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SIGNALING EVENTS DURING MALE GERM CELL DIFFERENTIATION : BASES AND PERSPECTIVES

Giovanna Berruti

Dipartimento di Biologia, Università di Milano, via Celoria 26, 20133 Milano, Italy

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1. ABSTRACT

In all species, reproductive function depends on the ability of the individual to produce functional differentiated gametes. Spermatogenesis is a cyclic process in which diploid spermatogonia differentiate into mature haploid spermatozoa. Thus from a genetic point of view, spermatogenesis can be divided into two phases, namely the diploid and haploid phase. Indeed, this complex differentiation process is still more intriguing since primary spermatocytes, if genetically diploid, are functionally tetraploid, while elongating spermatids, the germ cells undergoing the most dramatic morphological changes, if genetically haploid, become functionally anucleate due to ongoing condensation of chromatin resulting in an inactive nuclear DNA. This multi-step differentiative pathway is dependent on a specific environment provided by the anatomical and cellular relationships that take place in the testis and more specifically within the seminiferous tubules. Already, early anatomists (mind comes to Enrico Sertoli and Gustaf Retzius) were fascinated by the mixed cellular composition of the testis correctly deciphered as a whole of interacting and interdependent cell types despite the fact these belong to two well-established and different cell lineages, i.e, the somatic and germinal line. Since their time (the XIX century) up to-day a conspicuous bulk of experimental work and a relative massive bibliographic documentation have been provided. From this it stands out : a) a sophisticated role played by the cyclic hormonal control elicited by the hypothalamic-pituitary axis; b) the structural membrane specializations of Sertoli-germ cell communications; c) the existence and action of a paracrine and autocrine testicular regulative secretion; d) a regulation of germ cell gene expression, highly specialized both at transcriptional, posttranscriptional, and translational level; e) an active participation of the haploid genome in the final steps of cell differentiation. Each of these points has been the matter of several more and less recent reviews to which the present author hands back in the course of this note. However all these points, although topics of separate and extensive treatises, are conceptually jointed by a 'leit-motiv', that is, the intracellular transduction of an exogenous signal evoking a specific stimulatory/inhibitory, proliferative/differentiative event. The spirit with which the present author interpreted this minireview was to recall some points to which to draw attention having as a scenario the complex process of male germ cell differentiation in mammals.

2. HORMONAL CONTROL : BACKGROUND

As Richard Sharpe introduces his new and view "Do males rely on female hormones ?", appeared in Nature 1997 (1), it is true that 'hormones maketh the man'. The two major functions of adult testis are the production of fertile spermatozoa and the secretion of testosterone, the male androgen responsible for the male sex characteristics. Both these functions are under pituitary control, that, in turn, depends on hypothalamic stimulation. The hypothalamic gonadotrophin-releasing hormone was first recognized in mammals as the hypothalamic decapeptide responsible for the release of gonadotrophins, namely luteinizing hormone (LH) and follicle-stimulating hormone (FSH), by the anterior pituitary (2). Traditional knowledge acts that LH and FSH receptors are located on the Leydig and Sertoli somatic cells of the testis, respectively. The combined action of FSH and testosterone, secreted by the Leydig cells under LH stimulation, on Sertoli cells stimulates these last to secrete peptides promoting germ cell differentiation (paracrine regulation) and to develop specialized Sertoli-germ cell junctions (structural membrane specializations) that 'create an isolated environment' and mediate differentiating germ cell migration towards the lumen of the seminiferous tubule. This is, in summary, the canonical statement and this is conceptually and physiologically true. However, it appears

that it is not so straightforward. Whereas for a detailed documentation upon the 'LH-Leydig cells-testosterone' and 'testosterone/FSH-Sertoli cells' effects on spermatogenesis I send back to other reviews (3-6), here the attention is on two points, namely a) on the 'female' hormones, i.e., oestrogen, the lacking of whose receptor in the male results in male infertility, and progesterone, whose sperm putative receptor is involved in the acrosome reaction, an exocytotic event essential to mammalian fertilization; b) on a protein that has a direct role in determining the fate of male germ cells and whose appearance is intricately linked to FSH hormonal control, i.e., CREMt.

