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MODULATORS OF HYPOTHALAMIC-PITUITARY-GONADAL AXIS FOR THE CONTROL OF SPERMATOGENESIS AND SPERM QUALITY IN VERTEBRATES

Topic Editors
Rosaria Meccariello, Silvia Fasano,
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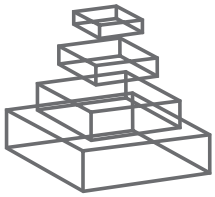

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MODULATORS OF HYPOTHALAMIC-PITUITARY-GONADAL AXIS FOR THE CONTROL OF SPERMATOGENESIS AND SPERM QUALITY IN VERTEBRATES

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Spermatogenesis is a process highly conserved throughout vertebrate species and is mainly under hypothalamic-pituitary control. It occurs in the testis in a stepwise fashion so that committed spermatogonia develop into spermatocytes and enter meiosis to produce round spermatids. These undergo a morphological transformation (spermiogenesis) into mature spermatids (i.e.: spermatozoa), which are differentially released from Sertoli cells (spermiation) depending on the species. In mammals, further transformations are necessary to form mature spermatozoa, suitable for fertilization. Gonadotropins, mainly responsive to gonadotropin-releasing hormone, control spermatogenesis through specific receptors located at the gonadal level. However, besides the endocrine route, the chemical mediators may also act locally in the gonad. Indeed, it is documented that testis physiology, including steroidogenesis and spermatogenesis, does not fully account for traditional endocrine control but an intragonadal network of autocrine and/or paracrine regulators also exists, whose activity, via cell-to-cell communication, regulates germ cell progression and development of qualitatively mature spermatozoa. Of note, a number of testicular modulators, such as gonadotropin releasing hormone, Kiss-peptin, endocannabinoids, has been early isolated in the brain and latest in the gonads. To fully understand precise mechanisms underlying the functional interaction of this intricate network, needless to say, it is crucially required to have detailed information about modulators and target cells.

Through synergy between the respective specializations of all the authors, this topic reviewed emerging knowledge about neuroendocrine and local mediators controlling germ cell progression and maturation.

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EDITORIAL
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Modulators of hypothalamic–pituitary–gonadal axis for the control of spermatogenesis and sperm quality in vertebrates

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In both male and female, gametogenesis is regulated by hypothalamus–pituitary–gonadal axis (HPG) that corresponds to the hormonal axis, gonadotropin-releasing hormone (GnRH)–gonadotropins–steroids. Indeed, the main target of GnRH is the gonadotrope cells, located in the adenohypophysis. These, in turn, release two gonadotropin hormones, the follicle stimulating hormone (FSH) and the luteinizing hormone (LH), that through the main circulation reach gonads to regulate gametogenesis via the synthesis of steroid hormones. It is now accepted that further non-steroid factors support germ cell progression via intragonadal action (1).

The first evidence of relationships between pituitary and gonad came out in 1905 from a study on castrated animals, which showed hypertrophy of the pituitary gland (2). Later in 1910, Homans and co-workers (3) showed that the "experimental hypophysectomy" in prepubertal animals induced persistence of gonadal infantilism. Surprisingly, only in 1930 the reciprocal relationship between gonads and pituitary via feedbacks was elucidated (4). Later in 1954, the long feedback connecting the hypothalamus and the gonad was described (5), but only in the 1970s did the picture become complete through the description of the short- and ultrashort-feedback mechanisms. It was at the end of the 1970s that paracrine and autocrine communications were described as being carried out also by "classic" hormones (6). In particular, it was observed that chemical messengers acting through the bloodstream could be produced in multiple tissues, not necessarily including any of the traditional ductless glands. This observation led to the new definition of what constitutes a hormone by considering its function (ὁρμάω, to excite) rather than its source (ductless glands). A hormone may now be considered as a chemical messenger acting through endocrine (bloodstream), paracrine, and/or autocrine (local) routes. Furthermore, any chemical mediators, not only hormones, besides the endocrine route may also act locally in the gonad (7, 8).

In the testis, it has been demonstrated that a network of intragonadal endocrine, paracrine, and autocrine factors converge in a complex stage-specific multi-factorial control of spermatogenesis (6). Indeed, it has been documented that traditional endocrine control does not fully account for testis physiology, including steroidogenesis and spermatogenesis, and an intragonadal network of autocrine and/or paracrine regulators also exist, which regulates germ cell progression and development of qualitatively mature spermatozoa via cell-to-cell communication (9, 10).

The aim of this Research Topic is to give a comparative track on HPG axis activity for the control of spermatogenesis and quality sperm production. Through synergy between the respective specializations of all the authors, this Research Topic reviews the emerging knowledge about neuroendocrine and local mediators controlling progression and maturation of germ cells in male vertebrates.

The Research Topic firstly reports the description of a primitive HPG in hagfish, one of the only two extant members of the class of agnathans – the most primitive vertebrates known, living or extinct – providing evidence that there are neuroendocrine–pituitary hormones that share common structure and functional features compared to later evolved vertebrates (11). A complex set of neuronal network converges information concerning environmental, stressors, and metabolic cues onto the centers governing the reproductive axis. In this respect, the most recent discoveries in the central pathways integrating metabolism and reproduction in teleost fish have been reviewed here (12). However, the list of central and local modulators of HPG is growing up and currently comprises gonadotropin-inhibiting hormone, firstly identified in Japanese quail in 2000 (13) as an inhibitor of gonadotropin synthesis and release but subsequently identified in all vertebrates (14); classical female hormone such as estrogens that elicit their activity through genomic and non-genomic mechanisms (15); lastly endocannabinoids (16), a set of lipid mediators that share some of the effects with delta-9-tetrahydrocannabinol (Δ^9-THC), the active principle of marijuana plant, *Cannabis sativa*. The middle part of this Research Topic comprises a set of four review articles dedicated to the control of fetal and postnatal development of both Leydig and germ cells and to the intragonadal networks controlling the progression of the spermatogenesis (17–20); two original research articles point out the discussed involvement of new players such as kisspeptins in the local control of testis physiology (21) and the difficulties to reproduce the testicular environment *in vitro* to get a successful spermatogenesis (22). Lastly, in order

to gain the production of high quality sperm, the importance of antioxidant defenses (23), GnRH, kisspeptins, estradiol (24), and endocannabinoids (25) has been reported.

The last part of this Research Topic is focused on disease models such as Kallmann Syndrome (26), blindness (27), lysosomal storage disease (28), and cryptorchidism (29).

We hope that this contribution published in Frontiers in Endocrinology may represent a comprehensive guide in the plethora of data concerning the control of male reproductive activity and that readers might find new insights for the building of general models.

REFERENCES

1. Cobellis G, Meccariello R, Pierantoni R, Fasano S. Intratesticular signals for progression of germ cell stages in vertebrates. *Gen Comp Endocrinol* (2003) **134**:220–8. doi:10.1016/S0016-6480(03)00281-8
2. Fichera G. Sur hypertrophie de la gland pituitarie consécutive à la castration. *Arch Ital Physiol* (1905) **43**:26.
3. Crowe SJ, Cushing H, Homans J. Experimental hypophysectomy. *Bull Johns Hopkins Hosp* (1910) **21**:127–70.
4. Moore CR, Price D. The question of sex hormone antagonism. *Proc Soc Exp Biol Med* (1930) **28**:38–40. doi:10.3181/00379727-28-5145
5. Flerkó B. Zur hypothalamischen steuerung der gonadotropen function der hypophise. *Acta Morph Acad Sci Hung* (1954) **4**:475–92.
6. Pierantoni R, Cobellis G, Meccariello R, Fasano S. Evolutionary aspects of cellular communication in the vertebrate hypothalamo-hypophysio-gonadal axis. *Int Rev Cytol* (2002) **218**:69–141. doi:10.1016/S0074-7696(02)18012-0
7. Pierantoni R, Cobellis G, Meccariello R, Cacciola G, Chianese R, Chioccareli T, et al. Testicular gonadotropin-releasing hormone activity, progression of spermatogenesis and sperm transport in vertebrates. *Ann N Y Acad Sci* (2009) **1163**:279–91. doi:10.1111/j.1749-6632.2008.03617.x
8. Cacciola G, Chioccarelli T, Fasano S, Pierantoni R, Cobellis G. Estrogens and spermiogenesis: new insights from type 1 cannabinoid receptor knockout mice. *Int J Endocrinol* (2013) **2013**:501350. doi:10.1155/2013/501350
9. Cacciola G, Chioccarelli T, Altucci L, Ledent C, Mason JI, Fasano S, et al. Low 17beta-estradiol levels in CNR1 knock-out mice affect spermatid chromatin remodeling by interfering with chromatin reorganization. *Biol Reprod* (2013) **88**:1–12. doi:10.1095/biolreprod.112.105726
10. Cacciola G, Chioccarelli T, Altucci L, Viggiano A, Fasano S, Pierantoni R, et al. Nuclear size as estrogen-responsive chromatin quality parameter of mouse spermatozoa. *Gen Comp Endocrinol* (2013) **193**:201–9. doi:10.1016/j.ygcen.2013.07.018
11. Nozaki M. Hypothalamic-pituitary-gonadal endocrine system in the hagfish. *Front Endocrinol* (2013) **4**:200. doi:10.3389/fendo.2013.00200
12. Shahjahan M, Kitahashi T, Parhar IS. Central pathways integrating metabolism and reproduction in teleosts. *Front Endocrinol* (2014) **5**:36. doi:10.3389/fendo.2014.00036
13. Tsutsui K, Saigoh E, Ukena K, Teranishi H, Fujisawa Y, Kikuchi M, et al. A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochem Biophys Res Commun* (2000) **275**:661–7. doi:10.1006/bbrc.2000.3350
14. Ubuka T, Son YL, Tobari Y, Narihiro M, Bentley GE, Kriegsfeld LJ, et al. Central and direct regulation of testicular activity by gonadotropin-inhibitory hormone and its receptor. *Front Endocrinol* (2014) **5**:8. doi:10.3389/fendo.2014.00008
15. Chimento A, Sirianni R, Casaburi I, Pezzi V. Role of estrogen receptors and G protein-coupled estrogen receptor in regulation of hypothalamus-pituitary-testis axis and spermatogenesis. *Front Endocrinol* (2014) **5**:1. doi:10.3389/fendo.2014.00001
16. Bovolin P, Cottone E, Pomatto V, Fasano S, Pierantoni R, Cobellis G, et al. Endocannabinoids are involved in male vertebrate reproduction: regulatory mechanisms at central and gonadal level. *Front Endocrinol* (2014) **5**:54. doi:10.3389/fendo.2014.00054
17. Ivell R, Heng K, Anand-Ivell R. Insulin-like factor 3 and the HPG axis in the male. *Front Endocrinol* (2014) **5**:6. doi:10.3389/fendo.2014.00006
18. Rossi P, Dolci S. Paracrine mechanisms involved in the control of early stages of mammalian spermatogenesis. *Front Endocrinol* (2013) **4**:181. doi:10.3389/fendo.2013.00181
19. Ricci G, Catizone A. Pleiotropic activities of HGF/c-Met System in testicular physiology: paracrine and endocrine implications. *Front Endocrinol* (2014) **5**:38. doi:10.3389/fendo.2014.00038
20. Grimaldi P, Di Giacomo D, Geremia R. The endocannabinoid system and spermatogenesis. *Front Endocrinol* (2013) **4**:192. doi:10.3389/fendo.2013.00192
21. Mei H, Doran J, Kyle V, Yeo SH, Colledge WH. Does kisspeptin signaling have a role in the testes? *Front Endocrinol* (2013) **4**:198. doi:10.3389/fendo.2013.00198
22. Reda A, Hou M, Landreh L, Kjartansdóttir KR, Svechnikov K, Söder O, et al. In vitro spermatogenesis – optimal culture conditions for testicular cell survival, germ cell differentiation, and steroidogenesis in rats. *Front Endocrinol* (2014) **5**:21. doi:10.3389/fendo.2014.00021
23. Guerriero G, Trocchia S, Abdel-Gawad FK, Ciarcia G. Roles of reactive oxygen species in the spermatogenesis regulation. *Front Endocrinol* (2014) **5**:56. doi:10.3389/fendo.2014.00056
24. Meccariello R, Chianese R, Chioccarelli T, Ciaramella V, Fasano S, Pierantoni R, et al. Intratesticular signals regulate germ cell progression and production of qualitatively mature spermatozoa in vertebrates. *Front Endocrinol* (2014) **5**:69. doi:10.3389/fendo.2014.00069
25. Maccarrone M. Endocannabinoids as markers of sperm quality: hot spots. *Front Endocrinol* (2013) **4**:169. doi:10.3389/fendo.2013.00169
26. Garaffo G, Provero P, Molineris I, Pinciroli P, Peano C, Battaglia C, et al. Profiling, bioinformatic, and functional data on the developing olfactory/GnRH system reveal cellular and molecular pathways essential for this process and potentially relevant for the Kallmann syndrome. *Front Endocrinol* (2013) **4**:203. doi:10.3389/fendo.2013.00203
27. Bellastella A, De Bellis A, Bellastella G, Esposito K. Opposite influence of light and blindness on pituitary–gonadal function. *Front Endocrinol* (2014) **4**:205. doi:10.3389/fendo.2013.00205
28. Piomboni P, Governini L, Gori M, Puggioni E, Costantino-Ceccarini E, Luddi A. New players in the infertility of a mouse model of lysosomal storage disease: the hypothalamus-pituitary-gonadal axis. *Front Endocrinol* (2014) **4**:204. doi:10.3389/fendo.2013.00204
29. Cobellis G, Noviello C, Nino F, Romano M, Mariscoli F, Martino A, et al. Spermatogenesis and cryptorchidism. *Front Endocrinol* (2014) **5**:63. doi:10.3389/fendo.2014.00063

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Endocannabinoids as markers of sperm quality: hot spots

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Male reproductive health is under threat from a range of environmental and lifestyle assaults, including endocrine disrupters, toxic pollutants, and ionizing radiations, as well as lifestyle factors such as sexually transmitted infections, alcoholism, smoking, and anabolic steroid use. The latest potential hazard in our modern lifestyle is the use of plant-derived cannabinoids present in hashish and marijuana as recreational drugs, and more recently as therapeutic agents (1). In the last decade, a highly sophisticated endogenous cannabinoid system (ECS) has been discovered in mammals, where it regulates many physiological functions including human male reproduction (2–5). Here, I shall briefly discuss the activity of distinct ECS elements that can be useful to assess sperm function, and hence to potentially monitor sperm quality. Among others, these include the effect of type-1 cannabinoid receptor (CB_1) in regulating energy metabolism and motility of human sperm, and that of transient receptor potential vanilloid 1 (TRPV1) channels in controlling their fertilizing ability. Remarkably, both receptors share a common natural agonist, that is the endocannabinoid (eCB) *N*-arachidonoylethanolamine (anandamide, AEA); instead, another major eCB like 2-arachidonoylglycerol (2-AG) can activate CB_1, but is ineffective at TRPV1 receptors (6). The potential therapeutic exploitation of these ECS elements for the treatment of human infertility will be also addressed.

Human sperm express CB_1, and its activation by AEA affects motility and acrosome reaction (AR). Both processes require energy, and a major role for glycolysis in supplying ATP for sperm motility has been recognized. Recently, human sperm exposure to methanandamide, a non-hydrolyzable analog of AEA, has been shown to significantly decrease mitochondrial transmembrane potential without triggering any mitochondria-dependent apoptotic death, and such an effect was prevented by the CB_1 antagonist SR141716, but not by the CB_2 antagonist SR144528, nor by the TRPV1 antagonist iodoresiniferatoxin (7). Interestingly, in the presence of glucose human sperm exposure to methanandamide for up to 18 h failed to affect sperm motility, that instead was dramatically reduced by the same substance under glycolysis blockage; again, the latter effect was prevented by SR141716 (7). Overall, CB_1 activation induced a non-apoptotic decrease of mitochondrial potential, whose detrimental reflection on sperm motility could be revealed only when blocking glycolysis. These findings contribute to elucidate the relationship between CB_1, energetic metabolism and mitochondria, an issue that appears relevant well beyond sperm biology. Indeed, mitochondrial CB_1 activation has been recently reported to control energy metabolism in neurons (8), though the actual receptor localization on mitochondria remains controversial (9).

