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Mini Review

Spermatogenesis Read between the Lines: A Biochemical Perspective on Sertoli-Germ Cell Crosstalk

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Abstract

Spermatogenesis is the biological process that generates male gametes for the transfer of the paternal chromosomes to the oocytes, at the time of fertilization. Neuroendocrine hormones work in tandem with testicular genes to bring about the maturation of the male gamete or spermatozoon. While some of the genes involved in the differentiation of spermatozoa are well known others are 'not so obvious'. The aim of this article is to conceptualize the molecular pathway(s) through which the 'not so obvious' Sertoli cell 'mediators' communicate with germ cells for their differentiation within the seminiferous tubules of the mammalian testes.

Keywords: *CaM; ABP; Transcytosis; Endosomes; Transmembrane Receptor*

Introduction

Research on spermatogenesis has focused on two aspects 1) Endocrine Regulation of Spermatogenesis 2) Genetics of Spermatogenesis. The biological events underlying the maturation of germ cell into spermatozoa are well known, but the synergistic role of reproductive hormones in the expression of genes involved in germ cell differentiation is far from being completely elaborated [1-5]. It is well established that germ cell differentiation program is regulated by circadian release of hypophyseal luteinizing (LH) and follicle stimulating (FSH) hormones and testicular hormone-testosterone (T) [6-8]. Testicular gene expression is coordinated by the reproductive hormones. Hormonal synergism that regulates germ cell differentiation depends upon the expression of cognate receptors for FSH (FSHR) and androgen (AR) in Sertoli cells [9,10]. The identity of Sertoli cell 'mediators' of germ cell differentiation, however, awaits elucidation [11]. The expression of estrogen receptors (ERs) in Sertoli and germ cells suggests that estradiol (E2) too is involved in Sertoli-germ cell communication [12-16]. Besides LH, FSH, T and E2, hypophyseal hormone Prolactin (Prl) reportedly modulates spermatogenesis, in particular under conditions of stress [17-20]. The identity of the hormone-regulated Sertoli cell 'mediators' of spermatogenesis and the molecular pathway(s) through which Sertoli-germ cells interact remain largely unexplored [21].

Role of androgen-binding protein in Sertoli-germ cell communication

Androgen binding protein (ABP) remains an abiding enigma of male reproductive physiology. ABP is the only secretory protein of Sertoli cell origin reported to mediate communication between Sertoli-germ cells in mammals [22,23]. ABP is secreted bidirectionally in peripheral circulation and lumen of seminiferous tubules in rodents. Two promoters regulate tissue specific expression of androgen binding protein expression [24-29]. The observed upregulation of transition protein1 expression in rat spermatids, co-cultured with recombinant murine Sertoli cells secreting rat ABP, indicated its 'mediator' potential [30]. Estradiol valerate administration up-regulates ABP mRNA in rat testis [31]. Furthermore, blockade of rat Sertoli cell ARs with cyproterone acetate (CPA) also down-regulates testicular ABP [32]. Stimulation of the androgen-dependent mechanism for the autophagic clearance of ABP underlies the CPA-induced downregulation [33].

Thus, since FSH-induces aromatization of T to E2 in Sertoli cells, both T and FSH are potentially capable of upregulating ABP, purportedly for maintaining intratesticular T levels.

However, the regulatory role of ABP in germ cell differentiation is not clear. Massive germ cell apoptosis observed in the testis of ABP over-expressing transgenic mice presented a phenotype of androgen deficiency [34,35]. Apoptosis of germ cells, a typical estrogenic effect, occurred due to the arrest of spermatogenesis at meiotic stage [36,37]. Thus, ABP overexpression would have reduced the availability of testosterone at the Sertoli cell AR thereby expression of putative mediators, leading to anomalies of spermatogenesis. Presumably, ABP could induce changes in germ cells at the molecular level and direct their differentiation. Primate germ cells internalize sex hormone-binding globulin (SHBG), human homologue from peripheral circulation [22]. Intuitively, internalization of ABP is suggestive of a mechanism of T uptake for transport to germ cells for aromatization [38]. *Homo sapiens*, however, express a non-secretory variant of ABP transcripts in Sertoli cells and a novel variant of ABP protein in spermatids [39]. Presumably, an evolutionary shift might have occurred in ABP gene expression in *Homo sapiens* [40]. The expression of a non-secretory ABP variant indicates a shift in the identity of molecular 'mediator' and pathway(s) for transporting T to germ cells. In this context, an intriguing observation from murine Sertoli cell-specific reproductive homeobox on chromosome5 gene (Rhox5)/PEM (placenta and embryonic expression) gene knockout assumes significance. Ablation of androgen-dependent, Rhox5 transcription factor gene reduced by more than half the numbers of sonication resistant spermatids of late spermiogenesis [41]. Evidently, Rhox5, an androgen-dependent Sertoli cell factor mediated spermatidal chromatin condensation. Apparently, a Rhox5-dependent molecular mechanism of Sertoli cell origin underlies the affected expression of spermatid-specific chromatin condensation genes lacking AREs [42]. Therefore, Rhox5 ablation compromised the chromatin condensation process, which confers sonication resistance to elongating spermatids.

