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Signaling events during male germ cell differentiation: Update, 2006

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1. ABSTRACT

The intracellular transduction of exogenous and cell-autonomous stimuli triggers the transformation of a multipotent stem cell, the spermatogonion, into a highly differentiated, motile and fertile cell, the spermatozoon. This differentiation process is mediated by cell-cell contact and via key players including hormones, growth factors, and cytokines. Female hormones, estrogens and progestins, play a role in the production and functionality of spermatozoon. New findings, however, reconsider the direct action for estrogens on male germ cells while progestins work through non-canonical receptors. Similarly, testosterone, the male hormone, besides acting through its receptor expressed in the somatic cells of testis, seems to work by means of non-classical mechanisms. The recent identification of growth factors, transcriptional regulators, and media for *in vitro* growth of spermatogonial stem cells should now make it feasible to unravel the entire spermatogenic process. A peculiar feature of the meiotic cycle is the maintenance of condensed chromatin so that DNA duplication is prevented and reduction of genome is achieved. Recently, molecular mechanisms that lead to such a condensation have been discovered. Junctional intercellular complexes between Sertoli and germ cells are critical for coordinating spermatogenesis. Molecular players involved in such cell-cell communication have been identified in Sertoli cells. Now, there is also a need for unravelling the germ cell molecules involved. These issues are the major topics which are discussed here with the goal to suggest a possible answer.

2. INTRODUCTION

In the last decade of the 20th century, molecular mechanisms underlying the complex process of spermatogenesis were identified and for some of them, albeit still so far to be dissected step-by-step, it was possible to delineate a part of the cascade of signaling events which concur terminally in the production of a specific product and/or in a specific cellular reply (for a brief review, see 1). The fortuitous connection of various independent factors emerged more recently has led to an impressive impulse to study spermatogenesis, even from laboratories previously not directly engaged in male germ cell biology. To cite only some of the most meaningful factors: a) the availability of both mouse (Celera Genomics [http://www.celera.com]; Ensembl mouse genome server [http://mouse.ensembl.org]) and human (2) draft genomes; b) the development of methods to visualize gene expression monitoring by the DNA microarray techniques; c) the successfull efforts in preparing protein microarray formats (3-5) and, may be as a consequence of the ethical and legal issues on the embryonic stem (ES) cell research, d) the increasing interest in stem cell biology towards the adult stem cells (very recently it has been reported that not only sperm cells can develop from cultured mouse ES cells (6), but that spermatogonial stem cell lines can be maintained in stable conditions to allow stem cell propagation *in vitro* (7)); plus e) the alerting question of human infertility, a pathology that affects 15% of couple

with an equal contribution from both partners (8), which implies social, health, and psycologic consequences.

In this review, which is an updating to the previous one (1), hormonal control, function of some testis-specific trascriptional regulators and ligand-receptor complexes, and involvement of small G-proteins in post-meiotic sperm differentiation will be discussed.

3. HORMONAL CONTROL

3.1. Female hormones

3.1.1. Estrogens

In 1998, the news was that female hormones, particularly estrogens, would be essential for male fertility (9). This finding has opened an exciting area of research, i.e., that on the role of estrogens in spermatogenesis. Not only, but in those years epidemiological data were alerting about the adverse effects of the 'environmental estrogens' on the male reproductive system; the hypothesis of the 'endocrine disruptors' was often evoked among the causes of male infertility. Consequently, it was considered imperative to dissect the role/s estrogens play in the male. At the present, six-seven years later, the question is not yet clearly resolved.

Male germ cells express functionally active P450 aromatase (10, 11) and estrogen receptor (ER) isoforms (12, 13). Fertility problems of ER alpha knock-out male mice have been, however, shown to be due to the inability of epididymal epithelial cells to regulate the reabsorption of luminal fluid (9) or to the disruption of testis somatic cell function (14), while male germ cell differentiation goes on normally. Consequently, either male germ cells are not the direct target of estrogens or estrogen action in spermatogenic cells has to occur via alternative estrogen receptors, most likely ER beta. Krege and co-workers (15) generated, however, mice homozygous for a disruption of the ER beta gene and found that these mice develop normally, are fertile and exhibit normal sexual behavior. So, ER beta is not essential for both male and female fertility (for a recent review on the role of estrogens in male reproduction, including notices on mice lacking both ER forms, see (16)).

Indeed, a growing number of ER beta splice variants have been reported in human testis (13); only two correspond to ER beta wild-type transcripts and are expressed in testis somatic cells and/or primary spermatocytes, whereas the major part is due to unusual ER beta splice variants which are present, however, in high levels only in the haploid cells (13). This suggests that the diverse ER beta variant transcripts can exert specific functions during the different stages of spermatogenesis, particularly spermiogenesis. However, so far there is no experimental evidence to this respect.

In their work on transgenic mice overexpressing rat androgen-binding protein, Selva and co-workers (17) report that increased levels of ER beta mRNA and protein in pachytene spermatocytes are associated to a meiotic arrest and induction of germ cell apoptosis. Unfortunately, the Authors do not specify the ER beta variant, but most likely they refer to the wild-type species. Their conclusion is that ER beta may be involved either in regulating the progression of the first meiotic division or in favoring the entrance of primary spermatocytes to an apoptotic pathway. To study the mechanism of a supposed estrogen-induced death of spermatogenic cells, Nair and Shaha (18) used diethylstilbestrol (DES), an estrogen-like compound, to induce germ cell apoptosis *in vivo* in the male rat. The exposure to DES resulted in an increase in Fas-FasL expression. This increase was, however, confined to the spermatid population, that was just the cell type marked by increased apoptosis; spermatocytes, the cell type proposed as the possible target of the ER beta pro-apoptotic effect (17), were apparently unaffected (18). The apoptotic pathway identified by Nair and Shaha would be as follows: the increase in Fas-FasL expression leads to the cleavage of caspase-8 to its active form so that Bax translocates to the mitochondria and precipitates the cytochrome c that has been released by a drop in mitochondrial potential. Subsequent to this, activation of caspase-9 occurs and this, in turn, activates caspase-3 leading to the cleavage of poly(ADP-ribose) polymerase. So, these data delineate the cascade of events triggered by the increase of expression of the Fas-FasL system due to exposure of spermatogenic cells to DES. Unfortunately, it remains however unknown how DES directly works. Although DES has been reported to be able to bind to ERs (19), no notice about what kind of ER could be involved in the cascade of events leading to apoptosis is done. It might be, hypothetically, that DES has the ability to affect the expression of Fas-FasL system via a non-estrogenic pathway, as it has been already reported for other endocrine disruptors.

