 'transitional' gonocytes (Culty 2013). In the remainder of the review, which focuses on this specific phase of gonocyte development, we will refer to these cells as either 'transitional', or simply as 'gonocytes'. Of all of the processes involved in gonocyte development, transitional gonocyte differentiation is the least studied. This is surprising in light of the fact that gonocytes differentiate into SSCs, which are essential for the production of spermatozoa throughout the lifetime of a male. Morphologically, gonocytes and spermatogonia do not look similar. Fetal and neonatal gonocytes are large round cells with prominent nuclei, located at the centre of the seminiferous cords (Clermont & Perey 1957), whereas spermatogonia are semicircular in shape and are located at the periphery of the seminiferous tubules, with the flattest edge in apposition to the basement membrane. However, despite these differences and presence at different ages, it is during the transitional phase, especially at PND5 and PND6 in rat, when some perfectly round gonocytes still have not yet undergone differentiation but are already located at the basement membrane, that uncertainty can occur regarding the identity of the cell. At such times, morphology and location within the tubule alone are not sufficient to characterize the cells as being still gonocytes and not already transitioned to spermatogonia (Culty 2009). Owing to the lack of synchronization in gonocyte development, the absence of a marker exclusively expressed in gonocytes, and the several day-long transition time frame in rodents (weeks to months in humans), it is impossible to clearly determine a day at which gonocyte differentiation has ended and spermatogonial self-renewal has started, making the study of gonocyte differentiation a challenging topic.

Yet, over the last years, several genes have been characterized as indicators of gonocyte differentiation, simplifying the study of factors and pathways regulating this process. One of these markers is stimulated by RA8 (STRA8), first identified as a target gene of RA in P19 mouse embryonal carcinoma cells, and later, in F9 mouse embryonal teratocarcinoma cells, undifferentiated spermatogonia, and premeiotic male germ cells (Bouillet *et al.* 1995, Oulad-Abdelghani *et al.* 1996, Giuli *et al.* 2002, Zhou *et al.* 2008a). Since then, the role of STRA8 as an essential initiator of meiosis in male and female germ cells has been clearly demonstrated in *Stra8*

knockout mice that were infertile, due to disrupted meiosis and premature chromosome condensation (Baltus *et al.* 2006, Anderson *et al.* 2008, Mark *et al.* 2008). Although the exact mechanism through which STRA8 induces meiosis is not well understood, the protein has been shown to shuttle between the cytoplasm and the nucleus in different types of germ cells, and to display transcriptional activity in cell lines when it is expressed in the nucleus (Tedesco *et al.* 2009). Several groups, including ours, have used STRA8, alongside KIT (another marker of differentiating spermatogonia), as a marker for differentiating gonocytes (Wang & Culty 2007, Zhou *et al.* 2008b). As mentioned before (Culty 2009), transcript and protein expression changes in these markers of differentiation are convenient surrogates for determining whether a gonocyte has initiated differentiation, but these changes reflect only some aspects of the alterations in the complexes taking place during the transition from gonocyte to spermatogonia. Indeed, it is likely that RA-treated gonocytes do not recapitulate the entire differentiation process, especially considering that differentiation to spermatogonia is achieved only when the cells establish close contact with the basement membrane. Nonetheless, this is a convenient tool that allows dissecting some of the signaling pathways and molecular changes occurring in response to RA, which remains, as far as we know, the only demonstrated inducer of differentiation in gonocytes.

Our work has shown that PND3 rat gonocyte differentiation is induced *in vitro* by RA (Wang & Culty 2007). Although *in vivo* and freshly isolated gonocytes express little STRA8 mRNA and protein at PND3, the addition of RA, an active metabolite of vitamin A (Collins & Mao 1999), to isolated gonocytes induces a strong and consistent increase in *Stra8* mRNA (Wang & Culty 2007). This is followed by an increase in protein expression, mainly located in the cytoplasm, suggesting that STRA8 is maintained as an inactive form in gonocytes (Sarkar and Culty, unpublished observations). Similar to our findings in rats, studies have shown that mouse PND2 gonocytes also differentiate in response to RA, as indicated by increased *Stra8* mRNA levels (Zhou *et al.* 2008b). The importance of vitamin A and RA in spermatogenesis has been known for many years due to the use of a vitamin A-deficient mouse model (VAD mice). Studies have demonstrated that in VAD adult mice, all differentiated germ cells are lost from the seminiferous tubules and the only cells remaining are undifferentiated type A spermatogonia and Sertoli cells (Mitranond *et al.* 1979, Van Pelt & de Rooij 1990). However, once these rats are administered vitamin A, normal spermatogenesis can be rescued (Morales & Griswold 1987, Griswold *et al.* 1989). At first, studies demonstrated that normal spermatogenesis could only resume if vitamin A (retinol) was given to the deficient mice and that the same results were not observed upon

RA administration (Ahluwalia & Bieri 1971, Huang *et al.* 1983). However, later studies demonstrated that RA could also stimulate normal spermatogenesis in these deficient mice, but at a much higher dose than retinol, probably due to differences in the levels of binding proteins for each of these retinoids in Sertoli cells (Van Pelt & de Rooij 1991).

In order to induce differentiation, retinol is delivered to the gonocyte via the retinol-binding protein (RBP) from Sertoli cells or the serum (Hogarth & Griswold 2010, 2013). Retinol is then internalized after binding to a membrane receptor, STRA6 (Kawaguchi *et al.* 2007, Hogarth & Griswold 2010). Inside the cell, retinol then undergoes the two-step oxidation process in order to become RA (Theodosiou *et al.* 2010). It is also possible that RA is directly transported to the gonocytes from the serum (via the vasculature) or Sertoli cells (Hogarth & Griswold 2010, 2013). Although spermatids and testicular and epididymal sperm are known to be able to store retinoids, Sertoli cells are the main site of RA synthesis in the testis, which is then passed onto the germ cells (Livera *et al.* 2002). Interestingly, RA was shown to be present in patches along the seminiferous cords of neonatal mice, while CYP26B1, the enzyme degrading RA, was expressed in a non-uniform manner in germ cells along the tubules (Snyder *et al.* 2010). Although these studies were carried out using PND2 mice, this developmental period is similar to that of PND3–PND4 in rats. Thus, it is likely that these factors also play a role in the lack of synchronization of gonocyte progression to spermatogonia, within the few days encompassing this process. It is not known whether, *in vivo*, RA induces differentiation by acting directly on the germ cells or if it is an indirect process involving Sertoli cells, as both cell types express RA receptors (RARs) and retinoid X receptors (RXRs) at varying levels (Akmal *et al.* 1997, de Rooij & Russell 2000). It has been shown that at PND3, gonocytes express all isoforms of RARs and RXRs, mainly high levels of RARA and RXRA, whereas Sertoli cells express mainly RXRG (Boulogne *et al.* 1999). Other groups have reported that PND3 gonocytes do not express RARA and that, at PND3, this receptor isoform is expressed solely in Sertoli cells, while gonocytes express RARG (Vernet *et al.* 2006a,b). However, differences in expression levels could also be attributed to the different affinities of the antibodies used for their respective targets. In our own studies, we have found that rat PND3 gonocytes express high levels of both *Rara* and *Rarg* (Manku *et al.* 2014).