2.1 Female hormones

2.1.1 Oestrogen.

As testosterone is considered to be the 'male' hormone, oestrogen is the 'female' counter-party. However, both sexes make both hormones; it is sufficient to remember that circulating estrogens originate by the aromatization of androgens catalized by the cytochrome P450 aromatase. So the critical points in the determination of the sexual distinctions are the quantitative divergence in sex hormone concentrations and the differential expression pattern of steroid hormone receptors. These two concepts, i.e., hormonal quantitative divergence and differential receptor-expression, are indeed the two key-motifs underlying also gonadogenesis prior to sexual differentiation. Ingraham and co-workers in a recent work (7) provide molecular evidence that the dosages of Dax-1, as well as WT1, are important for male sex development. Since these two gene products are, generally speaking, less familiar than steroid hormones, I recall here some points. An obligatory gene for mammalian gonadogenesis is the autosomal Ftz-f1 gene which encodes the steroidogenic factor 1 (SF-1), an orphan nuclear receptor (8). SF-1 mutant mice display a series of alterations, as the lack of gonadal and adrenal development, loss of pituitary gonadotropins, and structural alterations of hypothalamus, that demonstrate the essential role of SF-1 in the development of the hypothalamic-pituitary-gonadal axis. In embryonic testis, SF-1 achieves male sex-specific expression by recruiting a cofactor (WT1), a zinc-finger transcription factor (9), with which acts in concert to direct Sertoli cell-specific expression of MIS (polypeptide hormone Mullerian inhibiting substance) (7). This triggers the regression of the Mullarian duct resulting in male sexual development. On the other hand, DAX-1, another orphan nuclear receptor (10), is the candidate to be a female-specific gene since it seems to be involved to direct (by default) ovarian development. And in the above mentioned work by Ingraham's group (7) it has been shown that the quantitative divergence between DAX-1 and WT1 is important for sexual development since DAX-1 and WT1 oppose each other to affect SF-1 mediated transactivation of male-specific genes. A parallel situation occurs for the sex hormones: adult female make large amounts of estrogens, while adult male make large amounts of androgens, but both sexes make both hormones. This means that estrogens have to exert, whatever minor, a role in the adult male. A part the experimental evidences that estrogens are produced by the somatic cell component of the testis (3-4), more recently it has been shown that P450 aromatase is present and active in germ cells of both adult mouse (11) and adult rat (12) testis; not only, but also epididymal sperm contain active P450 aromatase which decreases as sperm traverse the epididymis (11). These findings led the authors to conclude that spermatids can synthesize oestrogen and that the presence of oestrogen in sperm from efferent ductules and caput epididymis could be important in the process of sperm maturation. This observation is intriguing and relevant in view of what it is going to be discussed just below. It appears, in fact, that estrogens may even be essential for male fertility. Male mice lacking the alpha form of the estrogen receptor, i.e., ERKO mice, are infertile (13). In particular, adult male ERKO mice have atrophized testes with a progressive dilatation of the lumen of seminiferous tubules followed by degeneration, low sperm numbers, and reduced mating frequency. Hess and co-workers (14) have recently provided evidence of how this may occur. Briefly, these authors have found that oestrogen, present in high concentrations in semen and rete testis fluids, regulates the reabsorption of luminal fluid in the head of the epididymis. In fact, in ERKO mice the fluid is not reabsorbed, but accumulates thus impairing sperm production because of the increased fluid pressure within the testis. Not only, but those sperm cells that enter the epididymis are diluted and cannot mature properly since the 'maturation factors' secreted by the epididymal epithelium are consequently also diluted, i.e., reduced in their action. Also in the man, recently, clinical cases of congenital deficit by estrogens have been described (15-17). The phenotipical, hormonal, and diagnostic scenario appears to be more variegated, and so less conclusive, in these male patients as respect to the ERKO mice (particularly, if referred to the fertility potential). On the other hand, Ergun *et al.* (18) have recently found the presence of oestrogen receptors in the epithelium of the efferent and epididymal ducts of human epididymis; this finding is consistent with a role of oestrogen in the control of efferent ductule function also in the man. Thus it follows that a till now under-explored field of research, i.e., the study of the role of 'female' hormones in male fertility, has to be extended. As both Hess and co-workers in their paper on Nature (14) and Sharpe in his news and views in the same journal (1) remark, we are looking forward these new studies. Recent epidemiological data in humans and animals are alerting about the adverse effect of industrial and environmental toxicants, firstly the 'environmental estrogens', upon the development of male reproductive system and semen quality. It has been advanced the 'hormone disrupter hypothesis' (19), i.e, these chemicals are believed to exert an adverse effect since they might : a) antagonize the effect of endogenous hormones; b) mimic the effect of endogenous hormones; c) disrupt the synthesis and/or metabolism of hormone receptors. A knowledge of the role of estrogens and estrogen receptors in the male can provide evidential replies to what is so far speculation.

2.1.2. Progesterone

The other 'canonical' female hormone is progesterone. As to the putative presence of progesterone in the testis I send back to the review of Saez (20), recalling to the mind only that 17OH-progesterone is an immediate

precursor of androstenedione and testosterone, the classic testicular androgens. The 'hot' point is indeed not the hormone, but its receptor. This paragraph devoted to progesterone could have been inserted in the section dealing with signal transduction pathways in male germ cells; however, because the real molecular nature of the progesterone sperm receptor is far from being established – as it will be evident just below - I have choosen this section, devoted to female hormone action in the male, such as the most appropriate one. Since the pioneer work of Meizel's group on progesterone-induced acrosome reaction in human sperm (21), several other works from various laboratories have dealed with the stimulatory effects of progesterone in mammalian sperm (22). From these studies it is emerged that progesterone is, together with the oocyte zona pellucida ZP3 protein, the major physiological inducer of the acrosome reaction, the sperm exocytotic event resulting in the release of the acrosomal lytic content that promotes the sperm penetration through the egg investments (22-23). Consequently, sperm cells, i.e., the terminal product of male germ cell differentiation, are the cellular type target of the direct or undirect progesterone action and therefore they ought to have developed and maintained some signalling protein complexes able to transduce intracellularly the stimulatory effect of progesterone. In the classic model, steroid hormones bind to intracellular receptors, which are hormone-regulated transcription factors that, as a consequence of ligand-activation, migrate into the nucleus and elicit changes in gene expression; this pathway is known as the 'steroid genomic effects'. However a 'genomic effect' due to whatever steroid is quite improbable in sperm cells, since these cells are not only translationally inactive, but display an extremely packaged chromatin which shuts off all RNA synthesis. On the other hand, in the recent years it has been accumulated evidence that steroids can also exert 'signaling activity' by another way, the consequently so called 'non genomic action' (24). In the case of sperm cells the hypothesis of a nongenomic action of progesterone is more realistic since the extreme cellular differentiation has led these cells to be highly reactive to stimulators of events, as the acrosome reaction and the acquisition of a coordinated forward motility, that do not require the synthesis *ex novo* of proteins, but are centered on a Ca^{2+}-triggered signaling cascade (the former) and a cytoskeleton responsiveness to chemoattractans (the latter). But, how could be these nongenomic actions realized ? Firstly, by the interaction of progesterone with either a non-specific membrane receptor or a steroid-specific membrane receptor variant form. It falls in the first case what proposed by some authors that have described the possible involvement of a $GABA_A$ receptor/Cl^- channel complex in mediating progesterone action (25). According to these authors, progesterone triggers a rapid, transient increase in the free Ca^{2+} of the sperm head; this is by the way of the progesterone activation of a sperm amino acid neurotransmitter receptor/Cl^- channel which results in plasma membrane depolarization and thereby in a control over voltage-sensitive sperm Ca^{2+} channels and Ca^{2+} influx, which is important to the acrosome reaction. Intriguingly, very recently, Zingg and co-workers (26) have described in uterine cells another case of progesterone nonspecific nongenomic action; however, here, progesterone acts in the opposite direction, i.e., by contrasting intracellular free Ca^{2+} increase. These authors show that progesterone (P_4) binds with high affinity to recombinant rat oxytocin receptor and, as a consequence of the binding, suppresses oxytocin-induced inositol phosphate production and calcium mobilization in uterine cells. Thus, Zingg and collaborators provide the first experimental evidence for a direct interaction between a steroid hormone (progesterone) and a G-protein-coupled receptor (oxytocin receptor). Not only, but this nongenomic effect is highly steroid- and receptor-specific, because binding and signalling functions of the closely related human oxytocin receptor are not affected by P_4 itself but by the P_4 metabolite 5-beta-dihydroprogesterone (26). Similarly, awaiting experimental evidences, a situation of strictly specificity between progesterone/ progesterone derivative and interacting membrane receptor might be evocated also for the sperm progesterone/GABA receptor/Cl^- channel to justify some conflicting results reported when sperm from different mammalian species are compared (22).