Aravindakshan and Cyr used nonylphenol, an environmental contaminant with a weak estrogen-like activity, to study its detrimental effects on spermatogenesis (20). It was found that nonylphenol, but not 17beta-estradiol, alters the protein level of connexin 43, a component of the gap junctional intercellular complex that allows communication between Sertoli cells and between Sertoli and germ cells and which is considered critical for coordinating spermatogenesis. Moreover, nonylphenol affects the phosphorylation state of connexin 43 by p38-MAP kinase pathway, i.e., via an apparent estrogen receptor-independent mechanism (20). Indeed, recent results from an extensive research (21) on the effects of endocrine-active compounds, including those with estrogenic activity, have shown that sperm count levels vary with demography and the hypothesized correlation between xenoestrogens and global decrease in sperm counts and others disorders of the male reproductive tract is not supported by published data. So, at the present state of knowledge the conflicting results obtained about the role/s of estrogens and estrogen-like compounds and their putative receptors in spermatogenesis rise still confusion. To study the role/s that estrogens play directly in spermatogenesis, the lesson might be that it is better to assay the naive estrogens and identify their endogenous cell type-specific receptors so to well define the signalling pathways induced by the hormone/receptor binding . To this goal, animal models as the recently developed REA-deleted mice (22) could result to be

of help. REA (repressor of estrogen receptor activity) directly interacts with estrogen receptors (Ers) and represses ER transcriptional activity working as an ER coregulator. Homozigous REA-deleted animals do not develop, but heterozygous animals are viable and present REA mRNA and protein levels which are half those of the wild type (22). Park and co-workers used mutant female mice to study the functional activity of REA *in vivo* and the estrogen-driven gene expression under conditions of up-regulation of the estrogen-stimulated genes in the female. Thus, REA-heterozygous mutant male mice might be used to assay the effects of estrogen in spermatogenesis.

3.1.2. Progesterone

As to the other 'female hormone' discussed in the 1998 review, i.e., progesterone, and its relation with male fertility, the literature to date is so rich that it deserves a review a part. Indeed, the action of progesterone (P) in male germ cells is so far confined essentially to sperm cell function. Anyway, being this review an update to the previous one, I maintain a brief sub-section devoted to progesterone (P) where some news about the putative P receptors expressed in male germ cells, rather than the direct P action, are discussed.

It appears to be definitively clarified that mammalian spermatogenic cells express both the classic (read also cytosolic or genomic) and non-classic (read also membranous or non-genomic) progesterone receptors (23, 24). These last, indeed, seem to exist and/or co-exist in more splice variants and/or molecular species (25-27). Despite this accumulating and growing evidence for progesterone (P) receptors in male germ cells, the P-mediated signaling and the characteristics of P-receptors (PRs) in sperm cells remain elusive. It was suggested that different genes encode for genomic and non-genomic PRs (28) since spermatozoa from transgenic mice lacking the classic progesterone receptor (PR) show apparently normal surface progesterone receptors (mPRs). The classic genomic PR is due to two isoforms, PR-A and PR-B, which, at least in the human, are generated from a single gene by differential promoter utilization. There is an additional third isoform, PR-C, which is a N-terminally truncated form of PR-A/PR-B, identified only in some tissues (29); moreover, some additional variants have been described (among recent excellent reviews, see (30 – 32)). As to the non-genomic mPRs - the effects of which could not result in activation of gene expression, but in more rapid cell responses such as transient increase in free-exchangeable Ca^{++} - these are still molecularly poorly characterized, notwithstanding the efforts to determine their structures. The major tendencies of thought as to the non-genomic PRs are: a) they are non-P-specific membrane receptors; b) they belong to a novel type of P-specific membrane receptors. The prototype for the non-P-specific receptors in male germ cells was the $GABA_A$ receptor/Cl^- channel complex (33), as already reported in the previous review. The possible involvement of GABA receptors in mediating progesterone action in mammalian sperm, i.e., in inducing the acrosome reaction, was and is still a matter of debate, as both positive (33) and negative (25) results have been reported. Recently, it has been shown that in a multipotential glial progenitor cell line, progesterone exerts its mitogenic effect indirectly, i.e., through its conversion to 3alpha, 5 alpha-tetrahydroprogesterone (allopregnanolone) which is a potent positive allosteric modulator of $GABA_A$ receptors (34). This result reveals an effective link between progesterone and GABA signaling and support, but only in part, the thesis of progesterone-induced activation of sperm GABA receptors. In fact, the $GABA_A$ receptor-mediated mitogenic effect of progesterone in progenitor glial cells falls within the 'genomic action' rather than the 'non-genomic one' of P, i.e., the opposite of that is ascribed to P-, $GABA_A$ receptor-mediated, signaling in mammalian spermatozoa. In other words, the P-$GABA_A$ receptor signaling is and remains still controversial in male gem cells (25).