Although both mice and rat gonocytes are stimulated by RA to undergo differentiation, the signaling pathways in mouse gonocytes have not been identified yet. In rats, we have found that the PDGF receptor (PDGFR) signaling pathway probably plays an important role in gonocyte differentiation (Wang & Culty 2007, Manku *et al.* 2014). It is already known that PDGFR expression begins to steadily increase at 18 dpc and continues to

increase until PND5, at which time this expression begins to decline (Loveland *et al.* 1993). We have found that rat spermatogonia at PND7 have significantly lower PDGFR expression compared with PND3 gonocytes, indicating a possible preferential role of these receptors in gonocyte development (Li *et al.* 1997). In studies on isolated PND3 rat gonocytes, we found that RA induced the formation of variant forms of PDGFR α (v-PDGFR α) and PDGFR β (V1-PDGFR β), concomitant with the increases in *Stra8* expression (Wang & Culty 2007, Manku *et al.* 2014). We have previously shown that the variant V1-PDGFR β form has retained its tyrosine kinase domain, while lacking the ligand-binding domain, and thus, it is ligand independent (Wang & Culty 2007). We have recently found that the variant v-PDGFR α includes exons 12–23, corresponding to the C-terminal portion of the protein, but it has yet to be fully characterized (Manku *et al.* 2014). Moreover, the inhibition of the tyrosine kinase activity of PDGFR using tyrphostin compounds alongside RA treatment led to a significant reduction in the RA-induced *Stra8* mRNA expression, indicating that PDGFR activation is required for RA-dependent induction of gonocyte differentiation. The analysis of downstream pathways commonly activated upon PDGFR activation showed that activation of the SRC family of kinases and the JAK2/STAT5 signaling pathways were both involved in RA-induced gonocyte differentiation (Manku *et al.* 2014). Considering that STAT5 is a possible downstream target of SRC (Drayer *et al.* 2005), it will be important to determine whether it is activated via JAK2, SRC, or another pathway in gonocytes. The presence of a variant PDGFR α form in gonocytes is interesting in view of the existence of a similar variant transcript identified in seminomas, the most common type of TGCTs (Palumbo *et al.* 2002), and because of the possible relationship between improper gonocyte development and TGCT formation (Rajpert-De Meyts & Hoei-Hansen 2007).

Another system that plays a role in gonocyte differentiation is the ubiquitin–proteasome system (UPS), as revealed by studies where we have demonstrated that inhibiting proteasome activation with the specific inhibitors lactacystin and bortezomib significantly decreased the RA-driven induction of *Stra8* expression in rat gonocytes (Manku *et al.* 2012). In this study, we also identified several UPS proteins preferentially expressed in gonocytes, such as the E3 ligase RNF149, that might have a role in gonocyte development. Several UPS proteins have been implicated in the regulation of spermatogenesis, including the E3 ligase Huwe1 (Liu *et al.* 2007). The involvement of the ubiquitin–proteasome system in gonocyte differentiation is not surprising considering the large amount of remodeling taking place during gonocyte development, but further studies are needed to identify the functional enzyme–substrate partners and their respective roles in gonocyte development.

Several hormones have been examined for their potential role in gonocyte differentiation. For example, Zhou & Hutson (1995) have shown that human chorionic gonadotropin (hCG) does not have any effect on gonocyte differentiation in neonatal mouse testis organ cultures, refuting the hypothesis that this process was under the control of the hypothalamic–pituitary–gonadal axis. By contrast, this study showed that the addition of Mullerian inhibiting substance (MIS; also known as anti-Mullerian hormone (AMH)), a protein produced by fetal to juvenile Sertoli cells, significantly increased the number of gonocytes undergoing differentiation into type A spermatogonia, suggesting a role of this protein in the regulation of gonocyte fate (Zhou & Hutson 1995).

More recent studies have demonstrated the importance of NOTCH signaling in the timing of gonocyte differentiation. The NOTCH pathway has been shown to be involved in a multitude of processes, including cell proliferation, cell differentiation, and cell–cell communication. Furthermore, various components of the NOTCH pathway are expressed in neonatal and adult testes in both rodents and humans (Dirami *et al.* 2001). Recent studies have demonstrated that NOTCH signaling in fetal Sertoli cells is critical for the maintenance of fetal gonocytes in a quiescent state and to exert a brake on the ability of fetal gonocytes to undergo differentiation (Garcia & Hofmann 2013). Using a Sertoli cell-specific NOTCH gain-of-function mouse model (*Amh-cre;RosaNICD⁺*), in which the Notch intracellular domain NICD is constitutively activated, leading to the conversion of the transcriptional repressor RBPJ (recombining binding protein suppressor of hairless) to an activator, this study demonstrated that the constitutive activation of NOTCH signaling in Sertoli cells at E13.5 induced gonocyte exit from quiescence, and premature migration and differentiation. At the molecular level, there was a decrease in Sertoli-specific factors that generally help maintain germ cells in an undifferentiated state (i.e. glial-cell-derived neurotrophic factor (GDNF)) and, in POU5F1/OCT4, a transcription factor used as a marker of undifferentiated spermatogonia. Concurrent with these decreases, there was increased expression of markers of differentiating spermatogonia, such as KIT and spermatogenesis- and oogenesis-specific bHLH transcription factor 2 (SOHLH2), indicating that these gonocytes had acquired properties of differentiating spermatogonia (Garcia *et al.* 2013). Given their findings, the authors concluded that NOTCH signaling in Sertoli cells is important for germ cell development and that, when over-activated, NOTCH signaling is able to inhibit quiescence and push germ cell toward differentiation and meiosis (Garcia *et al.* 2013). Interestingly, adult mice in which a *Stra8-icre* transgene was used to induce NOTCH1 gain of function in germ cells also do not have proper spermatogenesis as witnessed by decreased sperm count, decreased testis weight, and abnormal seminiferous tubules

(Huang *et al.* 2013). These defects were presumably due to the failed differentiation of SSCs presenting abnormal activation of NOTCH1, further highlighting the importance of having proper levels of NOTCH1 activation not only in Sertoli cells but also in germ cells.

Interestingly, microRNAs (miRNAs), short untranslated RNA molecules of ~22 nucleotides that regulate the expression of specific mRNA targets, have also been shown to be involved in gonocyte differentiation. Although studies on testicular miRNAs are limited, it is known that there are miRNAs preferentially or specifically expressed within the testis (McIver *et al.* 2012). Using miRNA microarray analysis of isolated gonocytes and spermatogonia from mice, the authors identified miRNAs that were differentially expressed between gonocytes and spermatogonia. In particular, miR-126, miR-743a, and miR-463 were higher in spermatogonia, whereas miR-293, miR-291a-5p, miR-290-5p, and miR-294 were lower in spermatogonia. One of the miRNA clusters downregulated during the transition from gonocytes to spermatogonia, including miR-293, miR-294, miR-291, and miR-290-5p, has been previously shown to play a role in the maintenance of embryonic stem cell (ESC) pluripotency. Functional analysis of these miRNAs indicated a variety of different pathways to which these miRNAs belonged. Of those pathways, it was the PTEN and Wnt/ β -catenin signaling pathways that were associated with the majority of miRNAs, pinpointing to the cell cycle regulator cyclin D1 as a common target of both pathways. Given the known role of Cyclin D1 in promoting cell progression to S phase, the authors proposed that the observed miRNA changes between gonocyte and spermatogonia might be related to their role in gonocyte differentiation (McIver *et al.* 2012). However, exactly what role these signaling pathways are playing in gonocyte differentiation remains yet to be determined.

Finally, what determines which gonocytes will undergo differentiation, including SSCs or first-wave fates, and which ones are destined for apoptosis are not known. Using isolated rat gonocytes from PND0–PND4, phase-contrast microscopy, Annexin V staining, and transplantation studies, Orwig *et al.* (2002) have determined that gonocytes with pseudopods were probably destined to undergo migration and differentiation, whereas gonocytes without pseudopods were destined to undergo apoptosis and be eliminated. Thus, it might be possible to distinguish functional subsets of gonocytes according to their *in vitro* morphology. Different subsets of isolated neonatal rat gonocytes can also be distinguished according to their size, including a group of very large cells (> 12 μ m diameter) and a group of medium size (10–12 μ m diameter), both populations being much larger than the somatic cells (5–8 μ m diameter) isolated from the same testes (Culty, unpublished data). Moreover, we have observed that mitotic gonocytes present daughter cells with different

immunoreactions for a number of proteins, suggesting that gonocyte division is asymmetrical (Culty, unpublished data). Similarly, studies have shown that there is asymmetrical division in SSCs as well (Luo *et al.* 2009). Further studies will be required to determine whether these differences correspond to cells with distinct fates and/or functions within the transitional gonocyte population.