Another hot spot is the involvement of the AEA-binding TRPV1 receptor in human sperm fertilizing ability. Immunoreactivity for CB_1 has been localized in the post-acrosomal region and in the midpiece of human sperm, whereas for TRPV1 it was restricted to the post-acrosomal region (10). Capsazepine (CPZ), a selective antagonist of TRPV1, was shown to inhibit progesterone (P)-enhanced sperm/oocyte fusion, as evaluated by the hamster egg penetration test. This inhibition was due to a reduction of the P-induced AR rate above that of spontaneous AR, which was instead increased (10). Altogether, these data demonstrate that TRPV1 plays a key-role in the human sperm fertilizing ability, by impacting on its fusion with the oocyte membrane. In line with this, a marked decrease of the ability of TRPV1 to bind its ligands has been shown in infertile versus fertile sperm, again supporting a major role for this ion channel in sperm functionality (11). On this basis, one might speculate that the reduction of AEA causes infertile sperm to lose their quiescent state and with that, the ability to prevent premature capacitation. This could then precipitate a premature AR, rendering that sperm infertile because of a reduced ability to penetrate an oocyte *in vivo*, or in assisted conception such as in *in vitro* fertilization (IVF) protocols. This hypothesis has recently found grounds through a clinical study performed on men affected by asthenozoospermia and oligoasthenoteratozoospermia (12). Indeed, AEA levels in seminal plasma were found to be halved in patients with respect to normal subjects (∼0.08 versus ∼0.20 nM). Remarkably, these differences in AEA content in men with different pathological semen subtypes were associated with poor semen quality, such as decreased sperm count and abnormal sperm motility, as well as with alterations of CB_1 at transcriptional level (12). Therefore, evaluation of eCBs content in human sperm and/or in seminal plasma could be proposed as a novel diagnostic tool in reproductive medicine. In line with this, a marked reduction (down to ∼25%) of both AEA and 2-AG content in seminal plasma from infertile men has been recently documented (11). Instead, no significant alterations were found in sperm from infertile versus fertile men, neither for AEA nor for 2-AG (11). Collectively,

these data pinpoint eCBs (and AEA in particular) as new biomarkers to determine semen quality, thus opening new avenues for the treatment of infertility in humans.

Further points of interest in the regulation of sperm quality by ECS are related to the role of membrane properties and epigenetic control of chromatin activity.

Mammalian sperm become fertile after completing capacitation, a process associated with cholesterol loss and changes in the biophysical properties of the membranes, e.g., at the level of cholesterol-rich microdomains termed lipid rafts (13). Membrane raft dynamics prepares the sperm to undergo AR, and in addition it may have a role in sperm-egg membrane interaction (14). Interestingly, CB_1 and TRPV1 are affected by sperm membrane properties (15), and CB_1 signal transduction in enhanced by lipid raft disruption in different neuronal and immune cells (16). In addition, the AEA congener *N*-palmitoylethanolamine (PEA), that has been shown in the male reproductive tract, modulates plasma membrane polarity with an effect on Ca^{2+} influx during the capacitation process (17). Remarkably, PEA might also affect some physiological sperm kinematic parameters (like sperm motility), thus impacting on the development of hyperactivation during capacitation, ultimately leading to idiopathic infertility (18). Taken together, further investigations into the contribution of sperm membrane lipid composition to the control of eCB signaling, and hence to its relevance for sperm quality and fertilizing ability, hold promise for a better design of preventive and/or therapeutic strategies against infertility. In this context, it remains to be assessed whether (and to what extent) sperm functionality might be affected by accumulation of AEA and congeners in intracellular stores called adiposomes (or lipid droplets), that are present in sperm (19), and are important for eCB signaling in different cell types (20, 21).

The last hot spot that I would like to address concerns chromatin remodeling and epigenetic regulation of sperm functions. Because CB_1 activation plays a pivotal role in spermiogenesis (that is the developmental stage where DNA is remodeled), it has been recently hypothesized that regulation of the CB_1 gene (*Cnr1*) might also influence chromatin quality in sperm (22). By using *Cnr1* null mutant ($Cnr1^{-/-}$) mice, CB_1 activation was demonstrated to regulate indeed chromatin remodeling of spermatids, *via* either increasing the levels of the *Tnp2* gene (encoding for the transition protein 2, that stimulates DNA nick repair *in vitro*), or enhancing histone displacement. Comparative analysis of wild-type, $Cnr1^{+/-}$ and $Cnr1^{-/-}$ animals suggested the possible occurrence of haploinsufficiency for *Tnp2* turnover under CB_1 control, whereas histone displacement was disrupted in $Cnr1^{+/-}$ and $Cnr1^{-/-}$ mice to a lesser extent. Furthermore, flow cytometry analysis demonstrated that the genetic loss of *Cnr1* decreased sperm chromatin quality and was associated with sperm DNA fragmentation. Of note, this damage increased during epididymal transit, from caput to cauda (22). Collectively, these results demonstrate that the expression (and expectedly the activity) of CB_1 controls the physiological alterations of DNA packaging during spermiogenesis and epididymal transit, which might have major implications for male fertility, given the deleterious effects of sperm DNA damage (22). On a final note, it should be recalled that the epigenetic regulation of target genes by eCBs, and conversely that of ECS genes (in particular CB_1) by pathological conditions, are emerging as a major issue to understand the fine tuning of eCB signaling in human health and disease (23). Therefore, it can be anticipated that epigenetic studies on sperm quality and fertilizing capacity will open new avenues for preventing or curing (e.g., through a correct lifestyle) human infertility with innovative therapeutics.

In conclusion, distinct ECS elements like CB_1 and TRPV1, along with the endogenous levels of their common ligand AEA, hold the promise to represent useful diagnostic biomarkers and therapeutic targets of male fertility defects. It seems noteworthy that, while CB_1 has major effects also on female reproductive events (from oocyte development, to ovarian transport, and embryo implantation), apparently TRPV1 does not impact on female fertility (24), apart from generating hyperalgesia via primary sensory neurons during endometriosis (25). Therefore, the latter ion channel seems to represent an ideal target to specifically combat reproductive dysfunctions in males.

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REFERENCES

1. Lewis SE, Maccarrone M. Endocannabinoids, sperm biology and human fertility. *Pharmacol Res* (2009) **60**:126–31. doi:10.1016/j.phrs.2009.02.009
2. Schuel H, Burkman LJ, Lippes J, Crickard K, Forester E, Piomelli D, et al. *N*-Acylethanolamines in human reproductive fluids. *Chem Phys Lipids* (2002) **121**:211–27. doi:10.1016/S0009-3084(02)00158-5
3. Wang H, Dey SK, Maccarrone M. Jekyll and hyde: two faces of cannabinoid signaling in male and female fertility. *Endocr Rev* (2006) **27**:427–48. doi:10.1210/er.2006-0006
4. Maccarrone M. Endocannabinoids: friends and foes of reproduction. *Prog Lipid Res* (2009) **48**:344–54. doi:10.1016/j.plipres.2009.07.001
5. Bari M, Battista N, Pirazzi V, Maccarrone M. The manifold actions of endocannabinoids on female and male reproductive events. *Front Biosci* (2011) **16**:498–416. doi:10.2741/3701
6. Di Marzo V, De Petrocellis L. Endocannabinoids as regulators of transient receptor potential (TRP) channels: a further opportunity to develop new endocannabinoid-based therapeutic drugs. *Curr Med Chem* (2010) **17**:1430–49. doi:10.2174/092986710790980078
7. Barbonetti A, Vassallo MR, Fortunato D, Francavilla S, Maccarrone M, Francavilla F. Energetic metabolism and human sperm motility: impact of CB_1 receptor activation. *Endocrinology* (2010) **151**:5882–92. doi:10.1210/en.2010-0484
8. Bénard G, Massa F, Puente N, Lourenço J, Bellocchio L, Soria-Gómez E, et al. Mitochondrial CB_1 receptors regulate neuronal energy metabolism. *Nat Neurosci* (2012) **15**:558–64. doi:10.1038/nn.3053
9. Morozov YM, Dominguez MH, Varela L, Shanabrough M, Koch M, Horvath TL, et al. Antibodies to cannabinoid type 1 receptor co-react with stomatin-like protein 2 in mouse brain mitochondria. *Eur J Neurosci* (2013) **38**:2341–8. doi:10.1111/ejn.12237
10. Francavilla F, Battista N, Barbonetti A, Vassallo MR, Rapino C, Antonangelo C, et al. Characterization of the endocannabinoid system in human spermatozoa and involvement of transient receptor potential vanilloid 1 receptor in their fertilizing ability. *Endocrinology* (2009) **150**:4692–700. doi:10.1210/en.2009-0057
11. Lewis SE, Rapino C, Di Tommaso M, Pucci M, Battista N, Paro R, et al. Differences in the endocannabinoid system of sperm from fertile and infertile men. *PLoS One* (2012) **7**:e47704. doi:10.1371/journal.pone.0047704
12. Amoako AA, Marczylo TH, Marczylo EL, Elson J, Willets JM, Taylor AH, et al. Anandamide modulates human sperm motility: implications for men

with asthenozoospermia and oligoasthenoteratozoospermia. *Hum Reprod* (2013) **28**:2058–66. doi:10.1093/humrep/det232
13. Flesch FM, Brouwers JF, Nievelstein PF, Verkleij AJ, van Golde LM, Colenbrander B, et al. Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. *J Cell Sci* (2001) **114**(19):3543–55.
14. Miranda PV, Allaire A, Sosnik J, Visconti PE. Localization of low-density detergent-resistant membrane proteins in intact and acrosome-reacted mouse sperm. *Biol Reprod* (2009) **80**:897–904. doi:10.1095/biolreprod.108.075242
15. Botto L, Bernabò N, Palestini P, Barboni B. Bicarbonate induces membrane reorganization and CBR1 and TRPV1 endocannabinoid receptor migration in lipid microdomains in capacitating boar spermatozoa. *J Membr Biol* (2010) **238**:33–41. doi:10.1007/s00232-010-9316-8
16. Dainese E, Oddi S, Maccarrone M. Interaction of endocannabinoid receptors with biological membranes. *Curr Med Chem* (2010) **17**:1487–99. doi:10.2174/092986710790980087
17. Ambrosini A, Zolese G, Wozniak M, Genga D, Boscaro M, Mantero F, et al. Idiopathic infertility: susceptibility of spermatozoa to in-vitro capacitation, in the presence and the absence of palmitylethanolamide (a homologue of anandamide), is strongly correlated with membrane polarity studied by Laurdan fluorescence. *Mol Hum Reprod* (2003) **9**:381–8. doi:10.1093/molehr/gag056
18. Ambrosini A, Zolese G, Ambrosi S, Bertoli E, Mantero F, Boscaro M, et al. Idiopathic infertility: effect of palmitoylethanolamide (a homologue of anandamide) on hyperactivated sperm cell motility and Ca^{2+} influx. *J Androl* (2005) **26**:429–36. doi:10.2164/jandrol.04141
19. Bian X, Gandahi JA, Liu Y, Yang P, Liu Y, Zhang L, et al. The ultrastructural characteristics of the spermatozoa stored in the cauda epididymidis in Chinese soft-shelled turtle *Pelodiscus sinensis* during the breeding season. *Micron* (2013) **44**:202–9. doi:10.1016/j.micron.2012.06.010
20. Oddi S, Fezza F, Pasquariello N, De Simone C, Rapino C, Dainese E, et al. Evidence for the intracellular accumulation of anandamide in adiposomes. *Cell Mol Life Sci* (2008) **65**:840–50. doi:10.1007/s00018-008-7494-7
21. Maccarrone M, Dainese E, Oddi S. Intracellular trafficking of anandamide: new concepts for signaling. *Trends Biochem Sci* (2010) **35**:601–8. doi:10.1016/j.tibs.2010.05.008
22. Chioccarelli T, Cacciola G, Altucci L, Lewis SE, Simon L, Ricci G, et al. Cannabinoid receptor 1 influences chromatin remodeling in mouse spermatids by affecting content of transition protein 2 mRNA and histone displacement. *Endocrinology* (2010) **151**:5017–29. doi:10.1210/en.2010-0133
23. D'Addario C, Di Francesco A, Pucci M, Finazzi Agrò A, Maccarrone M. Epigenetic mechanisms and endocannabinoid signalling. *FEBS J* (2013) **280**:1905–17. doi:10.1111/febs.12125
24. Battista N, Meccariello R, Cobellis G, Fasano S, Di Tommaso M, Pirazzi V, et al. The role of endocannabinoids in gonadal function and fertility along the evolutionary axis. *Mol Cell Endocrinol* (2012) **355**:1–14. doi:10.1016/j.mce.2012.01.014
25. Liu J, Liu X, Duan K, Zhang Y, Guo SW. The expression and functionality of transient receptor potential vanilloid 1 in ovarian endometriomas. *Reprod Sci* (2012) **19**:1110–24. doi:10.1177/1933719112443876

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Paracrine mechanisms involved in the control of early stages of mammalian spermatogenesis

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Within the testis, Sertoli-cell is the primary target of pituitary FSH. Several growth factors have been described to be produced specifically by Sertoli cells and modulate male germ cell development through paracrine mechanisms. Some have been shown to act directly on spermatogonia such as GDNF, which acts on self-renewal of spermatogonial stem cells (SSCs) while inhibiting their differentiation; BMP4, which has both a proliferative and differentiative effect on these cells, and KIT ligand (KL), which stimulates the KIT tyrosine-kinase receptor expressed by differentiating spermatogonia (but not by SSCs). KL not only controls the proliferative cycles of KIT-positive spermatogonia, but it also stimulates the expression of genes that are specific of the early phases of meiosis, whereas the expression of typical spermatogonial markers is down-regulated. On the contrary, FGF9 acts as a meiotic inhibiting substance both in fetal gonocytes and in post-natal spermatogonia through the induction of the RNA-binding protein NANOS2. Vitamin A, which is metabolized to Retinoic Acid in Sertoli cells, controls both SSCs differentiation through KIT induction and NANOS2 inhibition, and meiotic entry of differentiating spermatogonia through STRA8 upregulation.

Keywords: primordial germ cells, spermatogonial stem cells, spermatogenesis, meiosis, growth factors, paracrine control, signal transduction, gene expression

BRIEF INTRODUCTION: PARACRINE CONTROL OF FETAL MALE GERM CELL DEVELOPMENT

The control of the germ cell fate by paracrine factors secreted by the surrounding somatic environment already starts in the fetal life in the period of germ cell specification, independently from the influence of the hypothalamic-pituitary axis. Bone Morphogenetic Protein 4 (BMP4) has been shown to induce primordial germ cell (PGC) formation, to act as a PGC survival and localization factor within the allantois (1) and as a mitogen in *in vitro* cultured PGCs (2). During PGC specification in the extraembryonic mesoderm, SOX2 induction is required for the transcriptional regulation of KIT expression in PGCs (3). KIT is a tyrosine-kinase receptor, which is activated by KIT Ligand (KL), a growth factor expressed by the surrounding somatic environment. KL/KIT interaction is essential in the fetal period both during the specification of PGCs and for their proliferation and migration [(3–7), and references therein]. KIT expression is then down-regulated both in fetal oocytes undergoing meiosis and in gonocytes, which stop to proliferate after germ cell sex determination. Sertoli cells can prevent meiotic entry of gonocytes through the production of paracrine factors acting as meiotic inhibiting substances. The best characterized meiotic inhibiting substance produced by fetal Sertoli cells is Fibroblast Growth factor 9 (FGF9). FGF9 is a SRY/SOX9-dependent growth factor crucial for male sex differentiation acting on the somatic compartment of the fetal testis (8, 9). However, FGF9 also acts directly on male fetal gonocytes by upregulating levels of the RNA-binding protein NANOS2 (10, 11). NANOS2 prevents meiosis through the post-transcriptional regulation of key genes involved in the meiotic program (10, 12, 13). Recently, it has been shown that the meiosis-preventing activity of FGF9 in the fetal testis is mediated, at least in part, by NODAL, a member of the TGF-β family, and its partner Cripto (14–16).