Role of estrogen receptors in Sertoli-germ cell communication

Germ cells express two forms of estrogen receptors, ESR1 (Erα) and ESR2 (ERβ) as well as Aromatase enzyme [38]. Available evidences indicate that estrogens arrest spermatogenesis leading to germ cell apoptosis [37,43,44]. Estradiol valerate administration inhibits rat sperm nuclear chromatin condensation at biological level [31]. ESR1 mediates the inhibitory effect on chromatin condensation by decreasing the expression of Tnp1 (Transition protein1), Tnp2 (Transition protein2) and Prm1 (Protamine1) rat spermatidal genes. ESR2 mediates apoptotic effects by decreasing the expression of Sod1 (Superoxide dismutase1), Cat (Catalase), Gpx1 (Glutathione peroxidase1), Prdx3 (Peroxiredoxin3), Bcl2 (B-cell lymphoma2), Bclw (Bcl2-like2), Ccna1 (Cyclin A1), Ccnb1 (Cyclin B1) genes while increasing that of Nos3 (Nitric oxide synthase3), Caspase-9 rat spermatocyte genes. ESR2 also mediates spermiation failure in rat by decreasing the expression of Arpc1b (actin-related protein complex 2/3 subunit 1b), Evl (ENA vasodilator phosphoprotein), Picalm (Phosphatidylinositol binding clathrin assembly protein) genes [45,46]. ESR1 also interferes with DNA methylation of imprinted genes in germ cells [47,48]. ERs modulate gene expression via EREs in promoters (personal communication) [46]. The physiological relevance of germ cell estrogen receptors is not evident. Estradiol could be playing an autoregulatory role during spermatogenesis since most ER mediated effects are anti-androgenic [49]. However, E2 stimulates mitosis in spermatogonia via ESR1 indicating a role in cell cycle progression and regulation of stage-length [50].

Synergism between ABP-ERs in Sertoli-germ cell communication

Spermatogenesis ensures the transformation of spermatogonia to spermatozoa. Germ cell lineages of successive cycles of seminiferous epithelium align in distinct stages, presumably to avail of a common hormonal milieu for stage-dependent gene expression [51]. Sertoli cells, which express receptors of reproductive hormones in a stage-specific manner, co-ordinate gene expression in juxtaposed germ cell lineages [9-10,38]. *In vitro* studies indicated that T and E2 downregulate ABP-induced tnp1 gene expression in rat spermatids, cocultured with recombinant mouse Sertoli cells expressing rat ABP [30]. ESR1 mediates the effect of E2 on Tnp1 gene suppression via EREs in its promoter [43,45]. Germ cells internalize ABP secreted by juxtaposed Sertoli cells [22]. It would be logical to assume that ABP transported T from Sertoli to germ cells and generated the endogenous ligand for estrogen receptors [52]. Exclusive expression of SHBG, homologue of ABP, in human spermatids suggests that it could be serving a crucial role of sequestering E2 [39].

Putative role of endosomes in Sertoli-germ cell communication

The mechanism through which testosterone regulates the expression of germ cell genes remains an enigma. Sertoli cells exclusively express AR [10]. Most of the germ cell genes are androgen-dependent but lack AREs in their promoters. Sertoli cells support germ cell differentiation through provision of maturational stimuli. Therefore, a mechanism for delivery of transducing androgen signals from Sertoli to germ cells awaits elucidation.