An alternative to non-P-specific membrane receptors is the existence of a novel type of P-specific membrane receptor/s. The prototype is the P-binding site at the plasma membrane in amphibian oocytes that, once activated, leads to resumption of meiosis (34). Thomas and co-workers (36) have recently shown that the action of P is mediated by a progestin membrane receptor, the structure of which is typical of G protein-coupled receptors, that is , distinct from that of the classical intracellular PRs. Genes in other Vertebrates homologous, including the human, to this novel (fish) gene have been identified (37). Of the three human mPR isoforms, the mPR alpha is specifically transcribed in reproductive tissues. This characteristic suggests that mPR alpha could be the sperm membrane P receptor which mediates the non-classical effect to induce the acrosome reaction. The mPR alpha protein, however, has not yet been identified and its signaling pathway is at all unknown. Thomas and Doughty (38), indeed, have reported that environmental xenoestrogens interfere with the rapid, nongenomic P action in spermatozoa of Atlantic croaker. According to these authors, the rapid P effect in these sperm is not, however, the induction of the acrosome reaction, but the stimulation of sperm motility. It is clear that the conflicting data about nongenomic P effects in spermatozoa raise still unresolved issues. The studies so far indicate the presence of a sperm membrane protein able to bind P and to elicit a signaling pathway. It is unclear, however, the true nature of this P-binding protein and which of the P-mediated signaling events are primary and which are activated by other downstream events or by a cross-talk with other signalling pathways.

3.2. Male hormone

This section is concluded with the canonical male hormone, testosterone, with a brief, but intringuing mention. Testosterone (T) and its derivate dihydrotestosterone are ligands for the androgen receptor (AR) and their binding leads to the nuclear translocation of AR so that this last could function as transcriptional activator. T is considered to be the major hormonal regulator of spermatogenesis and several genes are up-regulated in response to testosterone (39); in the testis, AR protein is expressed in the somatic Leydig, myoid and Sertoli cells (40). While AR expression is continuous in Leydig and myoid cells, it occurs in a stage-dependent fashion in Sertoli cells, i.e., its highest levels correspond

with the spermatogenic stages most acutely affected by androgen withdrawal. This, together with the fact that Sertoli cells are the only somatic cells in direct contact with germ cells, have led to retain Sertoli cells as the primary mediators of AR function during spermatogenesis. More specifically, AR activity from Sertoli cells would be responsible for the correct and efficient completion of meiosis (39). To definitely examine this general belief, Holdcraft and Braun (41) have created a conditional null allele of the *Ar* gene in mice which allows the selective removal of AR function from Sertoli cells only, thus obtaining unexpected results.

Sertoli cell AR expression does not appear to be required for the completion of meiosis and the transition of spermatocytes to haploid round spermatids, but it seems to be necessary for two other AR-sensitive steps of spermatogenesis (41). The first occurs in the late stages of spermatid differentiation near the time of spermiation. The second step occurs during the transition from the round to elongating stage of spermiogenesis. Upon the evidences provided by the two Authors, the primary role of AR function in Sertoli cells is to regulate spermatid adhesion to the seminiferous epithelium. Still more surprisingly, AR would function in opposite directions depending on the spermiogenetic step. AR would be required for maintenance of adhesion of round spermatids during their differentiation to elongated spermatids; conversely, AR function is apparently required for the execution of spermiation, i.e., for the release of mature sperm into the tubule lumen at the end of spermiogenesis. So, an unexpected scenario is opened by the conditional AR mutant mice: during spermatogenesis Sertoli AR has a role in governing cell-to-cell contacts between Sertoli and germ cells. It might be that upon androgen stimulation AR induces transcription of surface selectin-like molecules or other molecules acting as adhesion factors that trigger the activation of spermatid signaling pathways regulating spermatid adhesion. To explain the surprising lack of effect of Sertoli cell AR on the completion of meiosis, Holdcraft and Braun (41) put forward two interesting hypotheses: 1) in the absence of a functional AR, androgens could be capable of acting non-genomically as already shown for progestins; 2) the meiotic AR requirement has to be searched for outside of the seminiferous epithelium and the peritubular myoid cells could be possible candidates. As to the first hypothesis, it has to be noticed that Fix and co-workers (42) have recently shown that T can regulate Sertoli cell processes by means of non-classical mechanisms, i.e., leading to a rapid, and consequently non-genomic, activation of the MAP kinaase pathway. As to the second one, it is to remark that De Gendt and co-workers (43) generated a mutant mouse very similar to that of Holdcraft and Braun, i.e., a Sertoli cell-selective AR knock-out (SCARKO), but yielding conclusions that are rather dissimilar from the previous ones. According to De Gendt *et al.* (43) SCARKO mice, despite normal testicular descent, display a spermiogenetic arrest that prevents completion of meiosis and formation of round spermatids; in other words, the cell-autonomous action of AR in Sertoli cells plays a pivotal role in spermatocyte, and possibly round spermatid, development, while no mention is made about any involvement of AR in regulating cell-to-cell contacts between Sertoli and germ cells. So, further studies on both these conditional AR mutant mice and, may be, other animal models are necessary to dissect finely the role of androgen-dependent Sertoli cell AR in spermatogenesis.

4. SIGNAL TRANSDUCTION IN SPERMATOGENIC CELLS: COMMENT

The complex network of endocrine, paracrine, autocrine, cell-to-cell communication and intracellular events involved in male germ cell differentiation is coordinated through cell signaling. Highly dynamic mechanisms are essential for regulation of cell signaling and protein phosphorylation is generally accepted as the universal tool used to switch on or off dynamic processes. Recently, another protein post-translational modification has been recognized to play an important role in cell signaling, i.e., protein ubiquitination. The addition of ubiquitin tags to protein is resulted to be crucial for the down-regulation of signaling molecules as plasma membrane receptors, steroid hormone receptors, plasma membrane transporters, and ion channels (44). Moreover, as phosphatases switch off the signal triggered by protein kinases, deubiquitinating enzymes counterbalance the signaling by ubiquitination enzymes. Indeed, it could not be excluded a cross-talk, if not even a network, between the two highly dynamic mechanisms where, for instance, the phosphorylation of a deubiquitinase might act as a switch for inhibition of a step in the ubiquitination pathway. Whereas protein phosphorylation has been studied since the dawning of molecular biology of male germ cells, spermatogenic ubiquitin system is under investigation only recently (45); consequently, spermatogenic cell-specific ubiquitination/deubiquitination pathways have not yet been identified. Several researcher groups are, however, engaged in this intriguing field and some promising findings have already been obtained (46-50).