In the same period in which FGF9 is expressed during testis determination, Sertoli cells produce an enzyme, CYP26B1, which degrades Retinoic Acid (RA) of mesonephric origin, in order to block Stimulated by Retinoic Acid 8 (STRA8) expression, and, as a consequence, to prevent premature gonocyte entry into meiosis (17–20). Although the identification of RA as the CYP26B1 substrate in the fetal testis (required for STRA8 induction and meiosis initiation in the fetal ovary) has been questioned (21), most of the available data in the literature support the role of RA as a master inducer of the mitotic-meiotic switch in germ cells (22). In line with this evidence is the finding that RA treatment down-regulates NANOS2 expression in fetal gonocytes (10).

PARACRINE CONTROL OF POST-NATAL MALE GERM CELL DEVELOPMENT

Pituitary gonadotropins, FSH, and LH, were originally identified for their essential role in ovarian function, as the stimulator of follicular activity and the inducer of follicular luteinization, respectively (23). Later on, it became clear that the same hormones play important roles also in testicular function, FSH being involved in the induction of spermatogenesis at puberty, and LH being the main inducer of androgen production (24). Spermatogenesis is a highly ordered differentiative process that occurs under FSH and androgen control. Sertoli cells, the only known targets for these hormones in the seminiferous tubules, mediate hormone action on spermatogenesis by controlling the germinal stem cell niche and by creating a suitable environment for the complex developmental events of germ cell proliferation and differentiation. Sertoli

cells directly orchestrate these complex events through both membrane intercellular communications and the production of growth factors and cytokines that act directly on the germ cell compartment. In the following paragraphs we will focus on the better characterized Sertoli-cell controlled paracrine mechanisms acting on the early stages of mammalian spermatogenesis, which are schematically summarized in **Figure 1**.

MAINTENANCE OF THE GERM STEM CELL NICHE

Spermatogonial stem cells (SSCs) are the direct descendants of fetal gonocytes. In the testis, SSCs are a subpopulation of undifferentiated spermatogonia residing in the basal layer of the seminiferous epithelium. Their mitotic expansion allows continuous production of germ cells committed to differentiation. One of the specific properties of SSCs and other undifferentiated spermatogonia that distinguishes them from differentiating spermatogonia is the expression of the Glial cell line-derived neurotrophic factor (GDNF)-family receptor α1 (GFRα1) and the c-Ret receptor tyrosine-kinase, which are both required for signaling in response to the Sertoli-cell-derived GDNF (25–28). GDNF has been shown to be essential for fate determination of SSCs, since in aging males heterozygotes for GDNF deletion, testes appear devoided of germ cells and show a phenotype similar to Sertoli-cell-only syndrome (25). Furthermore, overexpression of GDNF in mouse testes appeared to stimulate self-renewal of stem cells and block spermatogonial differentiation, inducing a seminomatous phenotype (25, 27). GDNF-induced activation of AKT and MEK signaling pathways in SSCs leads to increased generation of reactive oxygen species (ROS) generated by NAPDH oxidase 1, and apparently (contrary to their alleged detrimental role for spermatogenesis) ROS stimulate proliferation and self-renewal of SSCs through the activation of p38 and JNK MAPKs (29). Thus, GDNF is important for SSCs self-renewal, and, at the same time negatively controls their differentiation. This notion has been recently challenged by the finding that GFRα1-positive chained spermatogonia (A paired and A aligned) are more numerous than GFRα1-positive

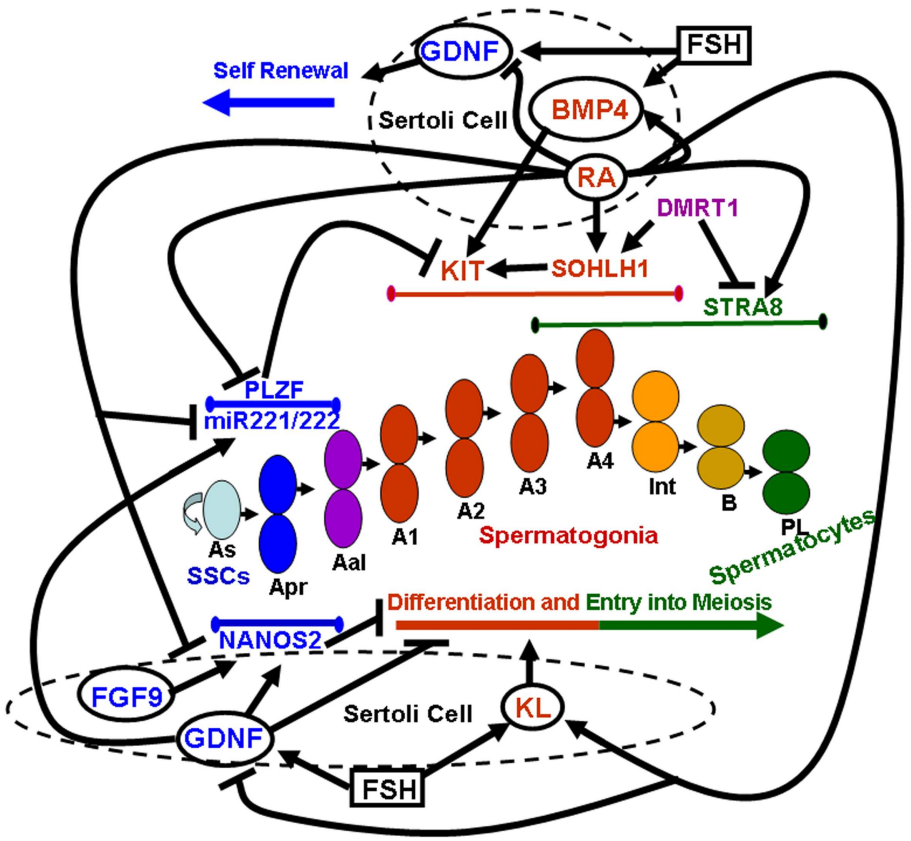

FIGURE 1 | Sertoli-cell controlled paracrine mechanisms acting on the early stages of mammalian spermatogenesis. Paracrine factors secreted by Sertoli cells (whose membrane is represented by dashed circles) are enclosed within solid line circles. Follicle stimulating hormone (FSH) is enclosed within a solid line square. Endogenous factors expressed by germ cells are represented by non-enclosed words. Blue colors refer to paracrine and endogenous factors that promote self-renewal of spermatogonial stem cells (SSCs) and inhibit spermatogonial differentiation and/or meiotic entry. Red colors refer to paracrine and endogenous factors that promote spermatogonial differentiation. Purple colors refer to endogenous factors which promote spermatogonial differentiation but at the same time inhibit meiotic entry. Green colors refer to endogenous factors that drive entry into meiosis. Lines delimited by small ellipsoids refer to the stage of expression of the germ cell endogenous factors involved in either self-renewal of SSCs and inhibition of differentiation (blue colors) or in differentiation (red colors) and meiotic entry (green colors). The succession of the various types of germ cells during the earliest stages of mouse spermatogenesis is represented in the center of the image: As, a single spermatogonia; Apr, a paired spermatogonia; Aal, a aligned spermatogonia; A1, A2, A3, A4, type A1–A4 spermatogonia; Int, intermediate spermatogonia; B, type B spermatogonia; PL, pre-leptotene spermatocytes.

A single spermatogonia, which are thought to represent the major SSCs reservoir in the mouse testis (30). However, GDNF signaling is essential to maintain NANOS2 expression in SSCs, and it has been proposed that this RNA-binding protein, besides its well-established role in preventing meiosis in fetal gonocytes, is also important to prevent spermatogonial differentiation in the post-natal testis (31). Overall, it is clear that GDNF mainly acts in positively regulating the proliferation of SSCs and maintenance of their undifferentiated state. Importantly, FSH and its second messenger cyclic AMP (cAMP) have been reported to stimulate GDNF expression in Sertoli cells (32, 33), which is instead down-regulated by RA treatment (33). These evidences suggest that that GDNF might be one of the paracrine factors that influences SSCs proliferation and population size under the control of the hypothalamic-pituitary axis.

CONTROL OF SPERMATOGONIAL DIFFERENTIATION

Undifferentiated SSCs (A single spermatogonia) have been described as single cells that are able both to renew themselves and to produce more differentiated A paired spermatogonia. The A paired cells then divide into A aligned spermatogonia that further differentiate into A1 spermatogonia (34). Appearance of A1 (differentiating) spermatogonia coincides with regain of the expression of KIT, encoding the receptor for KL (35–38). KIT mediates proliferation, survival, and differentiation in type A spermatogonia (33, 39–41). Upon KIT expression, spermatogonia become sensitive to KL produced by Sertoli cells (39, 42) and undergo a definite number of proliferative cycles, forming the A2-A4, intermediate, and B spermatogonia, before entering meiosis. The temporal appearance of KIT expression and of KL sensitivity in mouse spermatogonia, between 4 and 7 days postpartum (dpp) (33, 35, 36, 40), marks the switch from the A aligned spermatogonia to the A1-B differentiating cell types. Indeed, KIT is universally considered the most important marker that distinguishes differentiating spermatogonia from their undifferentiated precursors, including SSCs. Thus, paracrine factors in the testicular environment that stimulate KIT expression in mitotic germ cells play an essential role for the start of spermatogenesis at puberty. One of the paracrine signals involved in this event is BMP4, which is produced by Sertoli cells very early in the post-natal life, and whose expression is positively regulated by cAMP and RA (33, 43). Its receptor ALK3 and the SMAD5 transducer are expressed in undifferentiated spermatogonia, and *in vitro* treatment of these cells with BMP4 exerts both mitogenic and differentiative effects, inducing [^{3}H]thymidine incorporation and KIT expression both at the RNA and protein levels (43). As a result of the latter event, KIT-negative spermatogonia acquire sensitivity to KL (43). Since SSCs are able to renew themselves and at the same time to progress through differentiation (i.e., to the KIT-dependent stages of proliferation), BMP4 could be one of the factors that regulates such process. Alternatively, BMP4 could act on a subset of undifferentiated spermatogonia that have lost SSC features, i.e., that have entered the differentiative stage but are not yet KIT-positive. In agreement with the first possibility, BMP4 addition, on the opposite of GDNF, was shown to impair *in vitro* maintenance of mouse primary SSCs (44). Moreover, more recently BMP4 was shown to induce differentiation and KIT expression in a rat SSC cell line (45). In the adult testis, BMP4 has been reported to be produced by spermatogonia, but not by Sertoli cells (46), suggesting that it might work as a paracrine-autocrine factor modulating the establishment of the cycle of the seminiferous epithelium.

Another well-established paracrine factor involved in spermatogonial differentiation is the Vitamin A derivative RA. Mice kept on a diet deficient on vitamin A (VAD mice) or lacking vitamin A derivatives are sterile because the seminiferous tubules contain only undifferentiated KIT-negative spermatogonia, indicating a role of vitamin A in spermatogonia differentiation (38, 47). RA functions inside the nucleus recognizing two different classes of retinoid receptors. Both classes (RARs and RXRs) consist of three types of receptors, α, β, and γ, encoded by distinct genes and transduce RA signal by binding directly to RA-responsive elements. During post-natal development, each RAR is detected predominantly in a specific cell type of the seminiferous epithelium: RARα in Sertoli cells, RARβ in round spermatids and RARγ in type A spermatogonia (48). RARα conditional ablation in Sertoli cells showed germ cell apoptosis and seminiferous epithelium dysfunctions related to the disruption of Sertoli cells cyclical gene expression, which preceded testis degeneration (49). It has been reported that during the first, prepubertal, spermatogenic cycle RALDH-dependent synthesis of RA by Sertoli cells is indispensable to initiate differentiation of A aligned into A1 spermatogonia, and that this effect is mainly mediated by autocrine action of RA through RARα in the somatic compartment (50). However, RA (either the all-trans or the 9-cis Retinoic isomers) treatment *in vitro* exerts a direct effect on the differentiation of mitotic germ cell compartment by promoting KIT expression in undifferentiated spermatogonia (33, 51). This effect has been confirmed *in vivo* by the observation that targeted ablation of RARγ impairs the A aligned to A 1 transition in the course of some of the seminiferous epithelium cycles (52). Altogether these data indicate that RA favors spermatogonial differentiation through a direct action on spermatogonia and an indirect action mediated by changes in the expression pattern of paracrine factors such as KL, BMP4, and GDNF secreted by Sertoli cells (33).

Due to its importance for promoting expansion of differentiating spermatogonia, KIT expression in SSCs is subjected to a very tight transcriptional control. Promyelocytic Leukemia Zinc Finger (PLZF, also known as ZFP145, or ZBTB16) is a DNA sequence-specific transcriptional repressor that can exert local and long-range chromatin remodeling activity through the recruitment of DNA histone deacetylases and through the action of several nuclear corepressors (53). PLZF is specifically expressed in SSCs, and male PLZF knock-out (KO) mice show progressive spermatogonia depletion due to the deregulated expression of genes controlling the switch between self-renewal and differentiation (54–56). PLZF represses both endogenous KIT expression and expression of a reporter gene under the control of KIT regulatory elements (57). A discrete sequence of the KIT promoter, required for PLZF-mediated KIT transcriptional repression, was demonstrated to be bound by PLZF *in vitro* and also *in vivo*, by using chromatin immunoprecipitation (ChIP) of spermatogonia. Moreover, a 3-bp mutation in this PLZF binding site abolishes the responsiveness of the KIT promoter to PLZF repression In agreement with these findings, a significant increase in KIT expression

was found in the undifferentiated spermatogonia isolated from PLZF KO mice (57). Thus, one mechanism by which PLZF maintains the pool of SSCs is through a direct repression of KIT transcription, thus acting as a gatekeeper of spermatogonial differentiation. RA was shown to trigger downregulation of PLZF in SSCs (58), which might be part of the mechanisms which triggers up-regulation of KIT during spermatogonial differentiation.

Positive regulators of KIT transcription in spermatogonia are two b-Helix-Loop-Helix (HLH) transcription factors specifically expressed in germ cells, SOHLH1 (Spermatogenesis and Oogenesis HLH1), and SOHLH2. Both SOHLHs have been involved in the differentiation of spermatogonia and oocytes (59–64). In the male, deletion of each transcription factor leads to the disappearance of KIT-expressing spermatogonia in the prepuberal testis. An expression study of SOHLH1 and SOHLH2 during fetal and post-natal development showed a strong positive correlation between KIT and the two transcription factors in post-natal spermatogonia (65). SOHLH2 was found enriched mainly in undifferentiated spermatogonia, whereas SOHLH1 expression was maximal in KIT-dependent stages. Reporter gene expression driven by sequences contained within the KIT promoter and first intron was strongly up-regulated in transfection experiments overexpressing either SOHLH1 or SOHLH2, and co-transfection of both factors showed a cooperative effect (65). *In vivo*, co-immunoprecipitation results evidenced that the two proteins interact and overexpression of both factors increased endogenous KIT expression. Using ChIP analysis, SOHLH1 was found to occupy discrete bHLH binding site containing regions within the KIT promoter in spermatogonia chromatin (64, 65). Interestingly, expression of SOHLH1 was increased in post-natal mitotic germ cells by treatment with All-trans RA (65), which might be another mechanisms through which vitamin A derivatives triggers KIT up-regulation and spermatogonial differentiation. Using conditional gene targeting, it has been shown that loss of the Doublesex-related transcription factor DMRT1 in spermatogonia causes a precocious exit from the spermatogonial program and entry into meiosis (66). Apparently, DMRT1 acts in differentiating spermatogonia by restricting RA responsiveness, directly repressing transcription of the meiotic inducer STRA8, and activating transcription of SOHLH1, thereby preventing meiosis and promoting spermatogonial development (66). In agreement with the direct role played by SOHLH1 in regulating KIT transcription (65), a drastic reduction of KIT expression in spermatogonia was evident in testes from DMRT1 conditional KO mice (66).