Transmission electron microscopy (TEM) studies in rat demonstrated the direct internalization of ABP from Sertoli into rat germ cells [53,54]. An intriguing insight about the existence of a pathway emerged from Rhox5 gene ablation studies. Rhox5 is an androgen-dependent, Sertoli cell-specific, transcription factor. Rhox5 null mice are hypofertile. Compared to their wild type counterparts, Rhox5 null mice have two fold less caudal sperm and half the number of sonication resistant spermatids. Elongating spermatids develop sonication resistance when transition proteins replace histones during spermiogenesis [55]. Furthermore, elongated spermatids remain sensitive to sonication until the process of chromatin condensation is complete. Presumably, Rhox5 transcription factor transduced androgenic signals to complete chromatin condensation in germ cells, after reaching the spermatids via endosomes [41]. Expression of hormonally regulated Eea1 (Early endosomal autoantigen 1), Picalm, Stx5a (Syntaxin) genes with AREs in their promoters, and Arpc1b, Evl genes with EREs in their promoters affirm the relevance of endocytosis in Sertoli-germ cell communication [46,52]. Non-genomic Sertoli cell ARs are not known to initiate germ cell gene expression. Available evidences suggest that germ cells likely endocytose Sertoli cell 'mediators' transported in endosomes.

Transcytosis pathway exists for Sertoli-germ cell communication

Transcytosis is the process of direct transfer of mediators between cells in juxtaposition, via receptor-mediated endocytosis. Invagination of plasma membranes generates endocytosis endosomes for direct transfer of mediators into the adjacent cells, either for packaging in Golgi vesicles or eventual degradation in lysosomes [56-58]. Golgi vesicles extrude protein mediators via exocytosis. Endosomes are ferried on microtubules by Kinesins (motor proteins) from endoplasmic reticulum via Golgi vesicles to plasma membrane for exocytosis (anterograde). Endosomes are ferried on microtubules by Dynein (motor protein) from plasma membrane to lysosomes or Golgi cisternae and by Kinesins from Golgi to endoplasmic reticulum for endocytosis (retrograde) [59]. Kinesins recruit endosomes through Rab (RAS-related GTP-binding protein) GTPases. Dynein recruits endosomes through Dynactin adaptor proteins and Rab GTPases [60].

Several studies support exchange of 'mediators' between Sertoli and germ cells via transcytosis. FSH induces ABP secretion from cultured Sertoli cells only when cocultured spermatids and pachytene spermatocytes are in direct contact [30,61]. TEM studies of labelled T-ABP complex uptake by rat germ cells revealed endosomes and endocytic structures like clathrin-coated pits on plasma membrane. Fine processes emanating from Sertoli cell in the region of ABP internalization suggested direct uptake from its cytoplasm [53,54]. ABP secreted by recombinant Sertoli cells upregulates Tnp1 in cocultured rat spermatids, only when in direct contact, again suggests transcytosis from Sertoli cell cytoplasm [30].

Ablation of transmembrane receptor Megalin, expressed in sex-steroid target tissues, affects internalization of sex-steroids and leads to developmental defects in male reproductive tract [62]. Plasmatic Retinol, required for expression of Kit receptor, reaches germ cells bound to intracellular retinol-binding protein (CRBP), expressed exclusively in Sertoli cells [63,64]. Testicular transferrin (tTfn) transports plasmatic Iron (Fe) from Sertoli to germ cells via cognate germ cell receptors [65]. Megalin is not an exclusive receptor for a specific ligand. Sertoli cell mediators likely reach germ cells via Megalin-mediated transcytosis.

Role of cell adhesion proteins in Sertoli-germ cell communication

Cell to cell contacts are crucial to keep Sertoli and germ cells in juxtaposition, for sharing differentiation signals during spermatogenesis. Expression of cell adhesion molecules is necessary to form adhesion junctions that bind the Sertoli to germ cells. Several studies revealed the essential role of adhesion junctions in inducing elaboration of Sertoli cell mediators. It is imperative to coculture rat sperma-