On the other hand, it may be that sperm cells have indeed specific progesterone binding sites on their plasma membrane. By immunohistochemical studies carried out essentially on human spermatozoa using a fluoresceinated progesterone-BSA conjugate (27-28), a peroxidase-conjugated progesterone (29), and an antibody directed against the C-terminal portion of the human P-receptor (30), progesterone binding sites have been localized on the sperm surface. However, the 'molecular nature' of these putative P-receptors has not yet clear. In humans, the canonical, genomic, progesterone receptors are two distinct forms, i.e., PR-A, of 94 kDa, and PR-B, of 114 kDa, that are transcribed from distinct estrogen-inducible promoters within a single copy PR gene. The only difference between the two isoforms is that the first 164 amino acids of PR-B are absent in PR-A; this difference is however remarkable since PR-A functions as a transcriptional repressor, whereas PR-B functions as a transcriptional activator of progesterone-responsive genes (31). Indeed, recently Yeates and collaborators (32) have found that breast cancers (breast is one of the major progesterone target tissues) contain, in addition to PR-A and PR-B, a smaller PR protein of 78 kDa. This study shows that the 78 kDa protein is a truncated PR form, which is ligand-binding and thus may have a role in progesterone signaling, although this, if any, remains to be established. Very recently, Baldi and collaborators (33) have identified and characterized the first functional nongenomic progesterone receptor on human sperm plasma membrane. These authors, by using a panel of specific anti-PR antibodies, directed against either the P-binding domain of 'genomic' receptor or the DNA binding and NH2-terminal domains of the 'genomic' P-receptor, have been able to identify two proteins of similar molecular weight (54 and 57 kDa, respectively) in human sperm. These two forms, that represent likely truncated PR variants, are recognized only by antibodies directed against the P-binding domain of genomic receptor and are both localized in the human sperm plasma membrane. Thus, p54 and p57 could be considered as specific progesterone binding sites

on the sperm surface potentially involved in the induction of the acrosome reaction. This is a very intriguing finding. However, only the purification of these putative sperm membrane P-receptors and their amino acidic sequencing will provide the basis for delineating a biological role. Antibodies of new generation, possibly directed against specific not conserved regions of these receptors, could be employed in immunoprecipitation assays to check the co-presence of sperm interacting proteins, namely some known plasma membrane signal transducer, as a component of a ligand-gated ion channel (according to the studies dealing with the $GABA_A$ receptor/Cl^- channel) or a G-protein-coupled receptor (mimicking the progesterone/oxytocin receptor interaction discovered by Zingg *et al.* (26); as to this point, I remember that oxytocin has been found in the mammalian testis (34) where it has been postulated to play a role in modulation of seminiferous tubule contractility) or a receptor-associated/membrane-anchored tyrosine kinase (in agreement with the studies that report that progesterone increases protein tyrosine phosphorylation in acrosome-reacting sperm; see 22, 35-36)

At last, for a more correct information, it is to remember that steroid hormones, once inside a cell, can promote signalling pathways through their intracellular, 'genomic', receptors in a transcription-independent, i.e., 'nongenomic' , fashion. This is the case, for instance, of the estradiol-triggered reply in MCF-7 cells, a human mammary cancer cell line. Migliaccio and collaborators (37) have shown that estradiol, when complexed with its intracellular receptor, activates the tyrosine kinase/p21ras/MAP-kinase pathway in MCF-7 cells with kinetics similar to those of peptide mitogens. As Didier Picard suggests in his news and views appeared on Nature 1998 (38), the researchers have to not "underestimate the nature's wizardry – because steroids can reach specific receptors inside cells and elicit changes in gene expression, but this does not mean that they always have to do".