In summary, various signaling pathways, such as those involving TGF β superfamily ligands, RA, variant forms of PDGFRs, WNTs, and PTEN, have emerged as potential regulators of transitional gonocyte differentiation. However, more work is required to determine the target molecules of these pathways, and how these different pathways communicate with each other in order to fine tune gonocyte differentiation.

Spermatogonia and SSC differentiation

Although more commonly studied than gonocytes, most work carried out on SSCs have focused on the regulation of their self-renewal potential, a process required for maintaining an adequate pool of stem cells. One major challenge in studying the regulation of SSC differentiation has been the lack of means to isolate this specific subpopulation from other undifferentiated spermatogonia. A protein that has been instrumental over the last decade for the characterization of genes preferentially or highly expressed in SSCs is THY1 (CD90), a glycoprotein expressed at the surface of undifferentiated spermatogonia, including SSCs (Ryu *et al.* 2004). Indeed, Thy1 immunosorting has allowed for the preparation of spermatogonial populations enriched in SSCs. Yet, the gold standard for identifying true SSCs remains the functional transplantation assay carried out in germ-cell-depleted mice, which allows assessing whether transplanted cells can re-initiate spermatogenesis. This approach has been used to determine the impact of chemical or physical insults, such as chemotherapy and radiotherapy cancer treatments, as well as the role of genes following their silencing in germ cells (Brinster & Avarbock 1994, Brinster & Zimmermann 1994, McLean *et al.* 2003). However, it is a delicate and time-consuming method, impractical for the performance of large-scale assays. Moreover, transplantation studies provide only indirect information regarding the stemness nature of cells, as the presence of SSCs is deduced from the numbers of colonies formed over a period of 2–3 months in recipient testes. Thus, there is still limited information on the mechanisms behind SSC differentiation.

The identification of genes highly expressed in SSCs has also been critical in the study of SSC differentiation. Thus, genes involved in SSC self-renewal will be briefly mentioned below, because of their use in isolating SSC-enriched spermatogonia and characterizing SSCs, and because some of their gene products were shown to actively repress spermatogonial differentiation. Among

the most frequently cited SSC markers are the GDNF receptors GFR α 1 and RET, alongside OCT4 (POU5-F1/OCT3/4), PLZF (promyelocytic leukemia zinc finger; ZBTB16/ZFP145), LIN28A, BCL6B, NGN3, ID4, CDH1, UTF1, and SALL4 (reviewed by Nagano & Yeh (2013) and Song & Wilkinson (2014)). In contrast to the regulation of SSC maintenance, there are fewer proteins that have been implicated in the process of spermatogonial differentiation, such as the RA receptor RAR γ , and the RA-induced proteins STRA8 and KIT, SOHLH1 and SOHLH2, shown to be upregulated during SSC differentiation (Oatley & Brinster 2012). Moreover, the exact mechanisms through which these proteins act and their respective roles during differentiation remain yet to be determined.

It is known that RA is an upstream regulator of KIT and STRA8 expression during the transition from undifferentiated to differentiated spermatogonia, making them convenient markers in the distinction of these two germ cell phases (Schrans-Stassen *et al.* 1999, Zhou *et al.* 2008a,b). Similarly, we have shown that RA treatment upregulates *Stra8* and *Kit* mRNA expression in neonatal rat gonocytes (Wang & Culty 2007). As RA also stimulates the differentiation of PND5–PND6 mouse spermatogonia, it is presumed that it is inducing SSC differentiation. However, the definitive demonstration of this fact would require the ability to distinguish SSCs from first-wave spermatogonia during the first postnatal week in rodents, which, as explained above, still remains a challenge, due to the lack of a marker restricted to SSCs. Thus far, it is not clear whether predetermined subsets of gonocytes are destined to either the first wave or SSCs, or if specific combinations of factors, probably including RA, combined with differential intratubular locations, will dictate which gonocytes become SSCs or enter the first-wave process. Similarly, there is no information on whether the progression of the first-wave germ cells from type A to B spermatogonia involves the same factors as in subsequent postpubertal spermatogenic waves. However, several studies have described conditions, either knockout models or toxicant exposures, in which the germ cells of the first wave were spared and progressed through part or all of the first spermatogenic cycle, whereas subsequent cycles were impaired, leading to the inability to further generate differentiated germ cells. Rather than identifying mechanisms involved in the differentiation of first-wave spermatogonia, these studies provided information on molecules that are not required for this process, but are involved either in a later step of spermatogenesis or in SSC formation. However, to our knowledge, there is no report of an experimental or pathological condition in which the formation of the first-wave spermatogonia would be disrupted, but not that of SSCs. Despite these uncertainties, a number of genes and mechanisms have been proposed to induce SSC differentiation. This section will review some of the well-established and novel

inducers of spermatogonial differentiation during the first postnatal week in rodents, a period in which SSCs represent the highest proportion of germ cells, as distinct from later ages.

SSCs arise from RA-induced gonocyte differentiation, forming a stem cell reservoir located in a niche constituting somatic cells, principally Sertoli cells and peritubular myoid cells, which provides nutrients and regulatory factors to the SSCs (reviewed in Oatley & Brinster (2012)). At the same time, a small subset of gonocytes is believed to bypass the SSC phase to directly differentiate into the spermatogonia of the first spermatogenic wave (Yoshida *et al.* 2006). The first wave of spermatogenesis is less efficient and encompasses larger levels of apoptosis than subsequent steady-state cycles of spermatogenesis, and it is likely that the spermatozoa resulting from this first round may not be fertile (Kluin *et al.* 1982, Mori *et al.* 1997, Yoshida *et al.* 2006). Based on lineage analysis studies in association with transplantation assays, and using the basic helix–loop–helix transcription factor neurogenin 3 (*Ngn3/Neurog3*) as a marker of undifferentiated spermatogonia, including SSCs, and *Kit* as a marker of differentiating spermatogonia, Yoshida *et al.*'s study suggested that the first wave arises from the direct differentiation of NGN3[−] gonocytes into NGN3[−] spermatogonia, distinguishable from the pool of NGN3⁺ SSCs at the origin of steady-state spermatogenesis. In a subsequent study, these authors followed NGN3⁺ spermatogonia fate using pulse-chase experiments in tamoxifen-inducible *Ngn3/CreER;CAG-CAT-Z* transgenic mice, generated by crossing tamoxifen-inducible *Ngn3-CreER* mice with *CAG-CAT-Z* mice (with the *Cat* gene floxed by loxP-preventing *lacZ* expression) (Nakagawa *et al.* 2007). This strategy allowed the authors to perform pulse-chase labeling of NGN3-expressing cells for different periods of time, and to follow their fate in transplantation and regeneration studies using *lacZ* as a reporter gene for up to 1 year after labeling. These studies unveiled the co-existence of two distinct populations within the *Ngn3*⁺ undifferentiated (*A*_{single}, *A*_{pair} and *A*_{aligned}) spermatogonia, one constituting true SSCs and the other corresponding to a transit amplifying population of cells ('potential stem cells') that retained the ability to self-renew in specific conditions, where the replenishment of the SSC pool was required. However, a later study using additional markers proposed that true SSCs are included among a group of *Ngn3*[−] undifferentiated spermatogonia, and that NGN3⁺ spermatogonia represent transit amplifying progenitor spermatogonia issued from the SSCs (Suzuki *et al.* 2009). This difference illustrates a recurring problem linked to the difficulty of establishing definitive SSC identity (Hermann *et al.* 2011). Indeed, over the years, many genes labeled as SSC markers were subsequently found to be expressed in other types of undifferentiated spermatogonia apparently committed to differentiation. This incapacity of unequivocally delineating SSCs

among undifferentiated spermatogonia is consistent with the hypothesis that spermatogonia-type *A*_{pair} or *A*_{aligned} might be able in specific conditions to revert to SSCs (Nakagawa *et al.* 2007, 2010).