In line with the previous one, this review will deal with signaling events for each of the three spermatogenic phases - mitosis-meiosis-cytodifferentiation - where protein phosphorylation is the key tool for signal transduction.

4.1 Spermatogonial stem cells

4.1.1. Generation of a spermatogonial cell line

The goal of tissue engineering is to repair organ pathologies such as those acquired congenitally or due to external causes as trauma, infection, and inflammation. Embryonic stem (ES) cell research offers the hope of cell-replacement therapies; the remarkable scientific efforts in ES field are, however, complicated by ethical concerns about obtaining human ES cells from aborted fetuses. The heated debate upon this issue has delayed or stopped human ES cell research in some countries while, as a counterbalance, it has led to increase and strengthen the research on somatic stem cells. Two general observations, i.e., that a) the ideal autologous cells can often be found within the organ itself and b) male infertility is a serious health problem worldwide, together with the spermatologist interest to have, at long last, a male germ cell culture

technology, have boosted the research in spermatogonial stem cell biology.

Until recently, one of the major advances in spermatogonial cell culture technology has been the generation of an immortalized cell line. In 2002 Dym and co-workers (51) reported the generation of the first spermatogonial cell line, obtained from telomerase-immortalized mouse type A spermatogonia; these cells, under adequate stimulation, can differentiate *in vitro* into spermatocytes and round spermatids and, consequently, they could provide a powerful tool for elucidating some of the molecular mechanisms that regulate spermatogenesis. Since spermatogenic cells are difficult to transfect, these Authors used a retrovirus system to introduce the ectopic expression of the telomerase catalytic component. To be sure that the transfected cells are really spermatogonia and, consequently, the immortalized cell line could be used for studies on spermatogenesis - previous tries from other laboratories to generate immortalized male germ cells were, unfortunately, a failure - these cells were checked for 'spermatogonial type A' characteristic, i.e., c-kit immunopositivity. The transmembrane tyrosine kinase c-kit is in fact expressed specifically in spermatogonia A and the stem cell factor/c-kit system is well known to govern spermatogonia mitotic progression (52) and to trigger proliferation/differentiation of type A spermatogonia (53). In 2000 Hunter and co-workers (54) delineated a signaling pathway through which the SCF/c-kit system acts in spermatogonia of type A. Homozygous mutant mice carrying a selective point mutation in the c-kit gene revealed an early block of spermatogenesis. This mutation resulted to impair the SCF/c-kit-mediated activation of PI 3'-kinase, which on its own is known to activate Akt/PKB, a serine/threonine kinase with a crucial role in the control of cell survival. Although SCF/c-kit system controls also haematopoiesis and melanogenesis, the only defect found in the homozygous mutant mice was male sterility; this suggests indirectly that haematopoiesis and melanogenesis are not under a SCF/c-kit –PI 3'-kinase-dependent pathway. The SCF/c-kit/PI 3'-kinase/(Akt) signaling is, on the other hand, resulted to be essential for spermatogonial proliferation and, consequently, for male fertility. A confirmation that the SCF/c-kit-PI 3'-kinase pathway could be downstream extended to Akt has been successively given by Akt1-/- male mice which display attenuation of spermatogenesis and spontaneous male germ cell apoptosis (55).

4.1.2. Spermatogonial stem cell renewal

Recently, Braun and co-workers (56) and Pandolfi and co-workers (57) have independently shown that the transcriptional repressor Plzf , belonging to the POK (POZ and Kruppel) family, is essential for spermatogonial stem cell renewal. Plzf, originally identified in haematopoietic cells, is considered to be involved in stem cell maintenance. By studying two mouse mutants, both carrying specific mutations in the gene encoding Plzf, the two groups achieved the same result, i.e., loss of Plzf function shifts the balance between spermatogonial stem cell self-renewal and differentiation toward differentiation at the cost of self-renewal and leads to an increase of apoptotic cells which enter inappropriately into meiosis. By microarray analysis of isolated Plzf7Zfp145-null spermatogonia Costoya *et al.* (57) showed alterations in the transcriptomic profile; these alterations affect particularly the expression of genes for RNA-binding proteins, cyclins and *Tsx*. The function of the Tsx gene product is still unknown. It was originally reported to be expressed specifically in the testis; its expression in germ cells is, however, extremely transient being restricted uniquely to the premeiotic cells during the first wave of spermatogenesis (58). It has been suggested (59) that in spermatogonial stem cells Plzf could function as a cell-autonomous factor required for spermatogonial cell maintenance, but it might also interact with the c-kit/PI 3'-kinase signaling and/or GDNF/Ret (see below) signaling networks. Work in this direction is desirable. So far, in haematopoietic cells the transcriptional repressor activity of Plzf is resulted to be inhibited by activation of tyrosine kinase signaling pathways, such as the MAPK pathway triggered by the Fms-like tyrosine kinase 3 (60). As to spermatogonial stem cells (SSCs), Brinster's group has recently succeeded in identifying SSC-specific cell-surface markers (7). The major and surprising finding was that SSCs are not c-kit positive, whereas primordial germ cells and differentiated spermatogonia are known to be c-kit positive. Kubota and co-workers (7) have also developed a medium and identified the growth factors to maintain the SSCs indefinitely in culture, thus avoiding to immortalize spermatogonial cells by transfection technologies. So, it is emerged that GDNF (glial cell line-derived neurotrophic factor) is sufficient to sustain maintenance and self-renewal of SSCs. In the testis GDNF is produced by Sertoli cells (61) and SSCs express GDNF receptors, i.e., GFR alpha1 and c-Ret receptor tyrosine kinase (61, 62), which act downstream by activating PI-3' kinase and/or the Src family of tyrosine kinases (63). In the light of all these recent issues, it might be speculated that: a) in SSCs the GDNF/c-Ret/(PI-3' kinase) signaling works to allow that Plzf interacts with its corepressors (58) so to exert its repressional activity (this means either an active down-stream effect of the GDNF/c-Ret signaling on Plzf system or no GDNF/c-Ret involvement and the Plzf system acting autonomously); b) in differentiating/differentiated spermatogonia the SCF/c-kit/(PI-3' kinase) signaling could mediate the phosphorylation state of a Plzf associated corepressor so favoring its dissociation from chromatin and consequently leading to transcriptional derepression. This is reminiscent of what found for Fms-like tyrosine kinase in haematopoietic cells (58) or of what it has been very recently discovered in cultured cell lines; in these last the MAP kinase-activated protein kinase 3 (MAPKAPK3), phosphorylating a Polycomb Group Protein member (with which Plzf interacts), leads to chromatin dissociation of PcG complexes thus resulting in de-repression of target genes (64). (Figure 1) illustrates a simplified scheme where the intermediate cell stage (not more c-Kit $^{-}$ SSC, but not even differentiating c-Kit $^{+}$cell) is lacking.