2.2 CREMt

The cAMP-dependent signal transduction pathway plays a key role in regulation of mammalian testis function (39). LH and FSH interact with specific G-protein-coupled membrane receptors and thereby stimulate adenylate cyclase activity. The consequent activation of the cAMP-dependent protein kinase A results in phosphorylation of a family of transcription factors containing the basic domain/leucine zipper motifs which bind as dimers to cAMP-responsive elements (CREs) (40). This family of transcriptional regulators consists of a large number of members that may act as activators or repressors; anyway, the function of the CRE-binding proteins is modulated by phosphorylation by several kinases (39). In the testis the CREM (cAMP-responsive element modulator) gene products appear to play a pivotal role in the regulation of spermatogenesis (39). In fact, the CREM gene generates, by spermatogenic cell-specific splicing, alternative CREM isoforms; thus, whereas in premeiotic germ cells CREM is expressed at low amounts in the form of antagonists (CREM alfa, beta, gamma) of the cAMP transcriptional response, from the late pachytene spermatocyte stage onwards, an alternative splicing event generates exclusively an activator isoform, namely the CREMt, which accumulates in high amounts (41). Thus, in testis, CREM is the subject of a developmental switch in expression, which constitutes also a reversal of function. This CREM switch has been reported to be regulated by FSH acting through the Sertoli cells in the mouse, rat and hamster testis (42). Indeed, in hypophysectomized animals it has been observed the extinction of CREMt expression in testis, while the direct administration of FSH restores the expression (42). However, although Sertoli cells have FSH receptors - which after binding with the hormone interact with G-proteins giving rising to the activation of the cAMP-dependent pathway - up to date it is not clear how the 'signal' internalized in Sertoli cells is transduced into the germ cells. It is generally agreed that seminiferous tubule fluid is produced by Sertoli cells. Among the numerous different proteins and peptides secreted (on an estimation of about 100 different Sertoli cells products, only one-third have so far been identified, see (43)) it might be a not yet identified or characterized factor whose synthesis is FSH-induced and whose action is to trigger the CREM developmental switch in the interacting germ cells. Moreover, the factor has not to be necessarily a secreted product since the 'signal' may reside in a structural modification of a Sertoli plasma membrane component, as for instance a cell-cell adhesion molecule, which is picked up by the strictly cross-talking germ cells. For instance, it might be speculated that under appropriate stimulation Sertoli cells expose on their surface a selectin-like molecule that recognizes a particular carbohydrate moiety of a membrane signalling component of the spermatocyte; this would result in the nuclear translocation of the signal and, by last, in the CREM switch. This is in part reminiscent of the classical transitory cell-cell interactions triggered during regional inflammatory responses (44). However, even when identified the molecule, it remains to discover and delineate the germ cell signaling pathway elicited by the 'Sertoli factor' leading to the functional CREM switch. On the other hand, one can suppose a more direct effect of FSH on male germ cells, i.e., postulate the presence of FSH receptors on the germ cell surface itself. Indeed, even if some previous reports dealed with the presence of a not better defined FSH receptor on germ cells (45), this remains an unresolved question. Very recently, however, Baccetti and co-workers (46) have shown the presence of FSH receptor mRNA scattered in the cytoplasm of mouse male germ cells by *in situ* hybridization while the FSH receptor has been localized in endocytotic membrane vesicles by immunoelectromicroscopy; the cells with highest positivity range from spermatogonia to spermatocytes, but also round spermatids result positive. Moreover, a recent study of the Sairam's group (47) has revealed the existence of a testicular transcript encoding an FSH receptor variant with a truncated and modified carboxyl terminus. Thus, different putative testicular FSH receptor variants might be involved in different hormone binding and signaling mechanisms during the various phases of spermatogenesis. It is so clear that more aspects of the CREM transcriptional regulation during the male germ cell differentiation process require a deeper characterization. This is desirable in consideration of the

key role assigned to CREMt in regulating spermatogenesis. In fact, it has been shown indepedently from both the Sassone-Corsi's group (48) and Schutz's group (49) that male mice lacking the CREM gene (CREM KO) undergo a severe impairment of spermatogenesis, resulting in a post-meiotic arrest at the first step of spermiogenesis; not only, but this occurs despite the normal hormone (testosterone and FSH) levels found in the serum of the mutant animals (49). In a recent work Nieschlag and co-workers (50) report that also in human patients with round spermatid maturation arrest CREM expression is significantly reduced and CREM-negative spermatids fail to progress beyond stage III of spermiogenesis. Even more recently, Serio and co-workers (51) have shown a significative decrease in CREMt expression in the semen from oligozoospermic patients. Thus an altered CREMt expression seems to be a common aspect associated with cases of idiopathic male infertility. The spermatogenetic arrest, resulting in the lack of spermatozoa, that characterizes CREM-deficient mice seeems to be due to the non-expression of specific postmeiotic genes whose activation is CREMt-dependent as the genes for protamine 1 and 2, transition proteins 1 and 2, Krox-20 and Krox-24, MCS, and RT7 (48-49). The list of these haploid-specific genes is growing up. Through molecular cloning of the overall sequence, it is more recently resulted that : a) the promoter region of the mouse protamine 2 gene contains CRE and HRE elements, recognized respectively by CREMt and steroid hormones (52); b) the promoter of calspermin, a Ca^{2+}/calmodulin binding protein, contains two CREMt-binding elements which reside in the penultime intron of the gene codifying Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV), an enzyme that, intriguingly, can phosphorylate and activate CREMt in a manner analogous to protein kinase A (53); c) the promoter of the testicular variant of angiotensin-converting enzyme (ACE) is positively regulated by CREMtau (54); d) the promoter region of the testis-specific protein kinase 1 (TESK1) contains several potential binding sites for transcription factors, including AP-1, cMyc, SRY and CREMt (55); e) the transcript of a testis-specific actin-capping protein (ACP) contains a putative CREMt-binding motif upstream of the initiation codon (56); f) the expression of MSJ-1, a novel male germ cell-specific DNAJ homologue (57), is strongly reduced in CREMt-deficient mice (58).