tids with recombinant mouse Sertoli cells for secretion of ABP. The adherence of germ and Sertoli cells during coculture is indispensable for ABP-induced upregulation of spermatidal Tnp1 gene expression [30,61]. FSH and T synergise to form adhesion junctions between Sertoli and germ cells. The role of T emerged in studies of hypophysectomized rats. Testosterone replacement immediately after surgery maintains the junctional integrity between Sertoli and germ cells [66]. Delayed T replacement required priming with FSH for a minimum period of three weeks to avoid disruption of junctions [67]. Attachment of round spermatids to Sertoli cells between stages VII and VIII of rat seminiferous epithelial cycle depends upon T [68,69]. Estradiol reduces intratesticular T and disrupts imprinting, endocytosis and spermiation in rat [46,47,52,71,72]. Thus, expression of cell adhesion proteins would require both T and FSH. Testis expresses four cell adhesion molecular complexes (CAMS), namely cadherin-catenin, nectin-afadin, tubulobulbar and integrins-laminins. CAMS are involved in flagellar biogenesis, reorientation of spermatid polarity, nuclear chromatin condensation, nuclear reshaping and spermiation [70]. Establishment of junctional complexes initiates expression of transcription factors A-MYB-dependent Regulatory Factor X (Rfx2) and Cremτ in germ cells. These transcription factors transcribe several hundred genes required for cytodifferentiation of spermatids. Rfx2 is important for expression of adhesion proteins, RNA binding and processing, acrosome biogenesis, flagellar biogenesis, microtubule- associated intracellular vesicular transport genes and motor proteins [73]. Cremτ transcribes transition proteins (TPs) and protamines (P) genes implicated in nuclear chromatin condensation [74]. Coupling of gene transcription in germ cells and establishment of intercellular junctions suggests that Sertoli cells transport 'mediators' via transcytosis.

Role of calcium in Sertoli-germ cell communication:

Calcium plays a central role in regulation of male fertility [75]. L type Calcium channels on Sertoli cell membrane play a crucial role in germ cell differentiation [76]. Testosterone and FSH both regulate calcium influxes in Sertoli cells. Testosterone activates G protein linked phospholipase (PLC) via non-genomic AR. PLC hydrolyzes phosphatidylinositol bisphosphate (PIP2) to diacyl glycerol (DAG) and inositol triphosphate (IP3). Depletion of PIP2 depolarizes Sertoli cell plasma membrane, closure of K^{+}_{ATP} channel while opening the L type calcium channels. Testosterone thus induces Ca^{+2} influxes [77]. T also initiates expression of cyclic AMP response element binding protein (CREB) gene via genomic androgen receptors in Sertoli cell nucleus [78]. FSH activates Gq protein linked adenyl cyclase (AC) via FSH receptor (FSHR). Adenyl cyclase converts ATP to cAMP and activation of protein kinase A (PKA) [77]. Depletion of ATP modulates K^{+}_{ATP} channel while opening the L type calcium channels. FSH also facilitates Ca^{+2} influxes. Thus, blockade of Sertoli cell K+ATP channels causes membrane depolarization and facilitates Ca^{+2} influxes. Ca^{+2} is implicated in exocytosis [79]. K^{+}_{ATP} channel protein could ostensibly play a similar role in facilitating exocytosis by direct interaction with Syntaxin, an exocytotic molecule [80-83]. Thus, K^{+}_{ATP} channels could represent a novel perspective in hormonal regulated germ cell differentiation.

Calcium binds to intracellular receptor CaM (calcium-modulated protein). CaM activates CaM kinaseIV (Ca^{+2}/Calmodulin-dependent Protein KinaseIV) leading to CREB phosphorylation in Sertoli cell nucleus, the common target of T and FSH [84]. CREB phosphorylation initiates transcription of Sertoli cell genes related to germ cell differentiation. Hence, both T and FSH regulate germ cell gene expression via cognate receptors in Sertoli cells [34,85,86]. Ca^{+2} can exert pleiotropic effects in the expression as well as trafficking of Sertoli cell proteins [87,88]. Calcium is also crucial for activation of CaM KinaseIV in germ cells, involved in expression of CREM gene and phosphorylation of CREMτ. CREMτ expresses TPs and P1 in step 1-11 round spermatids and stage XI-XIV spermatocytes in rat via cyclic AMP response element (CRE) [74]. Stabilization and translation of repressed mRNAs in chromatoid body of differentiating spermatids also require CamIV Kinase [89]. Several reports describe that testicular cells communicate through nanotubes [90-92]. It is tempting to suggest that transcytosis could deliver Ca^{2+}/CaM to germ cells for hormonal regulation of spermatogenesis [93].