While the studies mentioned above analyzed the expression profiles of NGN3 in spermatogonial populations, others examined the role of NGN3 in spermatogonial differentiation. NGN3 was proposed to drive the differentiation of mouse SSCs and progenitor spermatogonia, as a downstream effector of STAT3-induced differentiation (Kaucher *et al.* 2012). Using THY1⁺ undifferentiated spermatogonia and transplantation assays, these authors showed that the transient inhibition of STAT3 or addition of GDNF both led to decreased NGN3 expression and increased self-renewal potential of Thy1⁺ cells. While STAT3 was shown to regulate *Ngn3* transcription, *Ngn3*-deficient THY1⁺ cells failed to differentiate in transplantation assays. These data concurred with earlier transplantation studies reporting that, upon decreased STAT3 activity, cells could not differentiate beyond the undifferentiated spermatogonia stage in recipient testes (Oatley *et al.* 2010). Taken together, these studies demonstrated the involvement of STAT3 and NGN3 in the differentiation of SSCs and progenitor spermatogonia. This is different from the role of STAT3 in *Drosophila*, where it is necessary for SSC self-renewal (Sheng *et al.* 2009). It is noteworthy that our studies in neonatal gonocytes showed that the RA induction of STRA8 expression also requires an active Stat, in this case, the JAK2/STAT5 pathway (Manku *et al.* 2014).

During SSC differentiation, RA downregulates ZBTB16/PLZF, which in turn probably leads to the increased expression of KIT in differentiating spermatogonia (Suzuki *et al.* 2012). Alongside increased KIT expression, RA-induced spermatogonia differentiation also results in increased expression of SOHLH1 (Suzuki *et al.* 2012), which in turn functions to increase KIT expression as well (Barrios *et al.* 2012). The role of RA in spermatogonial differentiation was further clarified using *Rbp4* null mice, in order to prevent the formation of RA from endogenous retinol pools (Ghyselinck *et al.* 2006). RA and retinol were reported to induce the *in vitro* differentiation of spermatogonia, using an organotypic culture system of fresh and frozen PND6 and PND7 mouse testes (Travers *et al.* 2013). Spermatogonia and primary spermatocytes were visualized using Tra98 immunostaining, which detects germ cell nuclear antigen (GCNA), while undifferentiated and differentiated spermatogonia were identified using Plzf⁺ and Kit⁺ immunostaining respectively. The authors concluded that *in vitro* SSC differentiation and subsequent spermatogenesis were feasible even using frozen testes, thus representing an attractive approach that could potentially be used in the future to restore fertility in patients. Barrios *et al.* have shown that PND4 isolated NGN3⁺, KIT⁺ mouse germ cells express high

levels of NANOS2, a RNA-binding protein involved in SSC self-renewal and in the suppression of spermatogonial differentiation (Suzuki & Saga 2008, Suzuki *et al.* 2009, Sada *et al.* 2012), in response to FGF9, a growth factor produced by Sertoli cells (Barrios *et al.* 2010). By contrast, the addition of RA reduced NANOS2 expression and concomitantly induced KIT and STRA8 expression in these cells (Barrios *et al.* 2010). Moreover, in PND7 mice, PLZF, a transcription factor required for SSC maintenance (Buaas *et al.* 2004), was shown to act as a repressor on the *Kit* promoter, preventing KIT expression, whereas isolated Thy1⁺ spermatogonia from *Plzf*^{-/-} mice showed more than a doubling in their KIT expression (Filipponi *et al.* 2007). Using cell line models and whole-mouse testes, Zhang *et al.* (2013a) reported that long and short transcripts of *Kit* are expressed in spermatogonia, the long transcript being mainly expressed in the cytoplasm and cell membrane, while the short transcript was found in nuclei as well as the cytoplasm and cell membrane. They also reported that the long transcript of *Kit* was not expressed in SSCs, in line with other studies (Payne 2013). This group concluded that there are extensive transcriptional and translational changes in KIT expression before and after SSCs differentiation (Zhang *et al.* 2013a).

The same authors reported that RA decreased the expression of BMP4 in PND5 mouse testes, but not at PND10 where it was increased by 0.7 μ M RA, while having no effect on BMP4 expression at PND60. These data suggest that RA exerts different effects on BMP4 in function of age. While the suppressing effect of RA on BMP4 expression at PND5 is surprising, the increase observed at PND10 agrees with earlier studies. Indeed, BMP4 was found to decrease the stem cell maintenance of adult mouse SSCs (Nagano *et al.* 2003), as well as to induce SSC differentiation by affecting cell adhesion pathways and cytoskeletal proteins in the rat cell line GC-6spg, used as an SSC model (Carlomagno *et al.* 2010). Moreover, it has been shown that when mouse SSCs undergo differentiation in the presence of BMP4, there is downregulation of PLZF, a pluripotency marker involved in SSC self-renewal, and upregulation of the differentiation marker KIT. During this process, SMAD1/5/8 proteins, common downstream components of BMP signaling cascades, are activated. BMP4 was also shown to exert its effect via upregulation of SOHLH2 (Hao *et al.* 2008), a transcription factor known to promote the differentiation of both SSCs and the cells these SSCs become in knockdown studies (Suzuki *et al.* 2012). Overall, these studies showed that RA increases BMP4 expression in SSCs, which in turn promotes SSC differentiation to progenitor cells by increasing SOHLH2 and KIT expression, while repressing the self-renewing potential of SSCs by decreasing PLZF expression. The importance of SOHLH1 and SOHLH2 in spermatogonial differentiation was highlighted in studies showing that SOHLH1/SOHLH2 KO mice had

functional spermatogonial proliferation but improper differentiation. It was also determined that SOHLH proteins were able to regulate *Gfra1*, *Sox3*, and *Kit* gene expression. Overall, this study found that SOHLH1 and SOHLH2 were able to induce genes necessary for spermatogonial differentiation, while at the same time suppressing genes involved in stem cell maintenance (Suzuki *et al.* 2012).

Regarding the receptors involved in these processes, Gely-Pernot *et al.* (2012) have shown that RARG (RAR γ) is expressed in A_{aligned} spermatogonia and during the A_{aligned} to A₁ transition, but not in A_s or A_p spermatogonia. RARG ablation in adult mice prevented the RA-induced transition from A_{aligned} to A₁ spermatogonia and led to testicular tubules missing germ cell layers. By contrast, in prepubertal mice, the absence of RARG could be compensated by RARA, as shown by the occurrence of a normal first spermatogenic wave, as well as the worsening of the phenotype in mutant mice with *Rara/g*^{-/-} spermatogonia (Gely-Pernot *et al.* 2012). Contrary to mice made VAD at birth, in which only Sertoli cells and undifferentiated GFRA1 and ZBTB16/PLZF-positive spermatogonia could be found, the testes from *Rarg*^{-/-} mice contained spermatogonia expressing KIT and STRA8, as well as GFRA1 and ZBTB16/PLZF, demonstrating more progression in germ cell differentiation. Similarly, the simultaneous inactivation of RARA and RARG in PND3 spermatogonia was not sufficient to totally prevent the formation of germ cells beyond the undifferentiated stage in all tubules. These data suggest that RA-dependent pathways other than those of RARA and RARG might play a role in prepubertal spermatogonial differentiation.