Retinoic acid can up-regulate KIT expression in spermatogonia also at the post-transcriptional level, by interfering with the action of two X-linked microRNAs, miR-221 and miR-222 (67). Since miR-221/222 negatively regulate both KIT mRNA and KIT protein abundance in spermatogonia, impaired expression of these microRNAs in mouse undifferentiated spermatogonia induces transition from a KIT-negative to a KIT-positive state and loss of stem cell capacity to regenerate spermatogenesis. Undifferentiated spermatogonia overexpressing miR-221/222 were found to be resistant to RA-induced transition to a KIT-positive state and incapable of differentiation *in vivo* (67). Moreover, growth factors that promote maintenance of undifferentiated spermatogonia, such as GDNF, were found to up-regulate miR-221/222 expression. On the contrary, exposure to RA down-regulates miR-221/222 abundance (67). In conclusion, RA promotes progression of SSCs to differentiating spermatogonia through different mechanisms, all of which positively influence KIT expression: downregulation of PLZF and of miR-221/222, and up-regulation of SOHLH1.

CONTROL OF SPERMATOGONIAL EXPANSION

KIT ligand/KIT interaction is essential during post-natal stages of spermatogenesis for the expansion of the differentiating spermatogonia pool. KL, expressed by Sertoli cells, stimulates proliferation of differentiating type A1-A4 spermatogonia both by inducing their progression into the mitotic cell cycle and by reducing their apoptotic rate. This effect is exerted by the activated KIT tyrosine-kinase using as signal transducers both PI3K-AKT and MEK-ERK1/2 (39, 40, 68). The role of KIT/KL in the maintenance and proliferation of differentiating spermatogonia has been highlighted by a mouse genetic model with a point mutation of KIT that eliminates the PI3K docking site (Y719F) through a single bp change (69, 70). While PGC specification and proliferation in both sexes is not compromised during embryonic development, KIT(Y719F)/KIT(Y719F) males are sterile due to the lack of spermatogonia proliferation during the prepuberal period and an arrest of spermatogenesis at the pre-meiotic stages. The KIT/KL system is also an important mediator of the influence of hypothalamic-pituitary axis on the spermatogenic process. Indeed, the expression of the mRNA for KL is induced by FSH in prepuberal mouse Sertoli cells cultured *in vitro*, through an increase in cAMP levels (39, 42). The cAMP-dependent increase in KL expression in Sertoli cells is mainly due to direct activation of transcription from proximal promoter elements within the KL gene (71). Stage-dependent induction of KL mRNA expression by FSH has also been observed in the adult rat testis (72), and the maximal levels of KL mRNA induction are observed in stages of the seminiferous epithelium which show the maximal sensitivity to FSH stimulation, and in which type A spermatogonia are actively dividing. Interestingly, the soluble and membrane forms of KL, produced by alternative splicing, are differentially expressed during testis development. Sertoli cells from prepuberal mice mainly express the mRNA encoding for the transmembrane form, while the mRNA encoding for the soluble form is expressed at higher levels later, in coincidence with the beginning of the spermatogenic process, and the two transcripts are expressed at equivalent levels in the adult testis (39). Moreover, FSH and/or cAMP analogs, beside increasing KL mRNA levels, also modify the splicing pattern of the two isoforms in cultured mouse Sertoli cells in favor of the mRNA encoding for the soluble form (39). In agreement with these observations is the finding that the highest levels of the transmembrane form of KL are detected immunohistochemically in stages VII-VIII of the mouse seminiferous epithelium (73), which are the less sensitive to FSH stimulation in the adult testis (74). It has been hypothesized that the transmembrane form of KL could be physiologically relevant for the progression through the blood-testis barrier of mitotic germ cells entering the first meiotic prophase at stages VII-VIII (5). Moreover, even though at the onset of meiosis KIT expression in male germ cells ceases at both the RNA and protein levels (5), KL/KIT interaction, besides its well-established role in the expansion of differentiating type A spermatogonia, is

also important for entry into the meiotic program, i.e., the transition from type B spermatogonia to pre-leptotene spermatocytes, as discussed in the next paragraph.

CONTROL OF ENTRY INTO MEIOSIS

Retinoic acid acts in a bimodal mode to promote the spermatogenic process. Indeed, besides its important role in promoting progression of SSCs to differentiating spermatogonia through activation of KIT expression, RA also promotes expression of the meiotic inducer STRA8 in spermatogonia (33, 51). Besides RA of Sertoli-cell origin, it has been reported that also RA synthesized by pre-meiotic spermatocytes cell autonomously induces meiotic initiation through controlling the RAR-dependent expression of STRA8 in the same cells (50). Targeted ablation of STRA8 revealed a crucial role for this gene in the initial stages of the meiotic process in post-natal male germ cells, either in the transition from type B spermatogonia/pre-leptotene to leptotene spermatocytes (75), or in slightly later stages of the meiotic prophase, with mutant leptotene spermatocytes undergoing a premature mitotic-like chromosome condensation (76). The mechanisms through which STRA8 regulates the initial stages of meiosis in both sexes are currently unknown. However, the role played by STRA8 in male meiosis appears to be different from that played in the induction of the meiotic process in the fetal ovary, in which STRA8 ablation leads to an arrest of pre-meiotic DNA synthesis in pre-leptotene oocytes (18), whereas the last round of germ cell DNA synthesis appears not be affected in STRA8-deficient pre-leptotene spermatocytes (75, 76). RA was found to increase meiotic entry of mouse KIT-positive differentiating spermatogonia *in vitro*, as evaluated by both morphological and biochemical criteria (33). Increased expression of STRA8 and of early meiotic markers, such as DMC1, accompanied the morphological switch from spermatogonia to pre-leptotene and leptotene spermatocytes. RA treatment also increased STRA8 expression in *in vitro* cultured KIT-negative undifferentiated spermatogonia, which included SSCs, but this was not followed by induction of meiotic entry, suggesting that spermatogonial competence to enter meiosis is acquired only during the differentiative stages in which they undergo KIT-dependent mitotic divisions (33). Transcriptome analysis of *in vitro* cultured differentiating spermatogonia stimulated with recombinant KL revealed a pattern of RNA expression compatible with the qualitative changes of the cell cycle that occur during the subsequent cell divisions in type A and B spermatogonia, i.e., the progressive lengthening of the S phase and the shortening of the G2/M transition (41). Moreover, KL treatment was found to up-regulate in differentiating spermatogonia the expression of early meiotic genes, and to down-regulate at the same time typical spermatogonial markers, suggesting an important role for KL/KIT interaction in the transition from the mitotic to the meiotic cell cycle, and also an active role in the induction of meiotic differentiation (41). Indeed, morphological and biochemical analysis of *in vitro* cultured spermatogonia treated with KL revealed an induction of STRA8 and DMC1 expression and of meiotic entry, evaluated as a dramatic increase in the number of pre-leptotene and leptotene spermatocytes similar to the one induced by RA treatment (33). The effect of RA and KL on meiotic entry did not appear to be additive, implying that these factors converge on common signal transduction pathways to exert this effect. Indeed, similarly to KL, RA treatment induced KIT autophosphorylation, MEK-ERK1/2 and PI3K-AKT activation, and selective inhibitors of any of these pathways inhibited the biochemical and morphological signs of meiotic entry. Thus, together with genomic effects leading to increased expression of KIT in spermatogonia and of KL in Sertoli cells, RA also exerts rapid non-genomic effects in differentiating spermatogonia and converge with KL on common KIT-dependent signaling pathways for the induction of meiotic entry (33).

In order to ensure the homeostasis of the spermatogenic process, paracrine mechanisms, and endogenous effectors which negatively regulate spermatogonial differentiation and the onset of the meiotic process in post-natal spermatogenesis must coexist with positive inducers such as RA and KL. One of these paracrine mechanisms is analogous to the one operating to prevent meiosis onset in the fetal testis, and involves FGF9 expression in the somatic environment of the seminiferous epithelium and expression of the RNA-binding protein NANOS2 in pre-meiotic germ cells. In the post-natal testis, NANOS2 was found to be specifically expressed at both the RNA and protein level in KIT-negative undifferentiated spermatogonia, but not in KIT-positive differentiating spermatogonia, nor in meiotic or postmeiotic germ cells (10). FGF9 stimulation of *in vitro* cultured differentiating spermatogonia resulted in a dramatic induction of NANOS2 expression and inhibition of the morphological and biochemical signs of entry into meiosis, without apparent effects on the expression of STRA8, whereas RA treatment resulted in a deep inhibition in the levels of NANOS2 expression in undifferentiated spermatogonia, together with the previously described stimulation of STRA8 expression (10). Thus, together with playing an essential role in preventing meiosis of gonocytes in the male fetal testis, FGF9 acts as an inhibitor of meiotic differentiation through the upregulation of NANOS2 also in post-natal male mitotic germ cells.

FUTURE PERSPECTIVES

Obviously there must be also a paracrine influence of germ cells on Sertoli-cell production of factors involved in the local control of spermatogenesis, but, up to now, little information is available in the literature about these germ cell-generated signals. On the other hand, Sertoli cells are clearly the only mediators of the influence of the hypothalamic-pituitary axis on the spermatogenic process. FSH drives both Sertoli-cell secretion of GDNF, on one side, and of BMP4 and KL, on the other side. This actually fits with the double role exerted by the pituitary hormones, as inducers of spermatogenesis at puberty (through the local mediation of BMP4 and KL), but at the same time as essential for its maintenance and quantitative output (through GDNF stimulation of SSCs self-renewal). The factors which locally control the balance between GDNF vs. BMP4 and KL secretion by Sertoli cells in response to FSH might be germ cell-generated signals, and they must be the object of further studies.

Another puzzling observation is that FGF9 exerts opposite effects in KIT-positive differentiating spermatogonia with respect to those elicited by RA and KL signaling. Indeed, it is intriguing to notice that KL and FGF9 act on the same germ cell type stimulating receptor tyrosine-kinase activities (and thus presumably partially shared signal transduction pathways), yet they exert

opposite effects (differentiation and promotion of meiosis vs. prevention of meiotic entry). It will be very important to dissect the differences in intracellular signaling elicited in differentiating spermatogonia by these two antagonistic growth factors and the downstream cascade of events that lead to RA/KL-mediated induction of meiotic entry and FGF9-mediated inhibition of the same process. For instance, it will be interesting to characterize the subtypes of FGF receptors expressed in spermatogonia, and to investigate whether activation of NODAL signaling is involved in FGF9 action in post-natal male germ cells as it has been reported for male fetal gonocytes (14–16). Preliminary results from our laboratory indicate that both FGF9 and KL stimulate transient ERK1/2 activation in spermatogonia, but PI3K-dependent AKT activation is elicited by KL, but not by FGF9 (V. Tassinari, P. Rossi, and S. Dolci, unpublished results). This might be of particular importance, in light of the notion that in the mouse testis, as mentioned previously, a point mutation of KIT that eliminates the PI3K docking site cause a total block of the spermatogenic process between 8 and 10 dpp (69, 70), coinciding with of the onset of meiosis in the male germ cell line, and that PI3K inhibitors completely block induction of meiotic entry elicited *in vitro* by RA and/or KL treatment of differentiating spermatogonia (33).

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REFERENCES

1. Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, et al. Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev* (1999) **13**:424–36. doi:10.1101/gad.13.4.424
2. Pesce M, Klinger FG, De Felici M. Derivation in culture of primordial germ cells from cells of the mouse epiblast: phenotypic induction and growth control by Bmp4 signalling. *Mech Dev* (2002) **112**:15–24. doi:10.1016/S0925-4773(01)00624-4
3. Campolo F, Gori M, Favaro R, Nicolis S, Pellegrini M, Botti F, et al. Essential role of sox2 for the establishment and maintenance of the germ cell line. *Stem Cells* (2013) **31**:1408–21. doi:10.1002/stem.1392
4. Dolci S, Williams D, Ernst MK, Resnick JL, Brannan CI, Lock LF, et al. Requirement for mast cell growth factor for primordial germ cell survival in culture. *Nature* (1991) **352**:809–11. doi:10.1038/352809a0
5. Sette C, Dolci S, Geremia R, Rossi P. The role of stem cell factor and of alternative c-kit gene products in the establishment, maintenance and function of germ cells. *Int J Dev Biol* (2000) **44**:599–608.
6. Gu Y, Runyan C, Shoemaker A, Surani A, Wylie C. Steel factor controls primordial germ cell survival and motility from the time of their specification in the allantois, and provides a continuous niche throughout their migration. *Development* (2009) **136**:1295–303. doi:10.1242/dev.030619
7. Rossi P. Transcriptional control of KIT gene expression during germ cell development. *Int J Dev Biol* (2013) **57**:179–84. doi:10.1387/ijdb.130014pr
8. Colvin JS, Green RP, Schmahl J, Capel B, Ornitz DM. Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell* (2001) **104**:875–89. doi:10.1016/S0092-8674(01)00284-7
9. Kim Y, Kobayashi A, Sekido R, DiNapoli L, Brennan J, Chaboissier MC, et al. Fgf9 and Wnt4 act as antagonistic signals to regulate mammalian sex determination. *PLoS Biol* (2006) **4**:e187. doi:10.1371/journal.pbio.0040187
10. Barrios F, Filipponi D, Pellegrini M, Paronetto MP, Di Siena S, Geremia R, et al. Opposing effects of retinoic acid and FGF9 on Nanos2 expression and meiotic entry of mouse germ cells. *J Cell Sci* (2010) **123**:871–80. doi:10.1242/jcs.057968
11. Bowles J, Feng CW, Spiller C, Davidson TL, Jackson A, Koopman P. FGF9 suppresses meiosis and promotes male germ cell fate in mice. *Dev Cell* (2010) **19**:440–9. doi:10.1016/j.devcel.2010.08.010
12. Suzuki A, Saga Y. Nanos2 suppresses meiosis and promotes male germ cell differentiation. *Genes Dev* (2008) **22**:430–5. doi:10.1101/gad.1612708
13. Suzuki A, Igarashi K, Aisaki K, Kanno J, Saga Y. NANOS2 interacts with the CCR4-NOT deadenylation complex and leads to suppression of specific RNAs. *Proc Natl Acad Sci U S A* (2010) **107**:3594–9. doi:10.1073/pnas.0908664107
14. Souquet B, Tourpin S, Messiaen S, Moison D, Habert R, Livera G. Nodal signaling regulates the entry into meiosis in fetal germ cells. *Endocrinology* (2012) **153**:2466–73. doi:10.1210/en.2011-2056
15. Spiller CM, Feng CW, Jackson A, Gillis AJ, Rolland AD, Looijenga LH, et al. Endogenous Nodal signaling regulates germ cell potency during mammalian testis development. *Development* (2012) **139**:4123–32. doi:10.1242/dev.083006
16. Wu Q, Kanata K, Saba R, Deng CX, Hamada H, Saga Y. Nodal/activin signaling promotes male germ cell fate and suppresses female programming in somatic cells. *Development* (2013) **140**:291–300. doi:10.1242/dev.087882
17. Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, et al. Retinoid signaling determines germ cell fate in mice. *Science* (2006) **312**:596–600. doi:10.1126/science.1125691
18. Baltus AE, Menke DB, Hu YC, Goodheart ML, Carpenter AE, de Rooij DG, et al. In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat Genet* (2006) **38**:1430–4. doi:10.1038/ng1919
19. Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD, Page DC. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc Natl Acad Sci U S A* (2006) **103**:2474–9. doi:10.1073/pnas.0510813103
20. MacLean G, Li H, Metzger D, Chambon P, Petkovich M. Apoptotic extinction of germ cells in testes of Cyp26b1 knockout mice. *Endocrinology* (2007) **148**:4560–7. doi:10.1210/en.2007-0492
21. Kumar S, Chatzi C, Brade T, Cunningham TJ, Zhao X, Duester G. Sex-specific timing of meiotic initiation is regulated by Cyp26b1 independent of retinoic acid signalling. *Nat Commun* (2011) **2**:51. doi:10.1038/ncomms1136
22. Griswold MD, Hogarth CA, Bowles J, Koopman P. Initiating meiosis: the case for retinoic acid. *Biol Reprod* (2012) **86**:35. doi:10.1095/biolreprod.111.096610
23. Fevold HI, Hisaw FL, Leonard SL. The gonad stimulating and the luteinizing hormones of the anterior lobe of the hypophysis. *Am J Physiol* (1931) **97**:291–301.
24. Steinberger E. Hormonal control of mammalian spermatogenesis. *Physiol Rev* (1971) **51**:1–22.
25. Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, Hess MW, et al. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* (2000) **287**:1489–93. doi:10.1126/science.287.5457.1489
26. Viglietto G, Dolci S, Bruni P, Baldassarre G, Chiariotti L, Melillo RM, et al. Glial cell line-derived neutrotrophic factor and neurturin can act as paracrine growth factors stimulating DNA synthesis of Ret-expressing spermatogonia. *Int J Oncol* (2000) **16**:689–94.
27. Yomogida K, Yagura Y, Tadokoro Y, Nishimune Y. Dramatic expansion of germinal stem cells by ectopically expressed human glial cell line-derived neurotrophic factor in mouse Sertoli cells. *Biol Reprod* (2003) **69**:1303–7. doi:10.1095/biolreprod.103.015958
28. Kubota H, Avarbock MR, Brinster RL. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc Natl Acad Sci U S A* (2004) **101**:16489–94. doi:10.1073/pnas.0407063101
29. Morimoto H, Iwata K, Ogonuki N, Inoue K, Atsuo O, Kanatsu-Shinohara M, et al. ROS are required for mouse spermatogonial stem cell self-renewal. *Cell Stem Cell* (2013) **12**:774–86. doi:10.1016/j.stem.2013.04.001
30. Grasso M, Fuso A, Dovere L, de Rooij DG, Stefanini M, Boitani C, et al. Distribution of GFRA1-expressing spermatogonia in adult mouse testis. *Reproduction* (2012) **143**:325–32. doi:10.1530/REP-11-0385
31. Sada A, Hasegawa K, Pin PH, Saga Y. NANOS2 acts downstream of glial cell line-derived neurotrophic factor signaling to suppress differentiation of spermatogonial stem cells. *Stem Cells* (2012) **30**:280–91. doi:10.1002/stem.790
32. Tadokoro Y, Yomogida K, Ohta H, Tohda A, Nishimune Y. Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. *Mech Dev* (2002) **113**:29–39. doi:10.1016/S0925-4773(02)00004-7
33. Pellegrini M, Filipponi D, Gori M, Barrios F, Lolicato F, Grimaldi P, et al. ATRA and KL promote differentiation toward the meiotic program of male germ cells. *Cell Cycle* (2008) **7**:3878–88. doi:10.4161/cc.7.24.7262
34. de Rooij DG. Proliferation and differentiation of spermatogonial stem cells. *Reproduction* (2001) **121**:347–54. doi:10.1530/rep.0.1210347