Schematic pathway for transport of rat Sertoli cell factors (Figure 1)

Sustentacular Sertoli cells nurture germ cell lineages by providing maturational signals. Maturational factors are transcytosed from peripheral circulation via Megalin and Cubulin cell-surface receptors consisting of an extracellular domain, a transmembrane domain, and a cytoplasmic domain [94]. The extracellular domain contains binding sites for Retinol binding protein (Rbp), serum Transferrin (sTfn), Al-

bumin, Haemoglobin, Apolipoprotein A1 (ApoA1), Intrinsic factor (IF)-vitamin B12, Apolipoprotein E (Apo E), Apolipoprotein M (Apo M), Transthyretin (Ttr), soluble form of the folate receptor (Folr1) and the morphogen sonic hedgehog (Shh). The cytoplasmic domain binds a cytosolic adapter protein (Dab2) for endocytosis that recruits Myo6 to clathrin-coated vesicles. The transcytosed molecules eventually reach the target cells. Sertoli cells internalize plasmatic T, Ca^{2+}, Fe and retinol required for germ cell differentiation. It is tempting to suggest transcytosis of plasmatic Ca^{2+} (bound to calcium-binding proteins/CABP), T (bound to ABP), retinol (bound to serum retinol-binding protein/sRbp) and Fe (bound to serum transferrin/sTfn) occurs via transmembrane receptors. Several studies in rat suggested hormonal regulation of ABPT and calcium uptake whereas mouse is preferred for gene ablation or overexpression studies. G protein-coupled Sertoli cell membrane receptors transduce the signals of reproductive hormones during spermatogenesis [95]. Sertoli cell cytoskeletal proteins undergo stage-specific spatiotemporal changes for intracellular transport conducive to germ cell differentiation. A hormone-regulated correlation was observed between the polymerization status of Vimentin and Vinculin and changes in Sertoli cell shape [96].

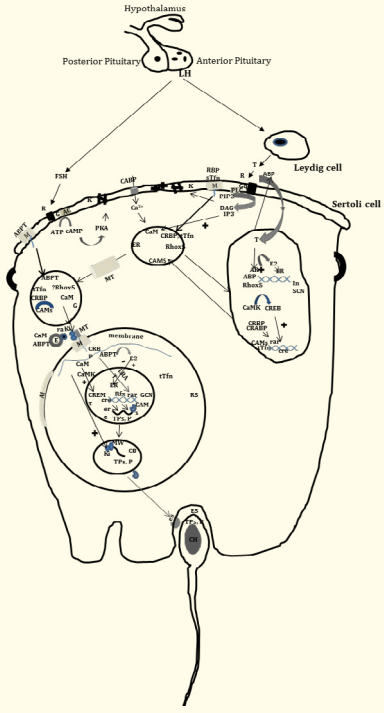

Figure 1: *Schematic pathway for movement of 'mediators' in Sertoli cell cytoplasm.*

Abbreviations: *ABP: Androgen-Binding Protein; ARE: Androgen Response Element; AC: Adenyl Cyclase; Activation: (+); AJ: Adhesion Junctions; AR: Androgen Receptor; Ca: Calcium Channel; CABP: Serum Calcium-Binding Proteins; CaM: Intracellular Calcium-Modulated Protein; CamKIV: Calmodulin KinaseIV; CRABP: Intracellular Retinoic Acid-Binding Protein; CRBP: Intracellular Retinol-Binding Protein; cre: Cyclic AMP Response Element; CREB: Cyclic AMP Response Element Binding-Protein; CB: Chromatoid Body; DAG: Diacylglycerol; Depolarization: (++); E: Endosome; ER: Estrogen Receptor; ERE: Estrogen Response Element; ER: Endoplasmic Reticulum; es: Ectoplasmic Specializations; FSH: Follicle Stimulating Hormone; GC: Germ Cell; GCN: Germ Cell Nucleus; G: Golgi Cisternae; G: Gprotein; Gq: Gprotein; IP3: Inositol Triphosphate; Inhibition: (-); K: Potassium ATP channel; Ki: Kinesin; LH: Luteinizing Hormone; LRAT: Lecithin-Retinol Acetyltransferase; M: Transmembrane Protein; MT: Microtubules; PIP2: Phosphatidylinositol Bisphosphate; PLC: Phospholipase; P: Protamine; ra: Rab GTPase (ras-like GTP-Binding Proteins); R: Receptor; Rhox5: Reproductive Homeobox on Chromosome5; Rfx: Regulatory Factor X2; RBP: Serum Retinol-Binding Protein; rar: Retinoic Acid Response Element; SC: Sertoli Cell; SCN: Sertoli Cell Nucleus; sTfn: Serum Transferrin; T: Testosterone; tTfn: Testicular Transferrin; TP: Transition Proteins.*