3. SIGNAL TRANSDUCTION IN MALE GERM CELLS : COMMENT

The task of presenting the argument of this section is, indeed, arduous. In the last decade there has been an imposing improvement in understanding the molecular mechanisms underlying the complex process of male germ cell differentiation so that the 'range' of possible worth mentioning examples is wide. It is, however, the purpose of this review to present in each section some of the recent exciting findings in a given field of the 'signal transduction related to mammalian spermatogenesis'. So, in consideration of this purpose and of, not last, space limitations, I have choosen three 'examples', each illustrative of a given germ cell type, i.e., diploid, meiotic, and post-meiotic, to talk about some molecular players surely involved or potentially involved in coordinating/controlling spermatogonial proliferation, meiotic cell cycle, and germ cell differentiation. Consequently, this section is by no means comprehensive and many topics and investigators are not included or cited.

3.1 Stem cell factor and its receptor c-kit protein in mammalian testis

The c-kit proto-oncogene encodes for a transmembrane tyrosine-kinase receptor with homology to the receptors for platelet-derived growth factor (PDGF) and colony-stimulating factor-1 (CSF-1) (59). The ligand of the receptor has been identified as stem cell factor (SCF), an integral membrane glycoprotein that exists in two forms generated by alternative splicing (60); one of these forms can be efficiently cleaved to release the extracellular domain as a soluble factor while the other form remains membrane bound. Loss or alteration of the expression of either the ligand or the receptor leads to anemia, albinism, and/or sterility in mice since the system SCF/c-kit influences haematopoiesis, melanogenesis, and spermatogenesis. Studies of mutant mice over the past decade have clearly established the importance of c-kit/SCF interactions for normal development of primordial germ cells (PGCs) and spermatogonia. Both the membrane-bound and soluble forms of SCF are produced in the testis by Sertoli cells, while the c-kit receptor is expressed by both spermatogonia and Leydig cells (61). By studies carried out with PGCs cultured in a variety of conditions (62-63) it has been shown that both SCF forms influence PGC survival, proliferation, migration and differentiation (60). In post-natal testis, hormonal stimulation of Sertoli cells by FSH-triggered/cAMP-dependent signaling results in an increase in the mRNA levels for both the SCF forms; the resulting soluble form is then able to promote DNA synthesis in mouse type A spermatogonia, the germ cell type which is the main site of c-kit expression in the adult testis (64). On the other hand, administration of ACK-2, a monoclonal antibody that blocks binding of SCF to c-kit, disrupts proliferation of mouse type A spermatogonia (65) whereas promotes apoptosis of both spermatogonia and primary spermatocytes (66). These findings indicate that signalling through c-kit is part of the mechanism that governs spermatogonia mitotic progression, a process indispensable for securing the renewal of the stem cell population. But it appears that during normal spermatogenesis spermatogonia undergo also a physiological apoptotic wave, which is reported as a necessary requirement for maintaining a proper cell number ratio between maturing germ cells and Sertoli cells (67). This apoptotic wave is coincident, in timing and localization, with a temporary high expression of the apoptosis-promoting protein Bax, which disappears at sexual maturity. Thus, it appears still more that the intracellular balance, likely hormonally controlled, between the levels of two key step-specific gene products acting in an opposite direction, i.e., in this case the c-kit and Bax proteins, plays a critical role for the progression of a correct spermatogenesis. However, which is the signaling pathway by which the SCF/c-kit system promotes spermatogonia proliferation? At the moment, this is not known. The pleiotropic effects of the c-kit receptor system

are mediated by the c-kit, ligand-induced, receptor autophosphorylation and its subsequent association with a panel of distinct second messengers activators such as phosphatidylinositol 3'-kinase (PI3'-K), p21ras and mitogen-activated protein kinase (MAPK). For instance, by studies of site-directed mutagenesis carried out in mast cells (68) it has been shown that substitution of tyrosine 719 in the c-kit kinase domain with phenylalanine abolishes PI3-K activation, diminishes c-fos and junB induction, and impairs SCF-induced cell adhesion to fibronectin. At the same time, this mutation has only partial effects on p21ras activation, cell proliferation and survival, while MAPK activation is not affected. On the other hand, substitution of tyrosine 821 impairs proliferation and survival without affecting PI3-K, p21ras and MAPK activation, while fibronectin-cell adhesion remains intact. Thus, by this and other studies (69) it follows that the diversity of molecular interactions between specific phosphotyrosine residues of the activated c-kit receptor and src homology-2 (SH2) domains of intracellular signal transducers - such as the regolative p85 subunit of PI3-K, the ras GTPase Activating Protein (GAP), the phospolipase C gamma-1, the tyrosine phosphatases SHP1 and SHP2, and the adaptor protein Shc - can provide a specific mode for c-kit signal definition and/or modulation. At the moment, our general knowledge of the molecular players involved in the signaling pathway regulated by the SCF/c-kit system in spermatogonia is very scanty. On the other hand, the spermatogonial c-kit interacting proteins have first to be checked among those cited above, the presence of some of which has recently been reported in male germ cells (70-72). The identification of the interacting proteins will help to find a link among these and downstream acting serine/threonine kinases and expression of cyclin genes and/or activation of cyclin dependent kinases in a manner which is reminiscent of the induction of the G1/S-phase transition by the integrin-linked kinase (ILK) (73).

At last, I wish to remind that alternative, shorter c-kit transcripts are expressed in post-meiotic male germ cells by developmental switch of a cryptic promoter (74). The same Geremia's group (75) has successively shown that microinjection of the recombinant truncated c-kit protein into methaphase II-arrested mouse oocytes causes oocyte activation and formation of a parthenogenetic pronucleus with progression through cleavage stages. These findings candidate the truncated c-kit as a putative sperm signaling factor for triggering mouse eggs activation at fertilization.