35. Manova K, Nocka K, Besmer P, Bachvarova RF. Gonadal expression of c-kit encoded at the *W* locus of the mouse. *Development* (1990) **110**:1057–69.
36. Yoshinaga K, Nishikawa S, Ogawa M, Hayashi S, Kunisada T, Fujimoto T, et al. Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. *Development* (1991) **113**:689–99.
37. Sorrentino V, Giorgi M, Geremia R, Besmer P, Rossi P. Expression of the *c-kit* protooncogene in the murine male germ cells. *Oncogene* (1991) **6**:149–51.
38. Schrans-Stassen BH, van de Kant HJ, de Rooij DG, van Pelt AM. Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. *Endocrinology* (1999) **140**:5894–900. doi:10.1210/en.140.12.5894
39. Rossi P, Dolci S, Albanesi C, Grimaldi P, Ricca R, Geremia R. FSH induction of steel factor (SLF) mRNA in mouse Sertoli cells and stimulation of DNA synthesis in spermatogonia by soluble SLF. *Dev Biol* (1993) **155**:68–74. doi:10.1006/dbio.1993.1007
40. Dolci S, Pellegrini M, Di Agostino S, Geremia R, Rossi P. Signaling through extracellular signal-regulated kinase is required for spermatogonial proliferative response to stem cell factor. *J Biol Chem* (2001) **276**:40225–33.
41. Rossi P, Lolicato F, Grimaldi P, Dolci S, Di Sauro A, Filipponi D, et al. Transcriptome analysis of differentiating spermatogonia stimulated with kit ligand. *Gene Expr Patterns* (2008) **8**:58–70. doi:10.1016/j.modgep.2007.10.007
42. Rossi P, Albanesi C, Grimaldi P, Geremia R. Expression of the mRNA for the ligand of c-kit in mouse Sertoli cells. *Biochem Biophys Res Commun* (1991) **176**:910–4. doi:10.1016/S0006-291X(05)80272-4
43. Pellegrini M, Grimaldi P, Rossi P, Geremia R, Dolci S. Developmental expression of BMP4/ALK3/SMAD5 signaling pathway in the mouse testis: a potential role of BMP4 in spermatogonia differentiation. *J Cell Sci* (2003) **116**:3363–72. doi:10.1242/jcs.00650
44. Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brinster RL. Maintenance of mouse male germ line stem cells in vitro. *Biol Reprod* (2003) **68**:2207–14. doi:10.1095/biolreprod.102.014050
45. Carlomagno G, van Bragt MP, Korver CM, Repping S, de Rooij DG, van Pelt AM. BMP4-induced differentiation of a rat spermatogonial stem cell line causes changes in its cell adhesion properties. *Biol Reprod* (2010) **83**:742–9. doi:10.1095/biolreprod.110.085456
46. Baleato RM, Aitken RJ, Roman SD. Vitamin A regulation of BMP4 expression in the male germ line. *Dev Biol* (2005) **286**:78–90. doi:10.1016/j.ydbio.2005.07.009
47. van Pelt AM, de Rooij DG. Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. *Biol Reprod* (1990) **43**:363–7. doi:10.1095/biolreprod43.3.363
48. Vernet N, Dennefeld C, Rochette-Egly C, Oulad-Abdelghani M, Chambon P, Ghyselinck NB, et al. Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. *Endocrinology* (2006) **147**:96–110. doi:10.1210/en.2005-0953
49. Vernet N, Dennefeld C, Guillou F, Chambon P, Ghyselinck NB, Mark M. Prepubertal testis development relies on retinoic acid but not rexinoid receptors in Sertoli cells. *EMBO J* (2006) **25**:5816–25. doi:10.1038/sj.emboj.7601447
50. Raverdeau M, Gely-Pernot A, Féret B, Dennefeld C, Benoit G, Davidson I, et al. Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. *Proc Natl Acad Sci U S A* (2012) **109**:16582–7. doi:10.1073/pnas.1214936109
51. Zhou Q, Li Y, Nie R, Friel P, Mitchell D, Evanoff RM, et al. Expression of stimulated by retinoic acid gene 8 (Stra8) and maturation of murine gonocytes and spermatogonia induced by retinoic acid in vitro. *Biol Reprod* (2008) **78**:537–45. doi:10.1095/biolreprod.107.064337
52. Gely-Pernot A, Raverdeau M, Célébi C, Dennefeld C, Feret B, Klopfenstein M, et al. Spermatogonia differentiation requires retinoic acid receptor γ. *Endocrinology* (2012) **153**:438–49. doi:10.1210/en.2011-1102
53. Barna M, Merghoub T, Costoya JA, Ruggero D, Branford M, Bergia A, et al. Plzf mediates transcriptional repression of HoxD gene expression through chromatin remodeling. *Dev Cell* (2002) **3**:499–510. doi:10.1016/S1534-5807(02)00289-7
54. Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, et al. Plzf is required in adult male germ cells for stem cell self-renewal. *Nat Genet* (2004) **36**:647–52. doi:10.1038/ng1366
55. Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, et al. Essential role of Plzf in maintenance of spermatogonial stem cells. *Nat Genet* (2004) **36**:653–9. doi:10.1038/ng1367
56. Payne C, Braun RE. Histone lysine trimethylation exhibits a distinct perinuclear distribution in Plzf-expressing spermatogonia. *Dev Biol* (2006) **293**:461–72. doi:10.1016/j.ydbio.2006.02.013
57. Filipponi D, Hobbs RM, Ottolenghi S, Rossi P, Jannini EA, Pandolfi PP, et al. Repression of kit expression by Plzf in germ cells. *Mol Cell Biol* (2007) **27**:6770–81. doi:10.1128/MCB.00479-07
58. Dann CT, Alvarado AL, Molyneux LA, Denard BS, Garbers L, Porteus MH. Spermatogonial stem cell self-renewal requires OCT4, a factor downregulated during retinoic acid-induced differentiation. *Stem Cells* (2008) **26**:2928–37. doi:10.1634/stemcells.2008-0134
59. Ballow D, Meistrich ML, Matzuk M, Rajkovic A. Sohlh1 is essential for spermatogonial differentiation. *Dev Biol* (2006) **294**:161–7. doi:10.1016/j.ydbio.2006.02.027
60. Ballow DJ, Xin Y, Choi Y, Pangas SA, Rajkovic A. Sohlh2 is a germ cell-specific bHLH transcription factor. *Gene Expr Patterns* (2006) **6**:1014–8. doi:10.1016/j.modgep.2006.04.007
61. Hao J, Yamamoto M, Richardson TE, Chapman KM, Denard BS, Hammer RE, et al. Sohlh2 knockout mice are male-sterile because of degeneration of differentiating type A spermatogonia. *Stem Cells* (2008) **26**:1587–97. doi:10.1634/stemcells.2007-0502
62. Pangas SA, Choi Y, Ballow DJ, Zhao Y, Westphal H, Matzuk MM, et al. Oogenesis requires germ cell-specific transcriptional regulators Sohlh1 and Lhx8. *Proc Natl Acad Sci USA* (2006) **103**:8090–5. doi:10.1073/pnas.0601083103
63. Toyoda S, Miyazaki T, Miyazaki S, Yoshimura T, Yamamoto M, Tashiro F, et al. Sohlh2 affects differentiation of KIT positive oocytes and spermatogonia. *Dev Biol* (2009) **325**:238–48. doi:10.1016/j.ydbio.2008.10.019
64. Suzuki H, Ahn HW, Chu T, Bowden W, Gassei K, Orwig K, et al. SOHLH1 and SOHLH2 coordinate spermatogonial differentiation. *Dev Biol* (2012) **361**:301–12. doi:10.1016/j.ydbio.2011.10.027
65. Barrios F, Filipponi D, Campolo F, Gori M, Bramucci F, Pellegrini M, et al. SOHLH1 and SOHLH2 control Kit expression during postnatal male germ cell development. *J Cell Sci* (2012) **125**:1455–64. doi:10.1242/jcs.092593
66. Matson CK, Murphy MW, Griswold MD, Yoshida S, Bardwell VJ, Zarkower D. The mammalian doublesex homolog DMRT1 is a transcriptional gatekeeper that controls the mitosis versus meiosis decision in male germ cells. *Dev Cell* (2010) **19**:612–24. doi:10.1016/j.devcel.2010.09.010
67. Yang Q-E, Racicot KE, Kaucher AV, Oatley MJ, Oatley JM. MicroRNAs 221 and 222 regulate the undifferentiated state in mammalian male germ cells. *Development* (2013) **140**:280–90. doi:10.1242/dev.087403
68. Feng LX, Ravindranath N, Dym M. Stem cell factor/c-kit up-regulates cyclin D3 and promotes cell cycle progression via the phosphoinositide 3-kinase/p70 S6 kinase pathway in spermatogonia. *J Biol Chem* (2000) **275**:25572–6. doi:10.1074/jbc.M002218200
69. Blume-Jensen P, Jiang G, Hyman R, Lee KF, O'Gorman S, Hunter T. Kit/stem cell factor receptor-induced activation of phosphatidylinositol 3′-kinase is essential for male fertility. *Nat Genet* (2000) **24**:157–62. doi:10.1038/72814
70. Kissel H, Timokhina I, Hardy MP, Rothschild G, Tajima Y, Soares V, et al. Point mutation in kit receptor tyrosine kinase reveals essential roles for kit signaling in spermatogenesis and oogenesis without affecting other kit responses. *EMBO J* (2000) **19**:1312–26. doi:10.1093/emboj/19.6.1312
71. Grimaldi P, Capolunghi F, Geremia R, Rossi P. Cyclic adenosine monophosphate (cAMP) stimulation of the kit ligand promoter in Sertoli cells requires an Sp1-binding region, a canonical TATA box, and a cAMP-induced factor binding to an immediately downstream GC-rich element. *Biol Reprod* (2003) **69**:1979–88. doi:10.1095/biolreprod.103.019471
72. Yan W, Linderborg J, Suominen J, Toppari J. Stage-specific regulation of stem cell factor gene expression in the rat seminiferous epithelium. *Endocrinology* (1999) **140**:1499–504. doi:10.1210/en.140.3.1499
73. Vincent S, Segretain D, Nishikawa S, Nishikawa SI, Sage J, Cuzin F, et al. Stage-specific expression of the Kit receptor and its ligand (KL) during male gametogenesis in the mouse: a Kit-KL interaction critical for meiosis. *Development* (1998) **125**:4585–93.
74. Parvinen M. Regulation of the seminiferous epithelium. *Endocr Rev* (1982) **3**:404–17. doi:10.1210/edrv-3-4-404
75. Anderson EL, Baltus AE, Roepers-Gajadien HL, Hassold TJ, de Rooij DG, van Pelt AM, et al. Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. *Proc Natl Acad Sci U S A* (2008) **105**:14976–80. doi:10.1073/pnas.0807297105

76. Mark M, Jacobs H, Oulad-Abdelghani M, Dennefeld C, Féret B, Vernet N, et al. STRA8-deficient spermatocytes initiate, but fail to complete, meiosis and undergo premature chromosome condensation. *J Cell Sci* (2008) **121**:3233–42. doi:10.1242/jcs.035071

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The endocannabinoid system and spermatogenesis

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Spermatogenesis is a complex process in which male germ cells undergo a mitotic phase followed by meiosis and by a morphogenetic process to form mature spermatozoa. Spermatogenesis is under the control of gonadotropins, steroid hormones and it is modulated by a complex network of autocrine and paracrine factors. These modulators ensure the correct progression of germ cell differentiation to form mature spermatozoa. Recently, it has been pointed out the relevance of endocannabinoids as critical modulators of male reproduction. Endocannabinoids are natural lipids able to bind to cannabinoid receptors and whose levels are regulated by specific biosynthetic and degradative enzymes. Together with their receptors and metabolic enzymes, they form the "endocannabinoid system" (ECS). In male reproductive tracts, they affect Sertoli cell activities, Leydig cell proliferation, germ cell differentiation, sperm motility, capacitation, and acrosome reaction. The ECS interferes with the pituitary-gonadal axis, and an intricate crosstalk between ECS and steroid hormones has been highlighted. This mini-review will focus on the involvement of the ECS in the control of spermatogenesis and on the interaction between ECS and steroid hormones.