At last as to Plzf , it is worth mentioning a promising experimental approach. By exploiting the strong transcriptional repression activity of Plzf, the production of Plzf-fusion with proteins acting as transcriptional regulators is becoming an useful tool for silencing the transcription

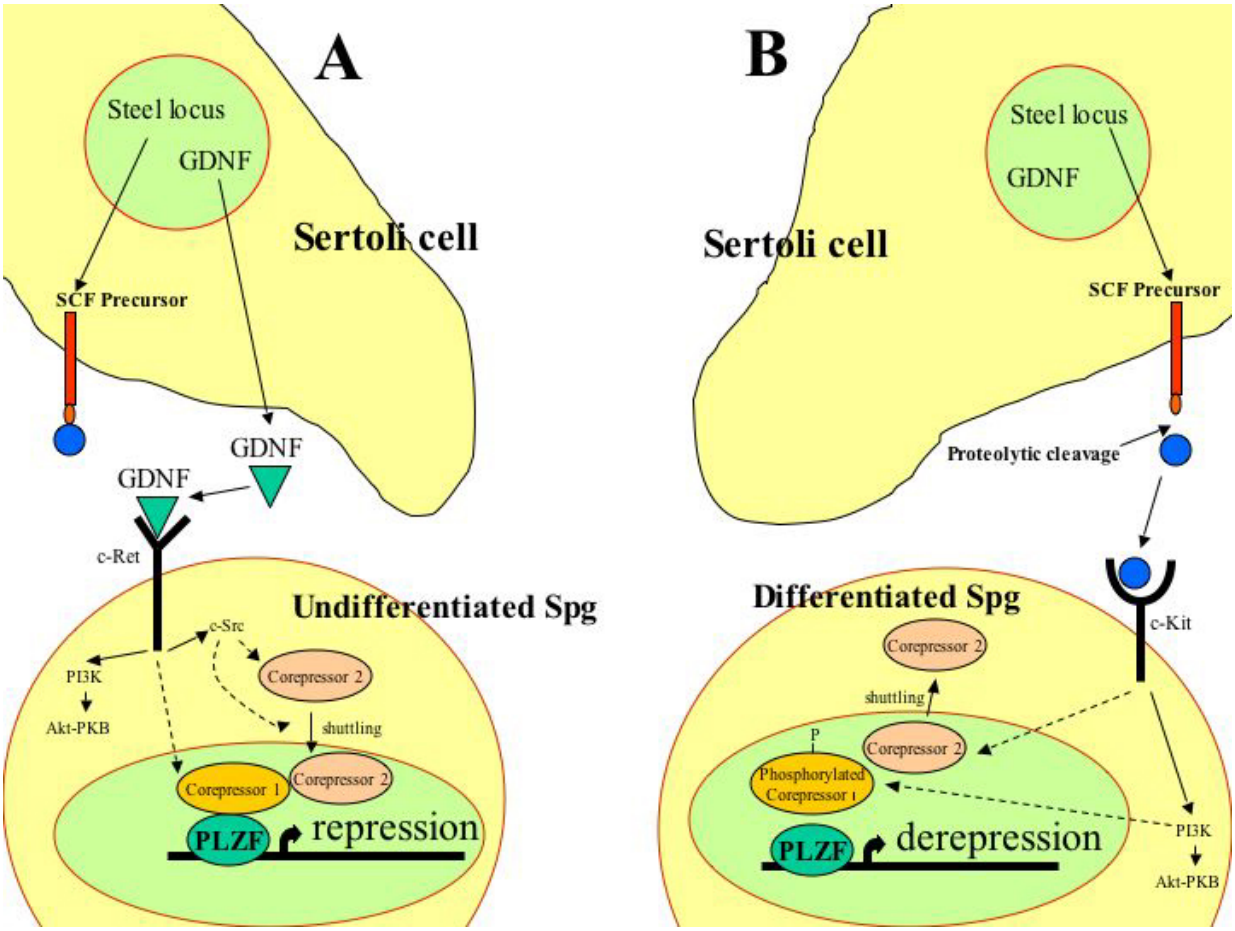

Figure 1. Effects of GDNF/c-Ret and SCF/c-Kit signaling on the transcriptional repressor activity of Plzf. A. A schematic drawing where GDNF, secreted by Sertoli cell, binds its receptor present on the surface of c-Ret+ spermatogonial stem cell: this triggers two signaling cascades, one that through PI3K/Akt-PKB regulates the survival-apoptosis decision, the other that through so far unknown mediators results in the enhancement of the binding of Plzf associated corepressors so to have transcriptional repression. B. A schematic drawing where SCF, secreted by Sertoli cell, binds its receptor expressed on the surface of c-Kit + differentiating spermatogonion: this triggers two signaling cascades, one that through PI3K/Akt-PKB regulates the survival-apoptosis decision, the other that through so far still unidentified mediators leads to phosphorylation of Plzf corepressor thus resulting in its dissociation from the transcriptional repressor complex and therefore in derepression.

regulated by the not Plzf moiety of the chimerical protein. So, to cite some examples, AR-Plzf fusion protein (65) and ER alpha-Plzf fusion protein (66) have been produced and used to inhibit androgen- and estrogen-regulated gene expression, respectively.