3.2 Hsp70-2, a gene product whose function is meiotic-specific.

From studies carried out on the cellular 'chaperone machines' (76) it comes out the implication that heat shock proteins (Hsps), or at least a subset of molecular chaperones, can be universally involved in peculiar cell functions not strictly related to heat shock protection, but centered on signal transduction mechanisms, as the cell cycle progression, the steroid hormone receptors function and the signaling by receptorial/intracellular tyrosine kinases (77). In fact, one important concept gained from the study of intracellular signaling is that formation of large protein complexes is essential for signal transmission and molecular chaperones are the intracellular players that selectively assemble such complexes and regulate their intracellular localization in response to extracellular stimuli. The 70 kDa heat shock protein (Hsp70) multigene family is universally expressed and is essential for growth either at high temperatures or at all temperatures (78). In mice, for instance, the Hsp70 family contains at least ten different proteins, including the constitutively expressed Hsc70 members and the environmental and/or physiological stress-induced Hsp70 members (79). In addition, two unique members are expressed during mouse spermatogenesis, i.e., namely Hsp70-2, expressed at high levels in pachytene spermatocytes, and Hsc70t, expressed in postmeiotic spermatids (79). Despite the conspicuous number of Hsp70 protein forms found in eukaryotic cells, ranging from yeast to insects and humans (78) , the Hsp70 genes isolated thus far are related at the level of greater than 50% identity over their entire length; the Hsp70 family is, therefore, highly conserved. On the other hand, if protein similarity is great, numerous are the functions assigned to Hsp70s, as the participation in protein folding, in assembling and disassembling of protein complexes, in the transport of proteins into cell organelles, in the uncoating of clathrin-coated vesicles, in the cell cycle-dependent formation of protein associations and other (76). To explain the apparent contrast between Hsp70 sequence similarity and Hsp70 function diversity, recent studies suggest that Hsp70s function in a wide variety of cellular processes as a result of their recruitment by specific partner proteins, acting as cochaperones (80-81). As to the two spermatogenesis-specific Hsp70s, Hsp70-2 is that better characterized. Hsp70-2 gene expression is developmentally regulated: it begins in leptotene-zygotene spermatocytes and occurs at a high levels in pachytene spermatocytes (82), under the direction of a promoter within 300 bp of the transcription start site (83) with which it seems to interact the heat-shock transcription factor HSF2 (84). Hsp70-2 shares over 80% amino acid sequence similarity with the other Hsp70s; gene homologous are expressed in the spermatocytes of rats (85) and humans (86). A polyclonal antiserum, raised against a synthetic peptide corresponding to an Hsp70-2 specific sequence, has been shown to react specifically with meiotic and post-meiotic male germ cells (82). To determine the function of Hsp70-2, Eddy and collaborators have used the gene targeting approach to disrupt Hsp70-2 gene in mice (79). This caused primary spermatocytes to arrest in meiosis I and undergo apoptosis, leading to male infertility, while fertility of the females, that indeed do not express Hsp70-2 also in the wild type condition, was unaffected. Intriguingly, the other Hsp70 proteins present in mouse Hsp70-2 knock out spermatocytes did not compensate for the loss of Hsp70-2, indicating that Hsp70-2 has unique functions required during meiosis I (79). Further studies from Eddy's laboratory have provide a series of novel evidences; among these there is : a) Hsp70-2 directly interacts with and functions as a chaperone for CDC2, the cyclin B-dependent kinase whose activity is essential for the G2/M-phase transition during the mitotic and meiotic cell cycles (87); b) Hsp70-2 is required for synaptonemal complex disassembly and the completion of meiosis I since in its absence

(Hsp70-2 knock out mice) synaptonemal complexes fail to desynapse, methaphase spermatocyets are not observed, and development of spermatids does not occur (88). Whether a link between the requirement for Hsp70-2 chaperone activity for assembly of a functional CDC2/cyclin B1 complex and the requirement for Hsp70-2 in synaptonemal complex disassembly exists, it is at the moment unknown. However, as to this point, Eddy and co-workers (88) have put forward a suggestive hypothesis, matter for further experimental work, that I recall here, i.e., : '… failure of synaptonemal complex desynapsis in Hsp70-2$^{-/-}$ spermatocytes might be due to loss of the ability of CDC2 to phosphorylate SCP1/SYN1, which is the major protein component of the synaptonemal complex transverse filaments and whose phosphorylation by CDC2 kinase would result in the signal for triggering the complex disassembly (in analogy with other cell cycle-dependent phosphorylation events) …'. As reported above, to explain the contrast between Hsp70 protein similarity and Hsp70 function diversity, other chaperone molecules have been called into question. However, the biochemical and molecular characterization of these Hsps protein partners that 'customize' the highly conserved Hsps for their specific roles (81) is still rather scanty. As to those identified in male germ cells, Watanabe and co-workers (89) reported the molecular cloning of a novel Ca^{2+}-binding protein specifically expressed during male meiotic germ cell development, called calmegin. Calmegin is a testis-specific endoplasmic reticulum protein that is homologous to calnexin, a ubiquitous reticulum chaperone that plays a major role in retaining incompletely folded or misfolded proteins (90). Okabe and collaborators have performed targeted disruption of calmegin gene obtaining very interesting results (91). Homozygous-null male mice are nearly sterile even though spermatogenesis is morphologically normal and mating is normal. These results have led the authors to suggest that calmegin functions as a chaperone for one or more sperm surface proteins that mediate the interactions between sperm and egg. Thus, calmegin mutant mice may provide a novel model for studying cases of unexplained male infertility despite the fact that spermatogenesis occurs normally, sperm numbers are normal and mating frequency is normal. Moreover, very recently it has been described a novel DnaJ homologue, named MSJ-1 for mouse spermatogenic cell-specific DnaJ first protein (59). DnaJ proteins are molecular chaperones universally considered as the specific regulators of Hsp70s (82). It would be interesting to check whether MSJ-1, expressed and translated only in male germ cells since MSJ-1 mRNA and MSJ-1 protein have not been detected in any somatic tissues and ovary (57-58), specifies the functions for the male germ cell-specific Hsp70s reported above, i.e., Hsp70-2 and Hsc70t (work in this direction has been planned by Eddy's and Berruti's laboratories).