Keywords: male germ cells, spermatogenesis, endocannabinoid system, sex hormones, cannabinoid

INTRODUCTION

Infertility affects 10–15% of couples, and it has been estimated that a male factor is responsible in approximately half of these cases. Male infertility is diagnosed with the analysis of several semen parameters, such as the number of total sperm, sperm motility, and percentage of sperm cells with a normal morphology. It is known that marijuana, the commonest recreational drug of abuse, has adverse effects on male reproductive physiology. Its use is associated with impotence, decreased testosterone plasma level, impairment of spermatogenesis, production of spermatozoa with abnormal morphology, reduction of sperm motility and viability and, more recently, with the occurrence of non-seminoma germ cell tumors (1). The identification of endogenous cannabinoids (ECBs) that mimic some effects of delta-9-THC, the active principle of *Cannabis sativa*, has opened new studies on the biological role of ECBs in male reproduction. In this mini-review we focused on the relevance of endocannabinoids and "endocannabinoid system" (ECS) in spermatogenesis and sperm functions, and on the interplay between ECS and sex hormone.

SPERMATOGENESIS

Spermatogenesis is a complex differentiative process starting from spermatogonial stem cells (SSCs), known as A-single (A_s). The A_s cells, similarly to other stem cells, have the capability to self-renew, producing daughter A_s cells, and to progress into "undifferentiated spermatogonia" known as A-paired (A_{pr}), and A-aligned (A_{al}) that represent committed cells. The A_{al} spermatogonia then differentiate into A1-4, intermediate (In) and B spermatogonia which undergo meiosis as pre-leptotene spermatocytes (2). Spermatocytes pass sequentially through leptotene, zygotene, pachytene, and diplotene phases of prophase I, and then quickly undergo two M-phase divisions, yielding haploid spermatids, that became spermatozoa through the morphogenetic process called spermiogenesis. Sperm released from the seminiferous epithelium into the tubule lumen are still immature and are not able to fertilize an egg. Sperm maturation occur in the epididymis. During spermatogenesis, germ cells, at each stage of differentiation, are in close contact with Sertoli cells which provide physical and metabolic support for their proliferation, meiosis, and successful progression into spermatozoa. Sertoli cells proliferate quickly during perinatal period and they switch to a mature, non-proliferative state, around the onset of puberty. Since only a limited number of germ cells can be supported by each Sertoli cell (3), in adult testis, the number of Sertoli cells will be a critical factor with obvious consequences on fertility.

Spermatogenesis continues throughout life and it is regulated by a complex assortment of hormones as well as numerous locally produced factors that include growth factors, cytokines, and chemokines, that act through autocrine and paracrine pathways. Sertoli cell-secreted growth factors are known to have direct effects mainly on spermatogonia: Gdnf acts on self-renewal of SSCs and inhibits their differentiation (4), Bmp4 has both a proliferative and differentiative effect on these cells (5), and Kit Ligand (KL), acts on the kit tyrosine-kinase receptor expressed by differentiating type A spermatogonia (6) stimulating their progression into the mitotic cell cycle and reducing apoptosis (7). The major hormonal control system of spermatogenesis is the hypothalamic-pituitary-gonadal axis, based essentially on the release of two gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), under the stimulation of hypothalamic GnRHs. Leydig and Sertoli cells, the somatic cells of the testis, are primary responders to circulating gonadotropin hormones and their failure to respond appropriately, results in male infertility (8). LH stimulates Leydig cells to synthesize testosterone (T) and FSH acts

on Sertoli cells stimulating their proliferation and expression of several trophic factors essential for spermatogenesis.

THE ENDOCANNABINOID SYSTEM

Endocannabinoids are lipid-signal molecules that are endogenous ligands for cannabinoid receptors, and together with enzymes responsible for their synthesis and degradation, they form the "ECS" (9). ECS is conserved from invertebrate to mammals and it assumes important role in physiological and pathological processes. The two best characterized endocannabinoids are *N*-arachidonoyl ethanolamine (AEA, anandamide) and 2 arachidonoyl glycerol (2-AG).

Endogenous cannabinoids bind to and activate their target receptors, causing several biological effects on different tissues. The main cannabinoid receptor targets type-1 (CB_1) and type-2 (CB_2) are seven trans-membrane G protein-coupled receptors (10). CB_1 is widely expressed in the nervous system mainly at the terminal ends of central and peripheral neurons, but it is also expressed in ovary, uterus, testis, vas deferens, and urinary bladder. CB_2 is mainly expressed in the cells of the immune system but it is also found in brainstem (11). ECBs are released from membrane phospholipid precursors by specific phospholipases, that are activated "on demand." AEA synthesis is catalyzed by an *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) (12). Similarly, the formation of 2-AG involves a rapid hydrolysis of inositol phospholipids by a specific phospholipase C (PLC) to generate diacylglycerol (DAG), which is then converted into 2-AG by an sn-1-DAG lipase (DAGL) (13). As lipid molecules, ECBs diffuse passively through the membrane, but the presence of a membrane transporter, EMT, that acts by a facilitated diffusion mechanism, has been hypothesized (14, 15). More recently an anandamide transporter named FLAT, which facilitates its translocation into cells has been identified in neural cells (16). The biological effects of ECBs depend on their lifespan in the extracellular space, which is limited by a re-uptake by cells. Once inside the cells ECBs are hydrolyzed by two specific enzymes: the fatty acid amide hydrolase (FAAH) cleaves AEA into arachidonic acid and ethanolamine, and the monoacylglycerol lipase (MAGL) (17) transforms the 2-AG into arachidonic acid and glycerol (18).

AEA, but not 2-AG, behaves also as an endovanilloid binding to the type-1 vanilloid receptor (transient receptor potential vanilloid 1, TRPV1) at an intracellular site (19). TRPV1 is a six trans-membrane spanning non-selective cation channel, whose expression is found mainly in specialized sensory neurons that detect painful stimuli (20). However it is now established that TRPV1 is expressed also in non-neuronal cells, such as keratinocytes and epithelial and endothelial cells, where it could play a wide variety of physiological functions.

THE ENDOCANNABINOID SYSTEM AND SPERMATOGENESIS

ECS AND GERM CELLS

Following the discovery of ECS, many studies about its expression and function in male reproductive system have been carried out (21). The presence of components of ECS has been demonstrated in the testis, in the reproductive fluids and tracts, in different organisms from invertebrates to mammals. All the components of the ECS have been identified in mammalian germ cells, from spermatogonia to spermatozoa.

First evidence of an effect of cannabinoid in male reproduction comes from a study in sea urchin in which it was demonstrated that exogenous cannabinoid THC directly reduced the fertilizing capacity of sperm (22) through the inhibition of the acrosome reaction (23). Next, endogenous cannabinoid AEA was shown to induce the same effects of THC on sea urchin sperm (24).

Endocannabinoids have been identified in human seminal plasma (25), in the amphibian cloacal fluid (26) and in mouse epididymis (27) indicating a role in the control of sperm functions. Most of the *in vitro* studies reported an adverse effect of AEA on sperm function with inhibition of motility, capacitation and acrosome reaction, and indicated a pivotal role of CB_1 receptor in mediating AEA effects. In humans, AEA inhibits sperm motility by decreasing mitochondrial activity and this effect was blocked by the CB_1 receptor antagonist SR141716 (28). In boar (*Sus scropha*), a stable AEA analog, methanandamide, reduces sperm capacitation and inhibits acrosome reaction (29). Also in frog *Rana esculenta* AEA has been shown to inhibit sperm motility through CB_1 receptor (26).

It has been described a role of CB_1 in spermiogenesis, when elongated spermatids are remodeled to form mature spermatozoa with a change in the chromatin structure. Indeed, genetic inactivation of CB_1 causes an inefficient histone displacement, poor chromatin condensation, and DNA damage in sperm (30), indicating a role of ECS in spermatid differentiation.

Further interesting findings supporting a role of AEA and CB_1 receptor on sperm function arise from the gene knockout animal models. In the absence of CB_1 signaling, sperm acquire motility precociously and the percentage of motile spermatozoa recovered from the caput of epididymis was higher with respect to wild-type mice, suggesting a physiological inhibitory regulation of endocannabinoids on sperm motility in the epididymis (31). Genetic loss of FAAH results in increased levels of AEA in the reproductive system and impairment of sperm fertilizing ability (32). These results lead to hypothesize that an "adequate tone" of AEA and the expression of CB_1 receptor are critical in the formation of morphologically and functionally normal sperm. In support of this observation, it has been recently reported that, in rats, *in vivo* administration of HU210, a synthetic analog of THC and a potent agonist of CB receptors, causes a marked impairment of spermatogenesis with reduction in total sperm count and motility, and a deregulation of the ECS, confirming the *in vitro* observations and indicating that the use of exo-cannabinoids may influence adversely male fertility (33).

Another molecular target of AEA is the vanilloid receptor TRPV1 (34), expressed in sperm cells of mouse (35), boar (29), bull (36), and humans (37). Activation of TRPV1 receptor by AEA, seems to play a role in the stabilization of the plasma membranes in capacitated boar sperm, preventing spontaneous acrosome reaction (29). Therefore, AEA can bring different signals in sperm cells, depending on the target receptor (CB_1 or TRPV1) that is activated.

Besides AEA, also the endocannabinoid 2-AG has been reported to affect male reproduction. Using mouse male germ cell populations at different stage of differentiation we highlighted a pivotal role of 2-AG and CB_2 receptor in mouse spermatogenesis (35).

We demonstrated that mammalian male germ cells, from mitotic to haploid stage, have a complete ECS which is modulated during spermatogenesis. Spermatogonia possess higher level of 2-AG that decreases in spermatocytes and drastically drops in spermatids. This correlates to higher level of biosynthetic (DAGL) and lower level of degrading enzymes (MAGL) in spermatogonia with respect to spermatocytes and spermatids. On the contrary, AEA levels remain unchanged during spermatogenesis and probably are crucial to maintain, locally, an appropriate "anandamide tone" for a correct progression of spermatogenesis as seen for normal development of mouse embryos (38). Interestingly, activation of CB_2 receptor in spermatogonia promotes their progression into meiosis as revealed by an increased number of cells positive for the meiotic marker SCP3 and by the expression of premeiotic and early meiotic genes. Thus, during spermatogenesis an autocrine endocannabinoid regulation of mitotic germ cell differentiation might occur as proposed in **Figure 1**.

Endocannabinoid 2-AG has been also found to play a role in regulating the ability of spermatozoa to become motile during their transit in the epididymis. 2-AG levels are high in mouse spermatozoa isolated from the caput of the epididymis, where they do not move regularly, and decrease dramatically in spermatozoa isolated from the cauda, where they acquire vigorous motility, suggesting that, along the epididymis, the decrease of 2-AG levels from caput to cauda promotes start-up of spermatozoa (27). Finally mouse sperm capacitation has been found to be linked to an enhancement of the endogenous tone of both AEA and 2-AG (39), underlying the important role of ECS in regulating important step of spermatogenesis and sperm functions.

With the aim to investigate, in humans, a possible relationship between male reproductive dysfunction and deregulation of the ECS, recent studies have shown a marked reduction of AEA and 2-AG content in the seminal plasma of infertile patients. This reduction in sperm from infertile versus fertile men can be determined by either an increased ratio of degradation/biosynthesis, or by lower levels of CB_1 mRNA expression (40, 41), indicating that the ECBs signaling is involved in the preservation of normal human sperm function.

ECS AND TESTICULAR SOMATIC CELLS

Endocannabinoid system components are expressed also in somatic cells of mammalian testis. Sertoli cells possess the biochemical machinery to synthesize, transport, degrade, and bind both AEA (42) and 2-AG (43). Mouse Sertoli cells express a functional CB_2 receptor, an AEA membrane transporter and the AEA-degrading enzyme FAAH (42). AEA has been shown to have a pro-apoptotic effect on Sertoli cells, inducing DNA fragmentation. Lower level of AEA correlates with higher level of FAAH protein and with a decrease in Sertoli cell apoptosis, suggesting a protective and pro-survival role of FAAH in Sertoli cells. More interestingly, FAAH activity and expression is hormonally up-regulated in Sertoli cells by FSH and estrogen (43, 44).

Rat Leydig cells express CB_1 which is modulated during development and it negatively correlates to cell division. Immature Leydig cells in mitosis were negative for CB_1, while immature non-mitotic Leydig cells were positive, indicating a negative effect of CB_1 on Leydig cell proliferation and suggesting that their differentiation may depend on the ECS (45).

THE ENDOCANNABINOID SYSTEM AND SEX HORMONE

As described above, the ECS is widely distributed in testicular cells and it is an important regulator of spermatogenesis and sperm functions. Recently, many evidence indicate the existence of interplay between ECS and sex hormones, testosterone and estrogen, thus stressing the relevant role of ECS in regulating male reproduction. Testosterone is produced by Leydig cells under the stimulation of LH and it is essential for the occurrence of events like blood-testis-barrier formation, germ cells progression beyond meiosis, mature sperm release. Sertoli cells are the major cellular target for the testosterone signaling and the absence of testosterone or of the androgen receptor, results in the failure of spermatogenesis and infertility. Several studies on human males smoking cannabis, reported a decrease in plasma levels of testosterone, FSH, and LH and this effect was also evident in animal studies after acute and chronic administration of THC (46, 47). Decreased levels of testosterone correlate to an inhibitory effect of cannabinoids on male sexual behavior (48). Moreover *in vitro* studies on Leydig cells showed a decrease in testosterone secretion induced by THC (49). Similarly endogenous cannabinoid AEA suppresses LH and testosterone levels in wild-type, but not in CB_1 knockout mice (50), providing evidence that the ECS acts to suppress testosterone levels.

It is now well documented that, beside testosterone, also estrogens are important modulator of male reproduction (51). The

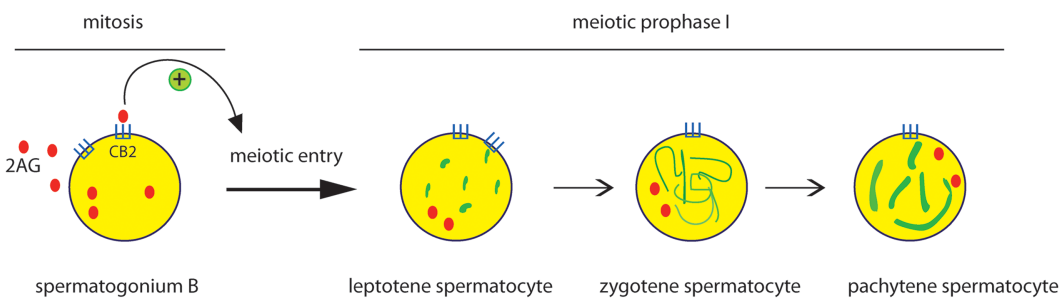

FIGURE 1 | Effect of CB_2 activation on the early steps of spermatogenic differentiation. Mitotic male germ cells express CB_2 receptor and high level of 2-AG. Activation of CB_2 receptor by 2-AG, through an autocrine pathway, promotes meiotic entry and progression of spermatogonia, as revealed by the meiotic organization of nuclear SCP3 (green) during the prophase I phases (leptotene, zygotene, pachytene).