4.2. Meiotic Progression

Once entered into meiosis, a single spermatocyte has to give rise to four haploid cells. Notwithstanding some recent progresses, few informations are so far available on the highly ordered control of the differentiation of spermatogonia into spermatocytes and on the molecular mechanisms of the progression to the metaphase of the first meiotic division. DNA microarray approaches have been used to identify genes specifically involved in the male meiotic program; by grouping these genes in clusters it might be possible to obtain useful information about their potential molecular interactions (67, 68). First meiotic prophase could be considered an extended G2 phase where germ cells transit from the mitotic to the meiotic cell cycle. During this prophase, the diverse stages follow one another with concomitant changes in both chromosomal behavior - as chromosome movement, pairing, synapsis and recombination - and chromosomal transcriptional activity, being known the pachytene as the most active stage; consequently, checkpoint mechanisms have to exist for monitoring all such events (69). A further peculiar feature of the meiotic cell cycle is the maintenance of condensed chromatin during the short interphase that separates metaphase I from metaphase II; in this manner DNA duplication is prevented and the genome could be reduced. Experimental evidences from Geremia's laboratory (discussed below) delineate a signaling cascade that very likely results in chromatin condensation in mouse spermatocytes. Insights into the molecular mechanisms of the progression of the first metaphase were obtained originally with studies about treatment of cultured spermatocytes with okadaic acid (OA), a

serine/threonine phosphatase inhibitor (70, 69); these studies suggested a role of the MAPK pathway in chromosome condensation. Successively, work by Sette and co-workers demonstrated that: 1) the MAP-kinase Erk1 is effectively involved in the meiotic progression of pachytene spermatocytes (71); 2) active Erk1 translocates into the nucleus where it phosphorylates and activates a specific isoform of the 90 kDa ribosomal S6 kinase, a downstream effector of the MAPK pathway, which is known as p90Rsk2 (72); 3) p90Rsk2, in turn, phosphorylates and activates the chromatin-bound Nek2 (72), a NIMA-like kinase whose activation was already shown to correlate with chromosome condensation in mouse spermatocytes (73); 4) active Nek2 phosphorylates high-mobility group protein A2 (HMGA2), a chromosomal protein without any intrinsic transcriptional activity, but able to regulate transcription by altering the chromatin architecture (74). The physical interaction between Nek2 and HMGA2 seems to be constitutive *in vivo*, but it is when Nek2 is activated in a MAPK-dependent manner, HMGA2 becomes phosphorylated thus losing its affinity for DNA. This phosphorylation could favor the release of HMGA2 from chromatin of late pachytene spermatocytes, possibly permitting to condensation factors to enter chromatin and to trigger chromosome condensation and thus allowing the transition to metaphase (74). Such a sequence of phosphorylation events correlates effectively with chromatin condensation; this so finely defined signaling pathway is, however, beheaded, i.e., defective of the original activator, which is the physiological molecule that triggers Erk1 activation. The same Geremia's group has shown that, by culturing spermatocytes under simulated microgravity, meiotic progression occurs spontaneously and is accompanied by Erk1/p90Rsk2/Nek2 pathway (75). This could be a significant validation of the previous results obtained with a drug, okadaic acid. On the other hand, spontaneous enzyme activation as well as alterations in signal transduction have been reported to occur under microgravity conditions (76). So, the physiological MAPK activator remains still unknown. Meiotic resumption in mammalian oocytes is under direct hormonal control (77). Above, in the chapter devoted to hormonal control, it has been reported that in Sertoli cells testosterone could act through alternative mechanisms to complement the classical AR action; the addition of physiological level of testosterone to cultured Sertoli cells stimulates in fact MAPK pathway in an AR-independent manner (42). Similarly, in primary prostate stroma cells the androgen activates MAPK pathway, increasing Erk phosphorylation, independently from AR (78). So, it might be that the lacking of a classical AR in spermatogenic cells does not mean a categorical lacking of T direct effects just in these cells.

As a scheme of signaling related to what discussed here, I remind essentially that proposed by Sette and co-workers (74), with the addition, however, of T as the putative upstream activator working through an AR-independent pathway.

4.3. A model of signaling in post-meiotic cells: players of a novel pathway ?

As to the signaling in haploid male germ cells, the title and the key molecular player are the same of the previous review. In the light of the new acquisitions and experimental evidences, novel scenarios are, however, opened. A topic which is acquiring a growing interest for spermatologists is that about the junction dynamic in the seminiferous epithelium during spermatogenesis. The movement of developing germ cells involves extensive adherens junction (AJ) restructuring between Sertoli cells as well as between Sertoli and germ cells (79). AJ between Sertoli cells is found at the basal compartment of the seminiferous epithelium; it, together with tight junction and basal tubulobulbar complex, creates the blood-testis barrier that preleptotene and leptotene spermatocytes must traverse to enter the adluminal compartment for further development (for a review, see (80)). AJ structures are also found between Sertoli cells and developing spermatids (round-elongating-elongated) at the apical sites of the seminiferous epithelium; these are known as apical ectoplasmic specializations (ES) (80). The extensive restructuring of apical ES is essential for the movement of spermatids across the epithelium and the release of fully developed spermatids/spermatozoa into the lumen of the tubule at spermiation. While since the 1970s testis junctions have been extensively studied at the morphological and ultrastructural level, attention is recently shifted on identifying the molecular architecture and the regulatory molecules that characterize AJ structures in the seminiferous epithelium. This novel trend can lead to possibly elucidate the mechanism of germ cell movement which is crucial for the completion of spermatogenesis. The work of some research groups, with that of Cheng (79, 80 and see below) among the leaders, has recently led to the identification of molecular components of the basal and apical ES. Curiously, albeit the cells that move are the germ cells, the progress in knowledge in AJ structures regards only the other partner of the junction, i.e., the Sertoli cell. Sertoli cells play clearly an important role in regulating junction dynamic, but developing male germ cells too.