As in gonocytes, miRNA species have emerged as important regulators of mouse SSC differentiation. However, the two developmental phases appear to involve different families of miRNAs. Previous studies had shown that miR-34c and miR-21 are highly expressed in SSC-enriched Thy1⁺ mouse spermatogonia, and showed a role of miR-21 in SSC self-renewal potential, but did not study the function of miR-34 in these cells (Niu *et al.* 2011). Other studies on ovarian primary epithelial and cancer cells had reported that miR-34 expression decreased cell proliferation upon p53-dependent activation (Corney *et al.* 2007). These data suggested that miR-34 might have an opposite role to miR-21 by promoting differentiation rather than self-renewal. Indeed, the over-expression of miR-34C in mouse PND6 undifferentiated spermatogonia was found to decrease the expression of NANOS2, leading to the upregulation of NANOS3 and STRA8, markers of spermatogonial differentiation, and premeiosis transition, and that of the meiotic marker SCP3 (Yu *et al.* 2014). These results suggest that spermatogonial differentiation requires not only the induction of differentiation factors, but also the removal of functional brakes such as NANOS2, probably responsible for preventing premature differentiation.

The roles of miRNAs in spermatogonial differentiation was further investigated by the team of Dr M Griswold, who showed that the expression of miR-17-92 (Mirc1) and miR-106b-25 (Mirc3) paralog clusters was significantly decreased during the RA-induced differentiation of PND7 THY1⁺-enriched undifferentiated mouse spermatogonia (Tong *et al.* 2012). Among the Mirc1 cluster, expression of miR-18a, miR-20a, and miR-92 was mainly observed at PND3, while miR-17 was the highest at PND3 and progressively decreased with age. RA-induced decreases in Mirc1 and Mirc3 in THY1⁺-enriched spermatogonia led to increased expression of *Bcl2l11* (*Bim*), *Kit*, and *Stat3*, important factors in spermatogonial differentiation (Tong *et al.* 2012). *Mirc1* KO mice had lower epididymal sperm but increased Mirc3 cluster, suggesting interactions between the two clusters. These studies positioned Mirc1 and Mirc3 clusters as negative regulators of undifferentiated spermatogonia. Another miRNA shown to regulate spermatogonial differentiation is miR-146, which was also downregulated in RA-treated undifferentiated spermatogonia (Huszar & Payne 2013). Alongside this decrease, there was a significant decrease in *ZBTB16/PLZF* expression and increase in *Kit* expression. Moreover, the over-expression of miR-146 led to decreased expression of *Stra8*, *Kit*, and *Sohlh2*, genes normally associated with spermatogonial differentiation (Huszar & Payne 2013).

Altogether, these studies demonstrated the participation of several miRNA species as either positive or negative regulators of undifferentiated spermatogonia differentiation. Considering that only a fraction of SSCs commit to differentiation at any given time, it would be interesting to examine whether the temporal and spatial fine tuning of SSC differentiation involves the expression of different sets of miRNAs at different locations and times within the seminiferous tubules.

Another potential regulator of SSC differentiation is activin A, encoded by the gene *Inhba*, for which contrasting studies are available. Nagano *et al.* (2003) have shown that activin A decreased stem cell maintenance in SSCs from PND5–PND8 mice co-cultured with TM4 or SF7 Sertoli cell lines, and the ability of these cells to form colonies in transplantation assays, similar to BMP4. As activin A has been previously shown to induce the proliferation of spermatogonia *in vitro* (Mather *et al.* 1990), the authors proposed that activin A may decrease the SSC pool by increasing their recruitment to the subset of cells committed to differentiation and induced to mitosis by activin A (Nagano *et al.* 2003). However, another group using PND0–PND7 *Inhba* knockin mice (*Inhba*^{BK/BK}) in which the mature coding subunit sequence of *Inhba* had been replaced by the lower affinity *Inhbb* domain sequence proposed that a reduction of bioactive activin A levels led to the premature increase in markers of differentiated germ cells such as KIT, and suggested that activin A is

critical for the proper coordination of germ cell maturation, as well as for Sertoli cell proliferation (Mithraprabhu *et al.* 2010). Interestingly, differentiating neonatal gonocytes were shown to be negative for activin A protein expression, with *Inhba* mRNA presenting a sharp decrease between PND3 and PND6 in mice, and instead to express the activin inhibitor follistatin (Meehan *et al.* 2000, Mithraprabhu *et al.* 2010). Simultaneous to the decrease in activin A during the first postnatal week, there was a gradual increase in its inhibitor follistatin, followed during the second postnatal week by an increase in inhibins (Barakat *et al.* 2008). These studies indicate that activin A and its negative regulators follistatin and inhibins are tightly regulated during the first postnatal week and probably play a critical role in controlling the commitment of cells to differentiate. Further studies are required to determine more precisely the timing of activin A expression and the mechanisms mediating its effects on the fate decision of SSCs.

Although the main function of GDNF, a member of the TGF β superfamily acting via the two receptor system of GFRA1 and RET, is to stimulate SSC self-renewal and survival, it has also been shown to promote differentiation in specific conditions (Meng *et al.* 2000). Indeed, GDNF expression levels are considered to play a pivotal role in controlling the fate of SSCs between self-renewal and differentiation, with high GDNF levels inducing SSCs self-renewal and low GDNF levels promoting differentiation (Meng *et al.* 2000). Other studies have demonstrated that the determination of which SSC undergoes self-renewal or differentiation is a completely random process that does not depend on its surrounding environment (Wu *et al.* 2009). Several studies have converged to support the existence of a signaling network in which PLZF and GDNF could interact at the level of mTORC1 to prevent SSC commitment to differentiation. First, GDNF was shown to stimulate the PI3K/AKT pathway in spermatogonia (Braydich-Stolle *et al.* 2007). Then, GDNF was reported to activate mTORC1 in SSCs, which was shown to deplete the SSC pool by favoring SSC differentiation rather than self-renewal (Hobbs *et al.* 2010). The connection was provided in the same study, where PLZF was shown to prevent mTORC1 activation by inducing its inhibitor, Redd1, promoting self-renewal against differentiation. Another level of regulation was added through the ability of mTORC1 to inhibit AKT (Sabatini 2006). Finally, PLZF was reported to negatively interact with SALL4, a pluripotency transcription factor preferentially expressed in spermatogonial progenitor cells, where it induces differentiation, as well as in germ cell tumors (Hobbs *et al.* 2012). Another factor that might regulate spermatogonial progenitor cell differentiation is the epithelial cell adhesion molecule EPCAM, which was originally proposed as a marker for the enrichment of SSCs. Using transplantation assays, Kanatsu-Shinohara *et al.* showed that EPCAM⁺-enriched cells contained

a limited amount of cells with stem cell potential, contrary to germ cells enriched by flow cytometry for the tetraspanin CD9⁺ that expressed stronger SSC potential. The study showed that EPCAM expression was increased in response to continuous exposure to GDNF, but not FGF2, in mouse spermatogonia. Moreover, overexpression of EPCAM in germ cells from Rosa mice did not affect their SSC potential in transplantation assays, while suppression of EPCAM increased the concentration of SSCs (Kanatsu-Shinohara *et al.* 2011). These findings implied that the suppression of EPCAM prevented spermatogonia from differentiating. The existence of these multi-layered signaling pathways, although not always identified in the same phase of spermatogonial development, illustrates how self-renewal and commitment to differentiation can be modulated at different interacting levels.

In search of factors involved in SSC differentiation, a study identified neuregulin 1 as inducing the formation of A_{aligned} spermatogonia chains in rat, when used in combination with GDNF (Hamra *et al.* 2007). This study took advantage of the different fates of spermatogonia when grown on a monolayer of the MSC-1 Sertoli cell line, which promotes stem cell maintenance, vs cells grown on the mouse embryonic fibroblastic SNL feeder layer cell line, which appears to induce differentiation. While the addition of exogenous GDNF alone to spermatogonia grown on Sertoli Cell-modified Laminin induced the formation of 4–8 cell chains, the simultaneous addition of GDNF and SNL cell-conditioned medium led to the formation of up to 32-cell long chains of A_{aligned} spermatogonia. The purification and mass spectrometry analysis of the substance in the cell-conditioned medium responsible for this effect revealed that it was neuregulin 1. Thus, neuregulin 1 was proposed to act *in vitro* as a spermatogonial amplification and differentiation factor in a GDNF-dependent manner, with GDNF targeting SSCs to become A_{paired} and neuregulin inducing A_{paired} to differentiate into A_{aligned} spermatogonia. This effect was attributed to the binding of neuregulin 1 on neuregulin receptor ErbB3, complexed to ErbB2, both present in undifferentiated spermatogonia (Hamra *et al.* 2007).