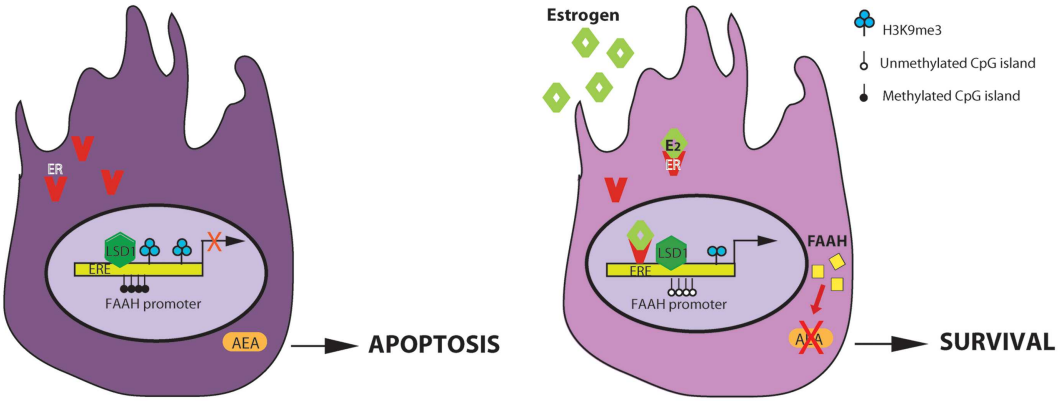

FIGURE 2 | Regulation of AEA-degrading enzyme FAAH expression by estrogen in Sertoli cells. E_2 regulates FAAH transcription by direct binding of estrogen receptor (ER) and epigenetic mechanisms including histone modification and DNA methylation. On the left: in the absence of estrogens, faah proximal promoter is methylated at DNA CpG sites and at lysine 9 of H3 histone and it is not competent for transcription. The final outcome is an increase AEA-induced apoptosis of Sertoli cells. On the right: estrogens activates the AEA-degrading enzyme FAAH transcription, through ER binding at ERE sites and reduction of DNA and H3K9me3 methylation. The direct/indirect interaction with histone demethylase LSD1, constitutively recruited at this site, is necessary for estrogen-induced transcription. The final outcome is a decrease of AEA-induced apoptosis of Sertoli cells (ERE, estrogen response element).

presence of estrogens in male reproductive tracts of numerous mammals has been reported (52). Aromatase is the enzyme that converts irreversibly androgen into estrogens and is expressed, in mammals, in all testicular cells except peritubular cells. The biological effects of estrogens are mediated by the estrogen receptors α (ERα) and β (ERβ), both expressed in mammalian testis. A role of estrogens in spermatogenesis is strongly supported by the observation that mice lacking estrogen receptors or aromatase are infertile and show impaired spermatogenesis in adulthood (53, 54).

Between all the components of ECS, the AEA-degrading enzyme *faah* gene has been demonstrated to be the only gene to be hormonally regulated in the testis. In Sertoli cells, FSH regulates FAAH expression and activity by triggering protein kinase A or aromatase-dependent pathway (42).

The PKA-dependent pathway enhances FAAH activity by inducing phosphorylation of other proteins that could activate the enzyme. On the other hand, the aromatase-dependent pathway, that leads to the conversion of testosterone into estrogens, induces FAAH expression at transcriptional level. Indeed we recently clarify the molecular mechanisms by which estrogens directly upregulate *faah* gene transcription (55). This involves direct binding of ER to the ERE sites in the *faah* promoter and the induction of epigenetic modifications in order to confer transcriptional competence.

As presented in **Figure 2**, in Sertoli cells, E_2 engages ER, which binds to ERE sites in the *faah* proximal promoter determining demethylation of both DNA, at CpG site, and histone H3, at lysine 9 (H3K9). The presence of histone demethylase LSD1, which is recruited at this site, ensures estrogens stimulation of *faah* transcription. LSD1 could interact with ligand-bound ER or with other different partners and activate gene transcription. The biological relevance of E_2-stimulation of FAAH expression consists in decreasing AEA levels in Sertoli cells and protect them against apoptosis induced by AEA. The pro-survival role of E_2 in Sertoli cells has a clear impact on spermatogenesis. In fact regulation of Sertoli cell apoptosis could be important to maintain their population size, and consequently, to sustain a normal spermatogenic output.

This is not the only example about the cross-talks between estrogens and ECS in the testis. Recent evidences reveal that estrogens affect spermiogenesis and regulate chromatin remodeling of germ cells (56). Indeed, in mice, genetic loss of CB_1 receptor causes a reduction in FSH and estrogen plasma levels and alteration in spermatid differentiation due to an inefficient histone displacement in the sperm. Estrogens treatment is able to rescue histone displacement suggesting a role in preserving chromatin condensation in spermatozoa.

CONCLUDING REMARKS

In this mini-review we highlighted the physiological role of ECS and its interplay with sex hormones, in male reproduction. A full comprehension of the molecular events regulated by ECS in the testis will allow to better define the "protective" role of this system in maintaining and ensuring the correct progression of spermatogenesis and the formation of mature and fertilizing sperm. Interfering with this system by exposure to exogenous cannabinoids, may alter the physiological function of ECS in male reproduction thus affecting male fertility.

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REFERENCES

1. Daling JR, Doody DR, Sun X, Trabert BL, Weiss NS, Chen C, et al. Association of marijuana use and the incidence of testicular germ cell tumors. *Cancer* (2009) **115**(6):1215–23. doi:10.1002/cncr.24159
2. de Rooij DG. Proliferation and differentiation of spermatogonial stem cells. *Reproduction* (2001) **121**:347–54. doi:10.1530/rep.0.1210347
3. Orth JM, Gunsalus GI, Lamperti AA. Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells

produced during perinatal development. *Endocrinology* (1988) **122**(3):787–94. doi:10.1210/endo-122-3-787
4. Meng X, Lindahl M, Hyvonen ME, Parvinen M, De Rooij DG, Hess MW, et al. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* (2000) **287**:1489–93. doi:10.1126/science.287.5457.1489
5. Pellegrini M, Grimaldi P, Rossi P, Geremia R, Dolci S. Developmental expression of BMP4/ALK3/SMAD5 signaling pathway in the mouse testis: a potential role of BMP4 in spermatogonia differentiation. *J Cell Sci* (2003) **116**:3363–72. doi:10.1242/jcs.00650
6. Rossi P, Dolci S, Albanesi C, Grimaldi P, Ricca R, Geremia R. Follicle-stimulating hormone induction of steel factor (SLF) mRNA in mouse Sertoli cells and stimulation of DNA synthesis in spermatogonia by soluble SLF. *Dev Biol* (1993) **155**:68–74. doi:10.1006/dbio.1993.1007
7. Dolci S, Pellegrini M, Di Agostino S, Geremia R, Rossi P. Signaling through extracellular signal-regulated kinase is required for spermatogonial proliferative response to stem cell factor. *J Biol Chem* (2001) **276**:40225–33.
8. Matzuk MM, Lamb DJ. The biology of infertility: research advances and clinical challenges. *Nat Med* (2008) **14**(11):1197–213. doi:10.1038/nm.f.1895
9. Di Marzo V, Melck D, Bisogno T, De Petrocellis L. Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci* (1998) **21**:521–8. doi:10.1016/S0166-2236(98)01283-1
10. Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, et al. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* (2002) **54**(2):161–202. doi:10.1124/pr.54.2.161
11. Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, et al. Identification and functional characterization of brainstem cannabinoid CB_2 receptors. *Science* (2005) **310**:329–32.
12. Okamoto Y, Morishita J, Tsuboi K, Tonai T, Ueda N. Molecular characterization of a phospholipase D generating anandamide and its congeners. *J Biol Chem* (2004) **279**:5298–305. doi:10.1074/jbc.M306642200
13. Bisogno T, Howell F, Williams G, Minassi A, Cascio MG, Ligresti A, et al. Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J Cell Biol* (2003) **163**(3):463–8. doi:10.1083/jcb.200305129
14. Beltramo M, Stella N, Calignano A, Lin SY, Makriyannis A, Piomelli D. Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science* (1997) **277**(5329):1094–7. doi:10.1126/science.277.5329.1094
15. Beltramo M, Piomelli D. Carrier-mediated transport and enzymatic hydrolysis of the endogenous cannabinoid 2-arachidonylglycerol. *Neuroreport* (2000) **11**(6):1231–5. doi:10.1097/00001756-200004270-00018
16. Fu J, Bottegoni G, Sasso O, Bertorelli R, Rocchia W, Masetti M, et al. A catalytically silent FAAH-1 variant drives anandamide transport in neurons. *Nat Neurosci* (2011) **15**(1):64–9. doi:10.1038/nn.2986
17. McKinney MK, Cravatt BF. Structure and function of fatty acid amide hydrolase. *Annu Rev Biochem* (2005) **74**:411–32. doi:10.1146/annurev.biochem.74.082803.133450
18. Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, et al. Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc Natl Acad Sci U S A* (2002) **99**:10819–24. doi:10.1073/pnas.152334899
19. Di Marzo V, De Petrocellis L. Endocannabinoids as regulators of transient receptor potential (TRP) channels: a further opportunity to develop new endocannabinoid-based therapeutic drugs. *Curr Med Chem* (2010) **17**:1430–49. doi:10.2174/092986710790980078
20. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* (1997) **389**:816–24. doi:10.1038/39807
21. Wang H, Dey SK, Maccarrone M. Jekyll and hyde: two faces of cannabinoid signaling in male and female fertility. *Endocr Rev* (2006) **27**(5):427–48. doi:10.1210/er.2006-0006
22. Schuel H, Schuel R, Zimmerman AM, Zimmerman S. Cannabinoids reduce fertility of sea urchin sperm. *Biochem Cell Biol* (1987) **65**(2):130–6. doi:10.1139/o87-018
23. Schuel H, Berkery D, Schuel R, Chang MC, Zimmerman AM, Zimmerman S. Reduction of the fertilizing capacity of sea urchin sperm by cannabinoids derived from marihuana. I. Inhibition of the acrosome reaction induced by egg jelly. *Mol Reprod Dev* (1991) **29**(1):51–9. doi:10.1002/mrd.1080290109
24. Schuel H, Goldstein E, Mechoulam R, Zimmerman AM, Zimmerman S. Anandamide (arachidonylethanolamide), a brain cannabinoid receptor agonist, reduces sperm fertilizing capacity in sea urchins by inhibiting the acrosome reaction. *Proc Natl Acad Sci U S A* (1994) **91**(16):7678–82. doi:10.1073/pnas.91.16.7678
25. Schuel H, Burkman LJ, Lippes J, Crickard K, Forester E, Piomelli D, et al. N-acylethanolamines in human reproductive fluids. *Chem Phys Lipids* (2002) **121**:211–27. doi:10.1016/S0009-3084(02)00158-5
26. Cobellis G, Cacciola G, Scarpa D, Meccariello R, Chianese R, Franzoni MF, et al. Endocannabinoid system in frog and rodent testis: type-1 cannabinoid receptor and fatty acid amide hydrolase activity in male germ cells. *Biol Reprod* (2006) **75**(82):89. doi:10.1095/biolreprod.106.051730
27. Cobellis G, Ricci G, Cacciola G, Orlando P, Petrosino S, Cascio MG, et al. A gradient of 2-arachidonoylglycerol regulates mouse epididymal sperm cell start-up. *Biol Reprod* (2010) **82**(2):451–8. doi:10.1095/biolreprod.109.079210
28. Rossato M, Popa FI, Ferigo M, Clari G, Foresta C. Human sperm express cannabinoid receptor CNR1, the activation of which inhibits motility, acrosome reaction and mitochondrial function. *J Clin Endocrinol Metab* (2005) **90**:984–91.
29. Maccarrone M, Barboni B, Paradisi A, Bernabò N, Gasperi V, Pistilli MG, et al. Characterization of the endocannabinoid system in boar spermatozoa and implications for sperm capacitation and acrosome reaction. *J Cell Sci* (2005) **118**:4393–404. doi:10.1242/jcs.02536
30. Chioccarelli T, Cacciola G, Altucci L, Lewis SE, Simon L, Ricci G, et al. Cannabinoid receptor 1 influences chromatin remodeling in mouse spermatids by affecting content of transition protein 2 mRNA and histone displacement. *Endocrinology* (2010) **151**(10):5017–29. doi:10.1210/en.2010-0133
31. Ricci G, Cacciola G, Altucci L, Meccariello R, Pierantoni R, Fasano S, et al. Endocannabinoid control of sperm motility: the role of epididymus. *Gen Comp Endocrinol* (2007) **153**(1–3):320–2. doi:10.1016/j.ygcen.2007.02.003
32. Sun X, Wang H, Okabe M, Mackie K, Kingsley PJ, Marnett LJ, et al. Genetic loss of Faah compromises male fertility in mice. *Biol Reprod* (2009) **80**(2):235–42. doi:10.1095/biolreprod.108.072736
33. Lewis SE, Paro R, Borriello L, Simon L, Robinson L, Dincer Z, et al. Long-term use of HU210 adversely affects spermatogenesis in rats by modulating the endocannabinoid system. *Int J Androl* (2012) **35**(5):731–40. doi:10.1111/j.1365-2605.2012.01259.x
34. De Petrocellis L, Bisogno T, Maccarrone M, Davis JB, Finazzi-Agro A, Di Marzo V. The activity of anandamide at vanilloid VR1 receptors requires facilitated transport across the cell membrane and is limited by intracellular metabolism. *J Biol Chem* (2001) **276**:12856–63. doi:10.1074/jbc.M008555200
35. Grimaldi P, Orlando P, Di Siena S, Lolicato F, Petrosino S, Bisogno T, et al. The endocannabinoid system and pivotal role of the CB_2 receptor in mouse spermatogenesis. *Proc Natl Acad Sci U S A* (2009) **106**(27):11131–6. doi:10.1073/pnas.0812789106
36. Gervasi MG, Osycka-Salut C, Caballero J, Vazquez-Levin M, Pereyra E, Billi S, et al. Anandamide capacitates bull spermatozoa through CB1 and TRPV1 activation. *PLoS One* (2011) **6**(2):e16993. doi:10.1371/journal.pone.0016993
37. Francavilla F, Battista N, Barbonetti A, Vassallo MR, Rapino C, Antonangelo C, et al. Characterization of the endocannabinoid system in human spermatozoa and involvement of transient receptor potential vanilloid 1 receptor in their fertilizing ability. *Endocrinology* (2009) **150**(10):4692–700. doi:10.1210/en.2009-0057
38. Wang H, Xie H, Guo Y, Zhang H, Takahashi T, Kingsley PJ, et al. Fatty acid amide hydrolase deficiency limits early pregnancy events. *J Clin Invest* (2006) **116**(8):2122–31. doi:10.1172/JCI28621
39. Catanzaro G, Battista N, Rossi G, Di Tommaso M, Pucci M, Pirazzi V, et al. Effect of capacitation on the endocannabinoid system of mouse sperm. *Mol Cell Endocrinol* (2011) **343**(1–2):88–92. doi:10.1016/j.mce.2011.01.022
40. Lewis SE, Rapino C, Di Tommaso M, Pucci M, Battista N, Paro R, et al. Differences in the endocannabinoid system of sperm from fertile and infertile men. *PLoS One* (2012) **7**(10):e47704. doi:10.1371/journal.pone.0047704
41. Amoako AA, Marczylo TH, Marczylo EL, Elson J, Willets JM, Taylor AH, et al. Anandamide modulates human sperm motility: implications for men with asthenozoospermia and oligoasthenoteratozoospermia. *Hum Reprod* (2013) **28**(8):2058–66. doi:10.1093/humrep/det232
42. Maccarrone M, Cecconi S, Rossi G, Battista N, Pauselli R, Finazzi-Agro A. Anandamide activity and degradation are regulated by early postnatal aging and follicle-stimulating hormone in mouse Sertoli cells. *Endocrinology* (2003) **144**:20–8. doi:10.1210/en.2002-220544