Rap1 is a GTPase belonging to Ras family; it was originally described to antagonize Ras (81) and then to be involved in cell differentiation (82, 83). Rap1 has recently attracted much attention because of its involvement in several aspects of cell adhesion, including integrin-mediated cell adhesion and cadherin-mediated cell junction formation (for a review, see (84)). I remember here that Rap1 regulates all integrins that are associated which actin cytoskeleton and controls both integrin activity and integrin clustering depending on the integrin and the cell type (85). Furthermore, Rap1 regulates cadherins, which are protein components of AJ known to form Ca2+ -dependent homotypic interactions thus stabilizing cell-cell contacts (86). The first documentation of this role of Rap1 was from Drosophila wings (87) in 2002 till to the most recent from human endothelial cells (88). Such an intriguing feature of

Rap1 biology is coming out essentially from cells actively engaged in a variety of temporary and selective cell-cell interactions and cell-cell adhesions as, for example, blood cells and endothelial cells lining blood vessels which are involved in the inflammatory reply. Surprisingly, this exciting yet rapidly developing field of research has not yet been extended to the testis junction dynamics.

Previously, the monomeric small G protein Rap1 was already suggested to be possibly involved in the postmeiotic male germ cell differentiation (1); in 1998 there was, however, no report on Rap1 in spermatogenic cells. At that time this laboratory was engaged in identifying the possible molecules able to interact with a male germ cell-specific isoform of 14-3-3 protein (14-3-3 proteins are a specific family of molecular adaptors that mediate the cross-talk between signaling molecules) cloned in Berruti's laboratory (89). By protein interaction assays, some spermatogenic 14-3-3 theta-binding proteins were identified and a protein complex, formed by the GTPase Rap1, the 14-3-3 theta, and the serine-threonine kinase B-Raf was found in postmeiotic mouse germ cells (90). This suggests that, in analogy with what was emerging about the role of Rap1 signaling in developing/differentiating cells (82, 83), Rap1 could be involved in spermiogenesis (90). Unfortunately, haploid male germ cells were not suitable as somatic cells to be cultured for transfection experiments and loss- or gain-of-function approaches; so, to test our hypothesis we should have used animal models. Conditional transgenic mice which express a dominant-negative form of Rap1, Rap1S17N, only in the testis, even more specifically, only in haploid male germ cells, have been generated; at present we are carrying on their phenotypic characterization. A common characteristic shared by the male transgenic mice is the unusual number of immature cells, essentially round spermatids, released into the lumen of both seminiferous tubules and epididymis (Berruti *et al.*, unpublished results). The phenotypic characterization is going on. It might be that the cells that express the mutant, inactive form of Rap1 are affected by alterations including disorganization of the AJ structures. In endothelial cells activation of Rap1 stabilizes cell-cell contacts and this activation is due to a cAMP-Epac-triggered signal (88). Rap1, in fact, like Ras, cycles between an inactive GDP-bound and an active GTP-bound conformation. Rap1 becomes the GTP-bound active form by guanine nucleotide exchange factor (GEF). GEFs for Rap1 include C3G, CalDAG-GEFs, Epacs, Dock4 (91); each type of Rap1-GEF is specifically activated, on its own, by a unique second cell messenger and for EPAC, acronym for exchange protein activated by cAMP, the signaling molecule is cAMP. Intriguingly, the cAMP-Epac-Rap1 signaling is emerging to govern cell-cell and cell-matrix contact/adhesion not only in endothelial cells. Gupta and Yarwood (92) have shown that in various cultured cell lines, as HEK293, COS1 and PC12 cells, the integrin-mediated cell adhesion to laminin is enhanced in LC2- (light chain 2 of MAP1A) and Epac1-transfected cells; this finding suggests a crucial activity of Rap1 in cells where differences in spatial stability and dynamics of the microtubular cytoskeleton are essential for cell processes, like neuron development. Similarly, Enserink and co-workers (85) have shown that in cultured cells cAMP acts as an upstream regulator of cell adhesion and cell spreading to laminin-5 through an Epac-Rap1-dependent pathway. Surprisingly, because seminiferous tubuli offer intriguing material for this kind of research, in literature there are no data about Rap1 activation mechanisms in male germ cells. Indeed, in 2003 it was reported that in cultured mouse spermatogenic cells cAMP is able to trigger Rap1 activation (93). This led us to search for the presence of Epac in spermatogenic cells: our published (93) and unpublished results confirm the presence of both Epac isoforms, i.e., Epac1 and Epac2, in mouse male germ cells, with a different intracellular distribution according to the spermatogenic cell type considered. So, a cAMP-Epac-Rap1 signaling has to take place very likely during spermatid differentiation. Which is/are the event/s triggered by such a signaling ? The data discussed here suggest that Rap1 could play a regulatory role in AJ/apical ES dynamics, although the precise cascade of events and the underlying regulatory mechanisms remain to be discovered. So below, a scheme of post-meiotic signaling is proposed (Figure 2); it is simplified in comparison to the potential signaling of Rap1 in spermatogenic cells being focused on the regulation of Sertoli-haploid germ cell contacts. In illustrating this model, I kept in mind that: a) male germ cells express both integrins that are associated to actin (94) and cadherins (95, 96) while Rap1 is lost in the latest stages of spermatid differentiation (90); b) testosterone suppression induces in the rat both sloughing of spermatids (step 8 and beyond) from the seminiferous tubules (97) and failure of spermiation (94), in a manner which is reminiscent of what found by Holdcraft and Braun (41) with conditional null AR mice; c) Sertoli cells express selective surface adhesion molecules as ICAM-1, an integrin substrate (85), when adequately stimulated (98).