The complexity of GDNF effects is further illustrated by studies showing that it can simultaneously promote SSC self-renewal and repress spermatogonial differentiation. This repressive effect on differentiation was proposed to be mediated by the GDNF-dependent upregulation of the expression of NUMB, a known repressor of NOTCH. NOTCH signaling regulates stem cell fate choices and can promote stem cell self-renewal or differentiation, depending on the surrounding cues (Braydich-Stolle *et al.* 2005). NUMB inhibits the NOTCH pathway by interacting with activated NOTCH receptor (NICD), leading to its degradation. Thus, the GDNF-regulated production of NUMB may be an important factor in the maintenance of the SSC pool by

inducing NOTCH1 degradation (Braydich-Stolle *et al.* 2005). GDNF is also important in maintaining the expression of NANOS2, which, as mentioned above, also prevents differentiation. Another key gene in the timing and extent of spermatogonial differentiation is *Dmrt1*, which was shown to repress the RA-induced transcription of *Stra8*, thus restricting the responsiveness of the cells to RA by limiting their differentiation to premeiotic changes (Matson *et al.* 2010). It is important to note that although factors such as BMP4, activin A, GDNF, and neuregulin 1 are probably involved in SSC differentiation, they must be properly controlled in order to keep a balance between the SSC self-renewal and differentiation processes.

In addition, Zheng *et al.* have recently used peptide tandem mass tag (TMT) labeling for the proteomics analysis of mouse neonatal testes from PND0.5–PND5.5, in an effort to identify proteins specifically involved in early phases of mouse spermatogenesis. The comparison of these data with existing databases led to the identification of several potential candidate proteins possibly involved in gonocyte and SSC maturation. The germ cell expression of some of these proteins was confirmed by immunohistochemical analysis of PND3.5 and adult testes, identifying the presence of NUP153, SUZ12, and scaffold attachment factor B2 (SAFB2) in P3.5 undifferentiated spermatogonia, while whole-testes immunoblot analysis indicated their upregulation between PND3.5–PND5.5, suggesting potential roles in SSC maturation (Zheng *et al.* 2014). NUP153 is a nucleoporin protein that has not been previously shown to be involved in spermatogenesis. Nuclear transport of regulatory factors was proposed as a key regulatory process in germ cell differentiation (Hogarth *et al.* 2005). Thus, it is not surprising that such proteins would be involved in SSC differentiation. Interestingly, we have found by gene expression array analysis that *Nup153* and *Safb2* mRNAs are both expressed in PND3 gonocytes, at high level in the case of *Nup153*, suggesting that these genes might also play a role in gonocyte development (Manku and Culty, unpublished data). Furthermore, SUZ12 is a component of the polycomb repressive complex (PRC2) and *Suz12*^{−/−} mice were found to have abnormal ESC differentiation. Thus, it is possible that a lack of SUZ12 would also negatively affect SSC differentiation. SAFB has previously been shown to be involved in SSC differentiation in rodents (Sergeant *et al.* 2007), in agreement with the postulate from the proteomic analysis.

Mouse SSCs are also able to transdifferentiate into hepatic-stem cell-like cells, which in turn, will differentiate into mature hepatocyte-like cells *in vitro*. This transdifferentiation requires activation of the ERK1/2 and Smad2/3 signaling pathways (Zhang *et al.* 2013b). Interestingly, we have previously shown that ERK1/2 activation is necessary for gonocyte proliferation, but that it does not participate in gonocyte differentiation

(Thuillier *et al.* 2010, Manku *et al.* 2014). The ability of SSCs to transdifferentiate into mature hepatocytes is quite remarkable, illustrating the high plasticity of these unipotent stem cells, capable of reverting to pluripotency under specific conditions, representing potential clinical use in the treatment of liver diseases (Zhang *et al.* 2013b). SSCs were proposed to have two potential therapeutic uses, one as a source of reprogrammable pluripotent stem cells that could be used for tissue regeneration (Nayernia 2007), the other as a reservoir of stem cells for the regeneration of spermatogenesis following cancer therapies (Valli *et al.* 2014).

Similar to gonocytes, in which WNT signaling pathways emerged as potentially involved in differentiation through miRNA studies, canonical and non-canonical WNT signaling pathways were proposed to play a role in spermatogonial functions. In particular, Wnt5a, probably produced by Sertoli cells, was shown to promote SSC survival and self-renewal via a non-canonical process while blocking β -catenin-dependent responses (Yeh *et al.* 2011), whereas WNT3A was found to be indirectly supporting SSCs by selectively stimulating the proliferation of progenitor spermatogonia committed to differentiation in a β -catenin-dependent manner (Yeh *et al.* 2012). These data suggest that WNT signaling pathways are involved both in gonocyte and spermatogonial functions, probably proliferation and differentiation, but further studies will be needed to clarify their exact functions.

Thus, although SSCs are an essential part of spermatogenesis, the processes and signaling mechanisms through which SSCs and other types of undifferentiated spermatogonia differentiate have not been sufficiently studied to fully comprehend the complexity of their regulation, due to their small cell number, SSCs being only 0.03% of the total testicular germ cells (Tegelenbosch & de Rooij 1993), and the difficulty of conclusively distinguishing them from other undifferentiated spermatogonia. However, the recent advancements in this field suggest that new technologies should soon become available to better study SSC differentiation.

Insights from non-rodent species

Although the most common models in the study of spermatogenesis are rodents, other animals have been used, including boars, pigs, fish, worms, and fruit flies. A small numbers of studies have also been carried out on fetal to juvenile primates, including humans. The majority of these studies focused on developing methods to isolate and culture germ cells, transplantation assays of SSCs into recipient testes from autologous or heterologous species, or the determination of gene markers to classify various germ cell types. Despite being a growing field of research, few studies have been performed on the differentiation of gonocytes, SSCs, and spermatogonia in these non-traditional animal models.

Similarities in gene expression profiles between rodent and primate gonocytes have been reported, in support of using rodent models to study early germ cell development. However, most of these studies focused on pluripotency genes that were also retained in carcinoma *in situ* and TGCTs. Typical examples are the transcription factors NANOG, OCT4, TFAP2C (AP2 γ), and the miRNA-binding protein LIN28A, which are expressed not only in rodent and human fetal PGCs and gonocytes (Culty 2009, Weber *et al.* 2010), but also in testicular tumors (Hoei-Hansen *et al.* 2005, Rajpert-De Meyts 2006, Sonne *et al.* 2009, Gillis *et al.* 2011, Aeckerle *et al.* 2012). In mouse and human ESCs, both NANOG and OCT4 were found to belong to a protein network regulating cell pluripotency, which included also Wnt and its downstream target β -catenin (Abu-Remaileh *et al.* 2010, Marucci *et al.* 2014). In ESCs, OCT4 was proposed to control ESC fate decision by inducing β -catenin degradation and preventing Wnt-regulated differentiation. As mentioned earlier, the role of the Wnt/ β -catenin pathway in spermatogonia is more complex. Interestingly, LIN28A was found to localize to the nuclei and cytosol of neonatal gonocytes, but to be restricted to the cytosol in spermatogonia, suggesting differential roles in gonocytes and spermatogonia (Gaytan *et al.* 2013). Moreover, despite their stem cell status, SSCs were found to express none or very little of NANOG, OCT4, and TFAP2C, while maintaining higher levels of two other ESC pluripotency genes, *Thy1* and *Plzf* (Culty 2009). These changes suggest that spermatogonial differentiation includes the active repression of selected pluripotency factors both in rodents and humans.