3.3. A model of signaling in male germ cells: players of a novel pathway

Spermiogenesis is a complex morphogenetic process of cell differentiation that involves the formation of the head and tail of the sperm, the condensation of the nucleus and the elimination of excess cytoplasm as part of the residual body. At the same time, spermatids have to organize their integral plasma membrane proteins not only laterally but also asymmetrically into specialized domains since the terminal product of the cell differentiation is a highly specialized and polarized cell, the spermatozoon. All this massive cellular remodeling, that implies of course also biochemical and functional modifications, requires the synthesis of new proteins whose developmentally regulated appearance has to be under transcriptional or post-transcriptional or translational level (92). This means, in other words, that diverse signaling pathways are evocated and transduced intracellularly in order to allow, for instance, to a transcriptional activator to become active and so to bind to Dna and promote the transcription of a given gene (an example, the triggered CREMt developmental switch discussed above and the consequent activation of haploid-specific gene expression), or to a stored mRNA, stabilized by interacting RNA-binding proteins that prevent its translation (93), to become temporally activated for translation (92). The 'scenario' of cell signaling in post-meiotic cells is clearly very open and wide. Here I am going to speak about a hypothetical signaling mechanism imagined to act in haploid male germ cells (so far it has been object of a congress presentation only). Which are the bases upon which it has been supposed ? Essentially two are the facts. First, many similarities have been observed between testis and brain: a) by a comparison of expression profiles of functional categories of gene expressed sequence tags (EST) in different tissues Hoog has shown that the relative frequency of expressed genes encoding for cell surface proteins and proteins involved in transcription, translation as well as in signal transduction is approximately the same between testis and brain (94); b) brain and testis share in common the tissue-specificity of expression of mRNAs for diverse 'signaling' proteins, from some protooncogenes (92, 95) and G proteins (96) or G-activating proteins (97) to cyclic-nucleotide-gated channels (98) and molecular adaptors of signaling proteins (99), to cyclin-dependent kinases (100) and deubiquitinating enzymes (101); c) both neurons and sperm cells are highly differentiated, not dividing, excitable, and polarized cells; spermatids, once rather ignored from this point of view, are now considered as adept as neurons at establishing their plasma membrane domains so that the sperm tail plasma membrane domain is suggested to be the equivalent of the neuronal somatodendritic plasma membrane domain while the sperm head plasma membrane domain is suggested to be the equivalent of the neuronal axonal plasma membrane domain (102). Thus, even if considerably less studied - mainly because of the lacking of long-term viable cell lines that represent the classic research tool for carrying out these studies - male germ cells appear to dispose of an armamentarium of cell signaling molecules which is reminiscent, at least in part, of that of neuronal cells. Secondly, some recent findings from my laboratory support this hypothetical signaling that, of course, requires further study and molecular characterization, which are anyway in progress in my lab.

Recently, it has been reported the molecular cloning and tissutal and developmental pattern of expression of the first 14-3-3 protein isoform found in male

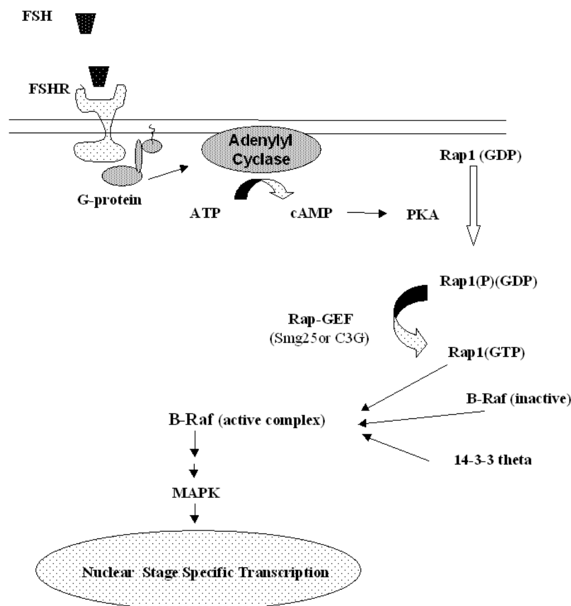

Figure 1. Model of signalling events in male germ cell differentiation. An FSH-mediated exogenous signal (here illustrated for schematic simplicity as a putative FSH/FSH receptor complex) is intracellularly transduced resulting in the activation of the germ cell cAMP-dependent protein kinase A (PKA). The PKA-dependent phosphorylation of the small G protein Rap1 stimulates the action of a Rap-specific exchange factor (Rap-GEF), i.e., Smg25 or C3G, resulting in the GTP loading of Rap1. This, in turn, induces the formation of an active Rap1/14-3-3 theta/B-raf complex that activates, via MEK-mediated phosphorylation, the MAP kinase (MAPK). The phosphorylated MAPK translocates into the nucleus where it promotes the activation of a stage-specific transcription factor (CREMt ?). Now, this last can bind DNA and trigger the expression of a subset of male germ cell-specific genes.