The hypophyseal hormone LH acts on interstitial Leydig cells via cognate LH receptors to synthesize and secrete T in circulation [87,88]. Testosterone acts via non-genomic, G protein-linked, ARs on Sertoli cell membrane to generate intracellular second messengers DAG and IP3 from PIP2 [78]. The depletion of PIP2 causes membrane depolarization and closure of membrane K^{+}_{ATP} channels. Depolarization induces influxes of Ca^{+2} via L type channel [80]. Intracellular CaM sequesters Ca^{+2} and eventually activates CamKIV Kinase. CaMIV Kinase activates transcription factor CREB that initiates Sertoli cell gene expression. Testosterone and its aromatization product E2 lead to expression of Sertoli cell ABP via genomic AR and ER [31,32]. Sertoli cells secrete ABP into peripheral circulation. AR expression is upregulated in Sertoli cells during stages VII-VIII of the cycle of seminiferous epithelium [9]. ESR2 expression is concomitantly upregulated in germ cells during stages VII-VIII (Round spermatids). ESR1 expression is upregulated in germ cells during stages VII-XIV (Round spermatids) [38]. E2 ostensibly plays an autoregulatory role during the androgen-dependent stages via ERs.

The hypophyseal hormone FSH acts on Sertoli cells via cognate FSH receptors to generate intracellular secondary messenger cAMP and PKA. The depletion of ATP ostensibly leads to closure of membrane K^{+}_{ATP} channel and induces influx of Ca^{+2} [77]. Calcium receptor CaM sequesters Ca^{+2} and eventually activates CamKIV Kinase. CamKIV Kinase activates transcription factor CREB and expression of Sertoli cell genes. FSH is also involved in expression of ABP. FSH receptor expression is upregulated in Sertoli cells during stages XII to II of seminiferous epithelial cycle [9]. Aromatase expression is maximally upregulated in germ cells at stages I-III (round spermatids) and XIII-XIV (Pachytene Spermatocytes) of the cycle of seminiferous epithelium. ESR1 expression is concomitantly upregulated in germ cells during stages I–II (Spermatogonia, Pachytene spermatocyte). ESR2 expression is concomitantly upregulated in germ cells during maturational stages I-III (Spermatogonia, Pachytene spermatocyte) and XII-XIII (Pachytene spermatocyte) [38]. E2 ostensibly plays a regulatory role in spermatogonial mitoses, spermatocyte meiosis and apoptosis regulated by FSH.

Sertoli cell proteins synthesized in endoplasmic reticulum sequester transcytosed plasmatic factors namely Retinol, Calcium, Fe and T and repackage bound to CRBP, CaM, tTfn, ABPT in Golgi cisternae. Rab proteins recruit endosomes containing Sertoli cell mediators to Kinesin motor proteins [60]. Microtubules transport endosome bound proteins to Sertoli cell membrane and transfer to differentiating germ cells. Presumably, germ cells internalize endosomal proteins via transmembrane receptor (M?). Hence, CaM (?) would activate CaM KinaseIV and transcribe CREM gene via cre to upregulate cremτ expression. Cremτ initiates expression of TPs and P germ cell genes [74]. TPs and P genes comprise EREs in their promoters and become accessible to E2 for autoregulation [46]. TPs and P mRNAs are stored in chromatoid body (CB) as repressed transcripts. CaM would also activate their temporal translation via miwi (MW) and Kinesin (Ki) [89]. ABP would transport T to germ cells for aromatization. Microtubules would also transport intracellular retinol-binding protein (CRBP) and Rhox5 (?) to germ cells via transcytosis [41]. CRBP transports retinol to germ cells [64]. LCAT converts retinol to retinoic acid. Retinoic acid acts via rarα and expresses Kit receptor [3]. Testicular Tfn transports Iron (Fe) from Sertoli to germ cells required for mitochondrial respiration [65]. Sertoli cells secrete Inhibin (In) and Leydig cells secrete E2 and T in peripheral circulation for feedback regulation of hypophyseal gonadotropins. Hence, FSH, T and E2 synergism depends upon the availability of their cognate receptors in Sertoli and germ cells during different stages of spermatogenesis [97].

Conclusion

Molecular pathway involved in the transport of FSH- and T-dependent Sertoli cell mediators of germ cell maturation awaits elucidation. A transcytosis mechanism could be operating between Sertoli and germ cells for exchange of maturational signals. Aberrations in the pathway for the transfer of Sertoli cell mediators like ABPT, CRBP, tTfn, CaM (?) and Rhox5 (?) to germ cells could adversely affect gene expression, differentiation and male fertility.

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Conflict of Interest

The author declares that there is no conflict of interest regarding the publication of this paper.

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