Given the limited availability of fetal and neonatal human testicular biopsies and the small numbers of germ cells that can be isolated from such samples, very few studies have examined the early steps of germ cell development in humans. At later ages, studies on SSCs have generally revolved around the maintenance of the SSC pool or the regeneration of spermatogenesis using SSCs isolated from pathological testes, after testicular insult/injury or chemotherapy treatments. Thus, although the steps leading to the formation of the SSC pool are critical for human spermatogenesis, these processes are seldom studied and the mechanisms that govern human gonocyte and spermatogonial differentiation are not well understood. Several studies have used primate models as surrogate for humans. Among them, studies in marmosets (*Callithrix jacchus*) have shown that germ cell differentiation occurs in a manner similar to that observed in humans, when analyzed at the level of common gene expression and cellular processes (Mitchell *et al.* 2008). At the molecular level, Mitchell *et al.* have shown that marmoset gonocyte differentiation results in decreased expression of the pluripotency markers OCT4 and NANOG and increase in *VASA* (*DDX4*) gene expression. Moreover, both humans and

marmoset gonocyte differentiation occurred in an asynchronous manner within individual tubules (Mitchell *et al.* 2008). Thus, marmoset was proposed to be an ideal model to study gonocyte differentiation. However, the exact molecular mechanisms and signaling pathways of differentiation in the human and marmoset also remain yet to be determined.

Although they follow similar developmental patterns and appear to involve the same sets of genes/pathways overall, human and rodent spermatogenesis differs in some aspects of their fetal to neonatal developmental phases. A major difference is that rodent testis presents better temporal segregation of germ cell sub-types during perinatal periods than humans or marmosets do. Indeed, despite the heterogeneity of rodent gonocytes and spermatogonial populations, one can still define major developmental phases in which one germ cell type is dominant (Culty 2009, 2013). By contrast, in human and marmoset, a survey of available data on gene expression profiles, cell behaviors (e.g. proliferation or migration), and cell positioning within seminiferous cords (central vs basal location) clearly shows large overlaps of gonocyte and spermatogonial sub-populations for a period spreading from late gestation to early infancy (Sharpe *et al.* 2003, Franke *et al.* 2004, Gaskell *et al.* 2004, Pauls *et al.* 2006, Jørgensen *et al.* 2012, Ewen *et al.* 2013, McKinnell *et al.* 2013, O'Shaughnessy & Fowler 2014).

Another difference resides in the fact that rodent gonocyte undergoes two phases of proliferation in the fetal and neonatal periods, respectively, separated by a proportionally long period of quiescence, whereas humans and marmosets lack this quiescent phase, and instead, one can find proliferative gonocytes in all periods surveyed, from gestation weeks (GWs) 12–14 until 2.5–4 months after birth in human, albeit at various levels (Hilscher & Engemann 1992, Berensztejn *et al.* 2002, Honecker *et al.* 2004, Ewen *et al.* 2013). Overall, these studies reported the highest rates of proliferation between the third and fifth gestation months, followed by decreased proliferation in late gestation and a small increase in proliferation in 2.5- to 6-month-old infants. Thus, despite the lack of total quiescence, human fetal gonocytes present a period of minimal proliferation similar to the quiescence phase observed in rodents. Moreover, it is interesting to note that the late gestation period of low proliferation in human fetal gonocytes partially overlaps with the phase of highest DNA methyltransferase expression (GW21–GW29), strikingly reminiscent of the high DNA methylation activities observed during late gestation in rodent gonocytes (Galetzka *et al.* 2007). In marmoset, in which gestation lasts 22 weeks, similar fluctuations in proliferation rates were observed, with maximal proliferation at GW11–GW14, a decrease at GW15–GW16, followed by an increase to 40% of proliferating gonocytes at GW17–GW20, and a return to a lower rate until 6 weeks after birth (Mitchell *et al.*

2008). A subsequent study by the same group concurred with a proliferation index of 29% in GW12–GW17 fetal gonocytes, decreasing to values of ~10% in infants from the first postnatal week to 5 months (McKinnell *et al.* 2013). Interestingly, we have observed an *in vivo* proliferation rate of ~30% in PND3 rats, similar to the values found in marmoset late fetal testes (Thuillier *et al.* 2009). Taken together, the transient decrease in proliferation rates and the concomitant high levels of DNA methylation in late gestation suggest that this period in human germ cell development is comparable to the fetal quiescent phase observed in rodents.

Another important discriminatory factor during gonocyte development is whether or not they have reached the basement membrane of the tubules, a prerequisite to differentiation. In rat, it is very rare to see a gonocyte not centrally located at PND3, whereas this can happen earlier in mice. This is very different from human and marmoset, in which germ cell relocation can be observed already *in utero*. In GW15–GW18 human testes, the quantification of more differentiated Vasa⁺ (30% of total gonocytes) and more pluripotent Oct4⁺ gonocytes (70% of total gonocytes), located either centrally or basally within the tubules, showed that one out of three Oct4⁺ cells had already relocated to the basement membrane, as well as two out of three Vasa⁺ cells (McKinnell *et al.* 2013). In marmoset, there were 26% of Vasa⁺ gonocytes located at the base of the tubules at GW12–GW17, while at birth, 31% of gonocytes were at the basement membrane (McKinnell *et al.* 2013). However, at postnatal weeks 18–24 (early infancy), there were still 56% of the cells with a typical gonocyte morphology centrally located, and their total numbers had increased by several fold, demonstrating the continuation of proliferation up to 6 months of age (Sharpe *et al.* 2003, McKinnell *et al.* 2013). These various studies provided indirect benchmarks on the timing of gonocyte and spermatogonial differentiation in comparison to rodents, and highlighted genes acting as potential gatekeepers that may need to be repressed for differentiation to proceed.

The study of gonocytes in Mongolian gerbils (*Meriones unguiculatus*) has revealed that, similar to rats, gonocytes must migrate to the seminiferous tubule basement membrane in order to differentiate. However, in the gerbil, the relocation period is much longer, extending to the second postnatal week (Pinto *et al.* 2010). This relocation also occurs simultaneously with a loss in androgen sensitivity (Pinto *et al.* 2010). The pig is another animal model in which early steps of germ cell development have been studied, because of their potential use in deriving pluripotent cells for the generation of transgenic farm animals (Alberio & Perez 2012). In pigs, gonocytes will proliferate, migrate to the basement membrane of the tubules, and differentiate to SSCs during the first 3 months of life. These gonocytes can be identified by the marker dolichos biflorus agglutinin

Figure 1 Summary diagram of the factors regulating transitional gonocyte and spermatogonial differentiation. In both cases, RA induces differentiation. Additional factors shown to induce gonocyte or spermatogonial differentiation are also shown. Green boxes: genes/proteins positively involved in differentiation. Red boxes: genes/proteins negatively involved in differentiation. Arrows with regular arrow heads indicate a positive regulation. Arrows with a blunt end indicate inhibition/negative regulation. Although SSCs and undifferentiated spermatogonia are combined for simplicity, effectors/pathways that are specific for the transition from SSCs to a more advanced phase of undifferentiated spermatogonia, or from undifferentiated to differentiating spermatogonia are described in more details in the text and Table 1.