germ cells, the 14-3-3 theta whose expression is high in the testis and brain only (99). 14-3-3 proteins, first described as abundant acidic mammalian brain-specific proteins of molecular weigth ranging around 30 kDa, have been successively identified in numerous tissues and eukaryotic species so that actually more than ten isoforms are known; they have been seen to associate with a number of different signaling proteins and have been proposed to be important in controlling mitogenic and differentiative signaling pathways (103-104). Relevant to the regulation of signal transduction pathways, 14-3-3 isoforms have been shown to interact with Raf-1 in different cell types (105), including oocytes (106). The serine/threonine kinase Raf-1 serves as a central intermediate in many signaling pathways, by functioning to connect upstream tyrosine kinases and Ras with downstream serine/threonine kinases (107). Raf-1 is the ubiquitously expressed member of the *raf* family of genes (95); the related genes A-raf and B-raf show a more restricted, tissue-specific pattern of expression (95, 108). As to mouse testis, Raf-1 mRNA has been detected in germ cells from type A and B spermatogonia through the early round spermatid stage; A-raf mRNA is almost undetectable in germinal cells whereas its expression is high in Leydig cells and B-raf mRNA, originally reported as the member of the *raf* gene family specific of neural tissues, is expressed as two transcripts of 4.0 and 2.6 kb, the former in pachytene spermatocytes and the latter in post-meiotic spermatids respectively (108). As to B-raf, whereas the number and size of transcripts, ranging between 2.6 and 12 kb, depend on the tissue analyzed (109), the identity, structure, and number of B-raf proteins have not been unequivocally established. A B-raf carboxyl-terminal specific antiserum revealed the presence of two proteins of 75 and 77 kDa in mouse testis and brain total protein lysates (95). At my knowledge, the work of Sythanandam *et al.* (95) is still today the only one where there is mention of a putative B-raf protein in mouse testis (inclusive of both somatic and germinal cell components; no immunolocalization was provided). On the other hand, in the rat pheochromocitoma PC12 cells, that differentiate into sympathetic neurons upon treatment with nerve growth factor (NGF), B-raf proteins were identified either as a single 95 kDa band or as two proteins of 67 and 95 kDa (110, 109), depending on the cell clones used. Intriguingly, Yamamori and collaborators (111), in their study addressed to molecularly characterize the bovine brain protein factor named REKS (Ras-dependent Extracellular signal-regulated kinase Kinase Stimulator), found that the activator factor is composed of B-raf (95 kDa isoform) and 14-3-3 proteins (two unidentified isoforms of 32 and 30 kDa respectively). This is the first report of an interaction between 14-3-3 proteins and B-raf. In addition, Eychene and collaborators (112), in their study addressed to identify signalling proteins interacting with B-raf by using the yeast two-hybrid system and by screening a mouse brain cDNA library with a B-raf bait, found that B-raf interacts with three members of the 14-3-3 protein family, namely 14-3-3 eta, theta and zeta isotypes. On the other hand, it is known that B-raf is activated by the small G protein Rap1 both *in vitro* (113) and *in vivo* (114). It is also well documented from the experimental point of view that Rap1 can be phosphorylated by cAMP-dependent protein kinase (PKA) and that this phosphorylation at the serine residue Ser179 results in Rap1 activation (115). Just as to the work of Vossler and co-workers (114), it is here shown that the PKA-mediated activation of Rap1, in B-raf-expressing cells, provides a mechanism for tissue-specific regulation of cell differentiation via MAP kinases since in PC12 cells (expressing the B-raf 95 kDa isoform) Rap1 is a selective activator of B-raf and can activate selected transcription factors and stimulate neuronal differentiation via an ERK-dependent, but Ras-independent, pathway. Even more recently, the same group in the work published on Nature (116) shows that in PC12 cells, and possibly, other neuronal cells that express B-raf, NGF-induced activation of Rap1 promotes a sustained activation of ERK and is required for the induction of a subset of neuron-specific genes. Christopher Marshall in his news and views on Nature (117) puts forward an attractive model to account for the different consequences of transient (Ras/Raf 1-dependent) versus sustained (Rap 1/B-raf–dependent) activation of ERKs, i.e., that sustained activation leads to persistent nuclear accumulation of ERKs, resulting in phosphorylation of transcription factors and changes in

gene expression. On the ground of part of these very intriguing findings (the first ones since the latter are too much recent and, consequently, were unknown to me when I started this kind of study in male germ cells) and on the ground of the finding of the presence of 14-3-3 theta in mouse spermatogenic cells (99), my laboratory was engaged in the search for the existence of both B-raf and Rap1 proteins and Rap1/14-3-3theta/B-raf protein complex in mouse male germ cells. Our unpublished results (manuscript is in preparation) indicate that not only B-raf , as the 95 kDa isoform, and Rap1 are present but that both B-raf and Rap1 interact both *in vitro* and *in vivo* with 14-3-3 theta and co-localize in early round spermatids. Moreover, kinase assays carried out on the immunocomplexes show that B-raf is able to phosphorylate exogenously added MEK; in addition we have found an endogenous MEK (MAP kinase kinase), actually under investigation to delineate a male germ cell MAP kinase cascade (here I remember that very recently Luconi *et al.* (35) have shown that MAP kinases, otherwise called ERKs, are present in human spermatozoa).

Thus, I conclude with the schematic representation of an hypothetical signaling pathway proposed upon the grounds of what discussed just above. I suggest it as a model for further experimental work and insert it here as a perspective for future directions in male germ cell signaling transduction.

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Send correspondence to: Prof. Giovanna Berruti, Dipartimento di Biologia, Università di Milano, Via Celoria 26, 20133 Milano (Italy), Tel: 39.2.26604469, Fax: 39.2.26604462, E-mail: berruti@imiucca.csi.unimi.it