5. PERSPECTIVE

Many genetic, behavioral and environmental factors affect male fertility. At present, treatments for male infertility are limited and a range of assisted reproductive technologies (IVF, ICSI, ELSI) are used to circumvent rather than treat male infertility problems. Unlike other biomedical procedures, assisted reproductive technology may produce generational consequences. On the other hand, owing to the complexity of spermatogenesis, at present germ cell maturation cannot be accomplished *in vitro*. The recent successes in spermatogonial stem cell technology, including stem cell transplantation (99), and the discovery of some of the signaling pathways that govern and regulate spermatogenesis - eventually supported by generation of mouse models for analyzing the role of a single individual molecule in the complex pathway - could lead step-by-step to our understanding of the entire process. By paraphrasing Camillo Golgi, the

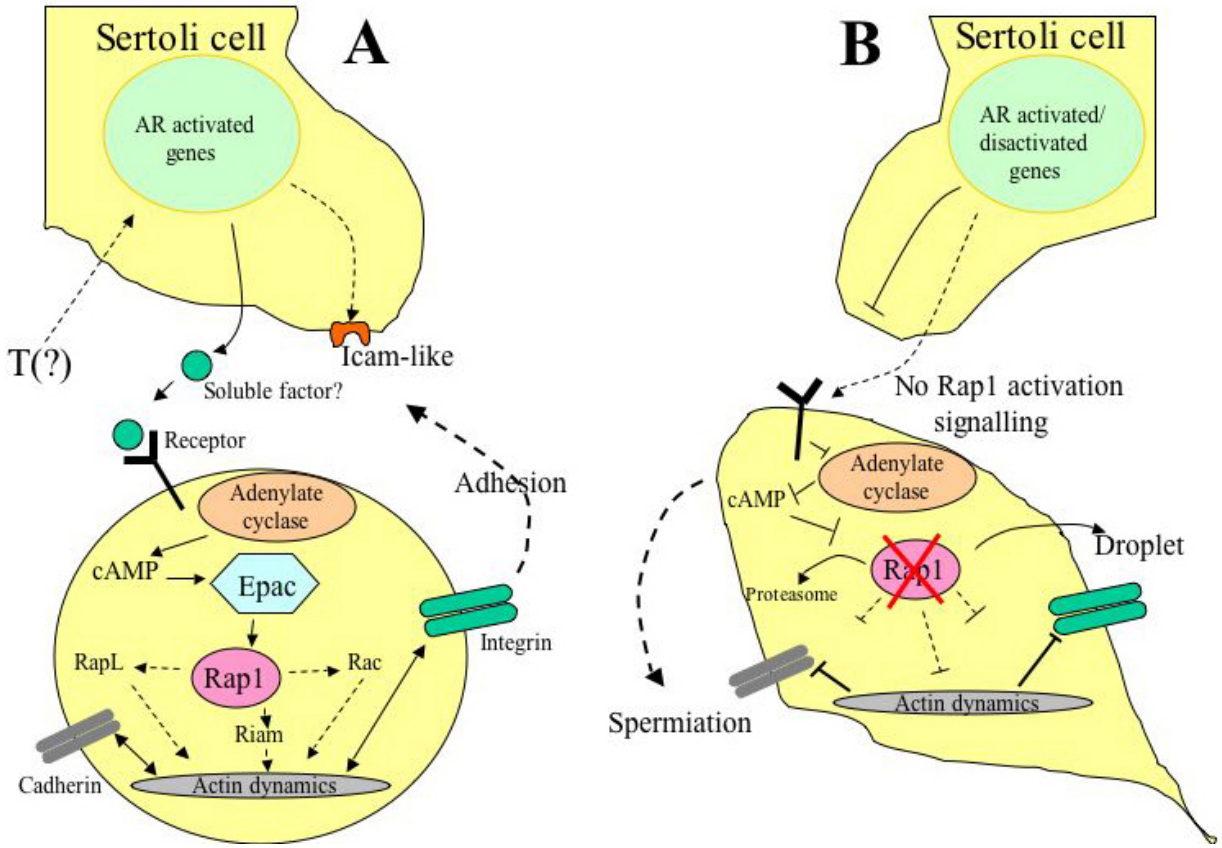

Figure 2. A schematic drawing illustrating how Rap1 may regulate the assembly and disassembly of the Sertoli- germ cell adhesion during spermiogenesis. A. A soluble or membranous factor (here simplified as a soluble secrete) expressed by Sertoli cell, might be under testosterone induction, triggers the activation, cAmp/Epac-mediated, of Rap1 in a round spermatid. The G-protein, through its intracellular effectors (RapL, Riam, Rac or other), regulates integrin-mediated germ-Sertoli cell adhesion and/or cadherin-stabilized germ-Sertoli cell contacts. As to the putative Sertoli molecular players, see the text and references. B. When Rap1 is not activated in spermatids or, even, Rap1 is no more present, as in late spermatids (being eliminated by the way of cytoplasmic droplet or degraded by proteasome), the loss of Rap1 activity causes dramatic alterations in germ-Sertoli cell association. This could lead to integrins and/or cadherins mislocalizations thus resulting either in sloughing of immature spermatids, such as induced by testosterone suppression, or in physiologic spermiation.

discoverer of the Golgi complex, who in his speech at the meeting of "Reale Istituto Lombardo di Scienze e Lettere" in 1914 said : ' We ought to be afraid of the unknown, not of the knowledge', the work of scientists has not to be prevented, but upheld so to promote only responsible, safe and ethical biomedical treatments.

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