(DBA), highly expressed not only before PND14, but also during gonocyte differentiation between 2 and 3 weeks of age and in subsets of spermatogonia (Klisch *et al.* 2011, Kim *et al.* 2013). Germ cells from 2- to 3-week-old, but not younger, prepubertal pigs also expressed stage-specific embryonic antigen 1 (SSEA1; Goel *et al.* 2007). Using SSEA1–FACS enrichment coupled to xenotransplantation assays, Kim *et al.* (2013) proposed that SSEA1 is a marker of undifferentiated pig spermatogonia, including SSCs. Approximately half of SSEA1 enriched cells co-expressed Uchl1 (ubiquitin carboxyl-terminal esterase L1/PGP 9.5) and more than 40% expressed the undifferentiated spermatogonial marker PLZF, as well as VASA, considered as an SSC differentiation marker in boars (Luo *et al.* 2009). In this model, Thy1, CD9, and $\alpha 6$ - $\beta 1$ -integrins appeared less effective for spermatogonial enrichment than they are in rodent models, suggesting differences in SSC markers between porcine and rodent models. Moreover, the mechanisms regulating porcine gonocytes and SSC differentiation are not understood yet.

A commonly used model for the study of germline stem cell (GSC) development is the *Drosophila* (see reviews by de Cuevas & Matunis (2011) and Spradling *et al.* (2011)). In this invertebrate, GSC formation from

PGCs relies on PGC migration, coinciding with gonad formation and the establishment of the hub, an embryonic center formed by somatic gonadal precursor cells located at the apex of the testes. Studies have shown that the Jak/Stat pathway activation is involved in PGC migration into the vicinity of the hub, where they will become GSCs, and that only germ cells with active Jak/Stat will form polarized adherent junctions with hub cells, defining the stem cell niche microenvironment essential for the maintenance of GSC self-renewal. Jak/Stat was found to be necessary for GSC maintenance, and to prevent further differentiation (Sheng *et al.* 2009). Differentiation appears to be regulated by interactions between germ cells and the cyst cells surrounding them. Early germ cells were shown to produce Spitz, an EGF receptor (EGFR) ligand, which acts on EGFRs on cyst cells, leading to MAPK activation and the promotion of germ cell differentiation. One of the genes involved in differentiation is *Bam* (bag of marbles). On the other end, cyst cells produce TGF β , which prevents *Bam* expression and differentiation (Davies & Fuller 2008). These animal models have also been instrumental in revealing the critical role of epigenetic processes such as chromatin remodeling and histone modifications in the regulation of germ cell differentiation (review in Eun *et al.* (2010)).

Table 1 Summary of genes/proteins/pathways involved in gonocytes or spermatogonial differentiation.

Cell type	Species	Gene/pathway	Function	Effect	References
Neonatal gonocytes	m	NOTCH1	Overexpression in fetal gonocytes leads to premature differentiation: ↑ Kit, ↑ SOHLH2	+	Dirami <i>et al.</i> (2001) and Garcia <i>et al.</i> (2013)
	m,r	RA, RAR	↑ Differentiation; ↑ Stra8, ↑ V1-PDGFRB, ↑ Dazl, ↑ Kit	+	Wang & Culty (2007) and Zhou <i>et al.</i> (2008a,b)
	r	Proteasome activity	Required for RA-induced differentiation	+	Manku <i>et al.</i> (2012)
	r	Variant V1-PDGFRB	↑ By RA; overexpression ↑ Stra8 expression	+	Sarkar, Manku and Culty (unpublished data)
	r	Variant PDGFRA	↑ By RA, unknown function	+	Manku <i>et al.</i> (2014)
	r	SRC, Jak2/Stat5	Activity required for RA-induced differentiation	+	Manku <i>et al.</i> (2014)
	m	MIS/AMH	↑ Differentiation to type A spermatogonia	+	Zhou & Hutson (1995)
	m	miR293, 294, 291, 290-5p	miRNA clusters downregulated during differentiation regulate PTEN and Wnt/β-catenin pathways	–	Mclver <i>et al.</i> (2012)
	r	Nup153 and Safb2	Pathways converging on cyclin D1 Strong mRNA expression	?	Manku and Culty (unpublished observations)
	m,r	RA, RAR	SSCs to undifferentiated, progenitors to differentiated	+	Schrans-Stassen <i>et al.</i> (1999) and Zhou <i>et al.</i> (2008a,b)
Spermatogonia SSCs	m	Stra8, Kit, Sohlh2, Bim, Dazl	Progression from SSCs to differentiating cells	+	Tong <i>et al.</i> (2012)
	m	Sohlh1	Increases Kit expression	+	Barrios <i>et al.</i> (2012), Oatley & Brinster (2012), and Suzuki <i>et al.</i> (2012)
	m	miR-34C	↓ Nanos2, ↑ differentiation, ↑ Nanos3, ↑ Stra8	+	Yu <i>et al.</i> (2014)
	r	BMP4	Increased by RA, ↑ SSC differentiation, ↑ SMAD1/5/8 activation, ↑ Sohlh2, ↑ Kit, ↓ PLZF	+	Carlomagno <i>et al.</i> (2010)
	m	Decreased activin A	↑ Recruitment of cells to differentiation, ↑ Kit	+	Nagano <i>et al.</i> (2003)
	m,r	Low level GDNF	Promotes SSC to A _{pair} differentiation	+	Meng <i>et al.</i> (2000) and Hamra <i>et al.</i> (2007)
	r	Neuregulin 1	Promotes A _{paired} to A _{aligned} differentiation	+	Hamra <i>et al.</i> (2007)
	m	STAT3	Induces Ngn3 expression	+	Oatley <i>et al.</i> (2010)
	m	Ngn3/Neurog	SSC to undifferentiated, transit amplifying progenitors	+	Kaucher <i>et al.</i> (2012)
	m	PLZF	In SSC to undifferentiated. Blocks Kit, SALL4, TORC1	–	Buaas <i>et al.</i> (2004)
	m	Nanos2	Regulated by Fgf9, prevents differentiation	–	Barrios <i>et al.</i> (2010) and Sada <i>et al.</i> (2012)
	m	Numb	Represses Notch1, ↓ differentiation	–	Braydich-Stolle <i>et al.</i> (2005)
	m	Mirc1, Mirc3 clusters	Negative regulators, ↓ by RA	–	Tong <i>et al.</i> (2012)
	m	miR-146	Negative regulators, ↓ by RA	–	Huszar & Payne (2013)
	m	DMRT1	Represses <i>Stra8</i> transcription; restricts RA responsiveness	–	Matson <i>et al.</i> (2010)
	m	Nup153, Suz12, Safb2	↑ Expression at PND3.5; in cells at basement membrane; in undifferentiated spermatogonia	+?	Zheng <i>et al.</i> (2014)

Upward pointing arrows indicate increased expression of a gene/protein or increased function. Downward pointing arrows indicate decreased expression of a gene/protein or suppressed function.

Although there is no defined ‘gonocyte’ stage in *Drosophila*, the postmigratory germ cells that make connections to hub cells and become GSCs demonstrate a behavior similar to the neonatal migratory gonocytes that need to establish contact with the basement membrane of the seminiferous cords in order to differentiate into SSCs. As mentioned earlier, we have recently found that Jak/Stat activation is required for RA-induced gonocyte differentiation *in vitro* (Manku *et al.* 2014). By analogy with the *Drosophila* model, it would be interesting to determine whether Jak/Stat

activation is involved in the cell adhesion processes allowing the anchoring of migratory gonocytes in the future niche, where they can become spermatogonia.

Conclusion

By presenting the state of the current knowledge on the mechanisms regulating gonocyte and spermatogonial differentiation (Fig. 1), this review emphasizes the broad gaps left in our understanding of these cells and the lack of available literature on these topics. Although the

development of these early germ cells dictates the subsequent status of spermatogenesis, the paucity of transitional gonocytes and SSCs within testes, together with the absence of specific markers for these phases of germ cell development, have hindered the pace of new findings in this field of research. Nonetheless, strides have been made, unveiling different genes and pathways that are critical for the differentiation of both cell types. The comparison of these processes also stressed how they involve different proteins and signaling pathways, despite both responding to RA (Table 1). This further suggests that the fine tuning and regulation of differentiation in time and space are carried out through crosstalk mechanisms between RA and other factors. However, more work needs to be carried out to determine how all of these pieces fit together to regulate proper early germ cell differentiation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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