43. Rossi G, Gasperi V, Paro R, Barsacchi D, Cecconi S, Maccarrone M. Follicle-stimulating hormone activates fatty acid amide hydrolase by protein kinase A and aromatase-dependent pathways in mouse primary Sertoli cells. *Endocrinology* (2007) **148**:1431–9. doi:10.1210/en.2006-0969
44. Grimaldi P, Rossi G, Catanzaro G, Maccarrone M. Modulation of the endocannabinoid-degrading enzyme fatty acid amide hydrolase by follicle-stimulating hormone. *Vitam Horm* (2009) **81**:231–61. doi:10.1016/S0083-6729(09)81010-8
45. Cacciola G, Chioccarelli T, Mackie K, Meccariello R, Ledent C, Fasano S, et al. Expression of type-1 cannabinoid receptor during rat postnatal testicular development: possible involvement in adult Leydig cell differentiation. *Biol Reprod* (2008) **79**(4):758–65. doi:10.1095/biolreprod.108.070128
46. Symons AM, Teale JD, Marks V. Effect of delta-9-tetrahydrocannabinol on the hypothalamic-pituitary-gonadal system in the maturing male rat. *Endocrinology* (1976) **68**:43–4.
47. Dalterio S, Bartke A, Burstein S. Cannabinoids inhibit testosterone secretion by mouse testes in vitro. *Science* (1977) **196**:1472–3. doi:10.1126/science.867048
48. Gorzalka BB, Hill MN, Chang SC. Male-female differences in the effects of cannabinoids on sexual behavior and gonadal hormone function. *Horm Behav* (2010) **58**(1):91–9. doi:10.1016/j.yhbeh.2009.08.009
49. Jakubovic A, McGeer E, McGeer P. Effects of cannabinoids on testosterone and protein synthesis in rat testis Leydig cells in vitro. *Mol Cell Endocrinol* (1979) **15**:41–50. doi:10.1016/0303-7207(79)90069-8
50. Wenger T, Ledent C, Csernus V, Gerendai I. The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. *Biochem Biophys Res Commun* (2001) **284**:363–8. doi:10.1006/bbrc.2001.4977
51. O'Donnell L, Robertson K-M, Jones M-E, Simpson E-R. Estrogen and spermatogenesis. *Endocr Rev* (2001) **22**:289–318. doi:10.1210/er.22.3.289
52. Hess RA. Estrogen in the adult male reproductive tract: a review. *Reprod Biol Endocrinol* (2003) **1**:52. doi:10.1186/1477-7827-1-52
53. Eddy E-M, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB, et al. Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* (1996) **137**:4796–805. doi:10.1210/en.137.11.4796
54. Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, et al. Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proc Natl Acad Sci U S A* (1999) **96**:7986–91. doi:10.1073/pnas.96.14.7986
55. Grimaldi P, Pucci M, Di Siena S, Di Giacomo D, Pirazzi V, Geremia R, et al. The faah gene is the first direct target of estrogen in the testis: role of histone demethylase LSD1. *Cell Mol Life Sci* (2012) **69**(24):4177–90. doi:10.1007/s00018-012-1074-6
56. Cacciola G, Chioccarelli T, Altucci L, Ledent C, Mason JI, Fasano S, et al. Low 17beta-estradiol levels in CNR1 knock-out mice affect spermatid chromatin remodeling by interfering with chromatin reorganization. *Biol Reprod* (2013) **88**(6):152. doi:10.1095/biolreprod.112.105726

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Does kisspeptin signaling have a role in the testes?

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Kisspeptins are a family of overlapping neuropeptides encoded by the *Kiss1* gene that regulate the mammalian reproductive axis by a central action in the hypothalamus to stimulate GnRH release. Kisspeptins and their receptor (GPR54 also called KISS1R) are also expressed in the testes but a functional role in this tissue has not been confirmed. We examined which cell types in the testes expressed kisspeptin and its receptor by staining for β-galactosidase activity using tissue from transgenic mice with *LacZ* targeted to either the *Kiss1* or the *Gpr54* genes. Expression of both genes appeared to be restricted to haploid spermatids and this was confirmed by a temporal expression analysis, which showed expression appearing with the first wave of haploid spermatid cells at puberty. We could not detect any kisspeptin protein in spermatids however, suggesting that the *Kiss1* mRNA may be translationally repressed. We tested whether kisspeptin could act on Leydig cells by examining the effects of kisspeptin on the immortalized Leydig cell line MA-10. Although MA-10 cells were shown to express *Gpr54* by RT-PCR, they did not respond to kisspeptin stimulation. We also tested whether kisspeptin could stimulate testosterone release by a direct action on the testes using explants of seminiferous tubules. The explants did not show any response to kisspeptin. The functional integrity of the MA-10 cells and the seminiferous tubule explants was confirmed by showing appropriate responses to the LH analog, human chorionic gonadotropin. These data suggest that kisspeptin signaling does not have a significant role in testes function in the mouse.

Keywords: kisspeptins, *Gpr54/Kiss1r*, testes, Leydig cells, testosterone secretion, spermatids

INTRODUCTION

Kisspeptins, encoded by the *Kiss1* gene, are an overlapping family of neuropeptides required for activation and maintenance of the mammalian reproductive axis [for review, see Ref. (1)]. Kisspeptins are encoded as a 145-amino-acid precursor protein in humans that is cleaved into shorter peptides (Kp54, Kp14, Kp13, and Kp10) that share a common RF-amide C-terminal decapeptide sequence. They all act as potent stimulators of GnRH release by signaling through the G-protein coupled receptor, GPR54 (also called KISS1R) expressed by GnRH neurons. Disruption of kisspeptin signaling causes hypogonadotropic hypogonadism in mice and humans (2–7). Mutant mice do not undergo sexual maturation at puberty and have low gonadotropic and sex steroid hormones levels caused by defective GnRH secretion from the hypothalamus. Conversely, activating mutations of *GPR54* cause precocious puberty in humans (8).

In addition to the role of kisspeptins in the central regulation of the reproductive axis, *Gpr54* expression has been detected in the testes of humans (9, 10), mice (3), rats (11), and frogs (12) raising the possibility that kisspeptins may also act at this location. Kisspeptins have been immunolocalized to Leydig cells in mice (13) and kisspeptin and GPR54 have been detected in human sperm, mainly localized to the head, neck, and the flagellum midpiece (14).

Although the expression profile of *Kiss1* and *Gpr54* suggests that kisspeptin signaling might have a role in the testes, very little has been done to test this hypothesis. The data to support a role for kisspeptin in the testes is largely circumstantial and based on discrepancies between the normally direct relationship of LH and testosterone levels. For example, in rats, chronic (13 days) subcutaneous administration of kisspeptin reduced testosterone secretion without a significant decrease in plasma LH (15). In Rhesus monkeys, continuous intravenous infusion of human kisspeptin over 4 days maintained plasma testosterone levels even after the LH stimulation levels had fallen (16). When circulating testosterone levels were expressed relative to LH levels, the [T]:[LH] ratios were significantly higher in the morning in the high dose kisspeptin treatment group compared to the vehicle group. This led to the suggestion that kisspeptins might augment the LH-induced secretion of testosterone. Support for this has come from kisspeptin administration in Rhesus monkeys pre-treated with acyline, a GnRH receptor antagonist, to allow the intratesticular actions of kisspeptin to be evaluated without the confounding effects of LH release from the pituitary (17). Kisspeptin administration significantly increased human chorionic gonadotropin (hCG)-stimulated testosterone levels in acycline treated monkeys compared to hCG treatment alone (17) suggesting that kisspeptin might enhance LH responses in Leydig cells.

To further investigate the possible function(s) of kisspeptin in the mouse testes, we used transgenic mice with *Kiss1* and *Gpr54* alleles targeted with a *LacZ* reporter gene to define the testicular cell expression profile of these genes. We also tested whether

kisspeptins can stimulate testosterone release from an immortalized mouse Leydig cell line and from primary testes explants in culture.

MATERIALS AND METHODS

MOUSE LINES AND MAINTENANCE

The 129S6/SvEv mutant mice with a targeted disruption of the *Gpr54* or *Kiss1* genes were generated as described previously (2, 5). All mice were maintained on a 12:12-h light-dark cycle (light on between 6:30 a.m. and 6:30 p.m.) with *ad libitum* access to food and water. Experimental procedures were performed under authority of a Home Office Project License and approved by a Local Ethics Committee.

MA-10 CELL CULTURE

The mouse Leydig tumor cell line MA-10 (18) was a generous gift from Dr. Mario Ascoli (University of Iowa, Iowa City, IA, USA). The MA-10 cells were maintained in RPMI-1640 medium (Sigma-Aldrich, Dorset, UK) containing 10% horse serum (Sigma-Aldrich, Dorset, UK) and 10% newborn calf serum (Sigma-Aldrich, Dorset, UK), and the cells were grown at 37°C in an humidified atmosphere of 5% CO_2. The growth medium was refreshed every 2 days to provide sufficient nutrition for cell growth.

RT-PCR GENE EXPRESSION ANALYSIS OF MA-10 CELLS

Total RNA was isolated from MA-10 cells using a NucleoSpin® RNA II kit (Cat No: 740955, MACHEREY-NAGEL GmbH & Co. KG) following the manufacturer's protocol. The time of the on-column DNA digestion was extended from 15 to 45 min to ensure complete removal of genomic DNA. The RNA was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Cat No: 18080-044, Invitrogen, UK) following the protocol provided by the manufacturer. Standard PCR was performed as follows: the samples were denatured for 5 min at 95°C and amplified for 44 cycles (30 s at 93°C, 1 min at 60°C, and 2 min at 70°C). The primer sequences were: *Kiss1* (Forward: tgctgcttctctctgtgtcg; Reverse: gccgaaggagttccagttgta, 310 bp product), *Gpr54* (Forward: gccttcgcgctctacaacctgctg; Reverse: aaggcatagagcagcggattgagc, 367 bp product), *GnRH* (Forward: cggcattctactgctgactgt; Reverse: catcttcttctgcctggcttc, 229 bp product), β-actin (Forward: ctgtattcccctccatcgtg; Reverse: gggtcaggatacctctcttgc, 113 bp product). RNA without a reverse transcription step was used as a negative control for identification of genomic DNA contamination and cDNA from wild-type hypothalamus was used as a positive control for *Kiss1* amplification.

X-GAL STAINING OF TESTES SECTIONS

Testes were fixed in 1% paraformaldehyde/PBS overnight at 4°C, cryoprotected with 30% sucrose/PBS overnight at 4°C, and cryosectioned at 20 μm onto poly-lysine coated slides. Sections were air dried at room temperature, rehydrated in PBS and β-galactosidase activity detected using a *LacZ* staining solution [1 mM $MgCl_2$, 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide in PBS] at 37°C overnight, and counterstained with 1% Saffronin.

IMMUNOHISTOCHEMISTRY TO DETECT KISSPEPTIN EXPRESSION

Testes were fixed in 4% paraformaldehyde/Tris-buffered saline (TBS) for 5 h at room temperature and transferred to 30% sucrose/TBS overnight at 4°C. The testes were then cryosectioned at 15 μM, air dried at room temperature, rehydrated in TBS, and slide-mounted immunohistochemistry was performed to detect kisspeptin expression. Polyclonal antibody AC566 raised in rabbits against mouse Kp10 was a generous gift from Alain Caraty, Tours, France. Characterization and specificity of AC566 has been described previously (5, 19–21).

Sections were treated with 3% hydrogen peroxide for 15 min to quench endogenous peroxidase and then washed in TBS. To visualize kisspeptin expression, sections were incubated with the antibody at 1:2000 dilution for 8 h at room temperature. For secondary antibody labeling, sections were incubated with biotinylated goat anti-rabbit (1:100; Cat No: BA-1000, Vector Laboratories, Peterborough, UK) immunoglobulins at room temperature followed by incubation with Vector avidin-peroxidase (1:50; Cat No: PK-4000, Vector Laboratories, Peterborough, UK). Finally, the sections were rinsed and immunoreactivity was revealed with glucose-oxidase and nickel-enhanced diaminobenzidine hydrochloride (12.5 mg/ml). Sections were counterstain with hematoxylin, dehydrated in ethanol followed by Histoclear, and then coverslipped with DPX.

PROGESTERONE RELEASE EXPERIMENTS FROM MA-10 CELLS

The MA-10 cells were seeded at 2.5×10^5 cells/well (24-well plates) 24 h before the hormone treatment. The cells were treated with increasing concentrations of Kp10 (human Metastin 45–54) (1, 10, or 20 μM) (Cat No: M2816, Sigma-Aldrich, Dorset, UK) or Kp10 followed by hCG (0.012 IU/ml as the final concentration) (Cat No: CG5, Sigma, Saint Louis, MO, USA). PBS was added as a negative control. Each condition was tested in triplicate. After 4 h, the media was collected for progesterone measurement. After collection of media, the MA-10 cells were rinsed twice with PBS and lysed in 1× lysis buffer (reporter lysis buffer, Cat No: E397A, Promega, UK) by a freeze-thaw cycle. The lysate was briefly centrifuged and the protein content of the supernatant determined with a Bio-Rad Bradford Assay following the standard protocol.

PRIMARY CULTURE OF TESTES EXPLANTS

The testes from adult wild-type mice were cut into two pieces (approximately 40 mg/piece) without removing the tunica and each piece was cut and flattened to a 1-mm thickness on a Nylon membrane (Cat No: 1417240, Boehringer-Mannheim, Indianapolis, IN, USA) floating in phenol red-free DMEM medium (Cat No: 21063, GIBCO) (300 μl/well for 12-well plate) supplemented with 1× penicillin/streptomycin. The tissues were immediately treated with vehicle, Kp10 (1 μM), hCG (0.6 IU/ml), or a mixture of Kp10 (1 μM) and hCG (0.6 IU/ml), respectively. Each condition was tested in at least four repeat wells. The tissues were cultured at 37°C in an atmosphere of 5% CO_2 and the media collected at different time points. Fresh media was added at each time point after media collection.

HORMONE ASSAYS

Testosterone and progesterone were measured using ELISA kits (Cat No: EIA1559 and EIA1561 from DRG International, USA)

according to the manufacturer's instructions. The testosterone ELISA kit had a sensitivity of 0.083 ng/ml, an inter-assay variation of 6.7%, and an intra-assay variation of 3.3%. The progesterone ELISA kit had a sensitivity of 0.045 ng/ml, intra-assay variation of 7%, and inter-assay variation of 5%.

RESULTS

Gpr54 AND *KISS1* ARE EXPRESSED IN THE MOUSE TESTES

The *Gpr54* and *Kiss1* alleles in the transgenic mice have been tagged with a *LacZ* gene that allows their gene expression patterns to be visualized by staining for β-galactosidase activity. Staining was observed within seminiferous tubules from both *Kiss1*$^{+/-}$ (**Figure 1A**) and *Gpr54*$^{+/-}$ mice (data not shown) but not in wild-type mice (**Figure 1A**). Background staining was observed in the epididymis and the vas deferens of wild-type mice as the epithelial cells in these tissues express an endogenous galactosidase-like enzymatic activity. To define the cells in which *Kiss1* and *Gpr54* are expressed, cryosection of testes were stained for β-galactosidase activity. The staining in cryosections was localized to the region of the seminiferous tubules that contained round spermatids (arrowed in **Figures 1B,C**). The spermatids are easily recognized as they have smaller nuclei than spermatocytes and are four-times more abundant as they have just completed meiosis. Very faint β-galactosidase staining was also found in the Leydig cells in the *Gpr54*$^{+/-}$ mice (**Figure 1D**) but not in the *Kiss1*$^{+/-}$ mice (data not shown).

To confirm that the β-galactosidase expression was localized to spermatid cells, the time point at which expression was first observed during the first spermatogenic cycle was determined. Expression of *Kiss1* and *Gpr54* could not be observed prior to 3 weeks of age but staining was seen from 1 month of age which corresponds to the time at which the spermatids first appear in mice (**Figure 2**).

Kisspeptin protein expression in the mouse testes was visualized using a well characterized rabbit antiserum highly specific for mouse Kp10 (20). Strong immunoreactivity was found in Leydig cells with no staining in spermatids (**Figure 1E**). As a control for antibody specificity, testes sections from *Kiss1* mutant mice lacking kisspeptin protein were used and no immunoreactivity was observed (**Figure 1E**). This Leydig staining may be non-specific however, as no kisspeptin protein was detected in *Gpr54* mutant mice (data not shown).

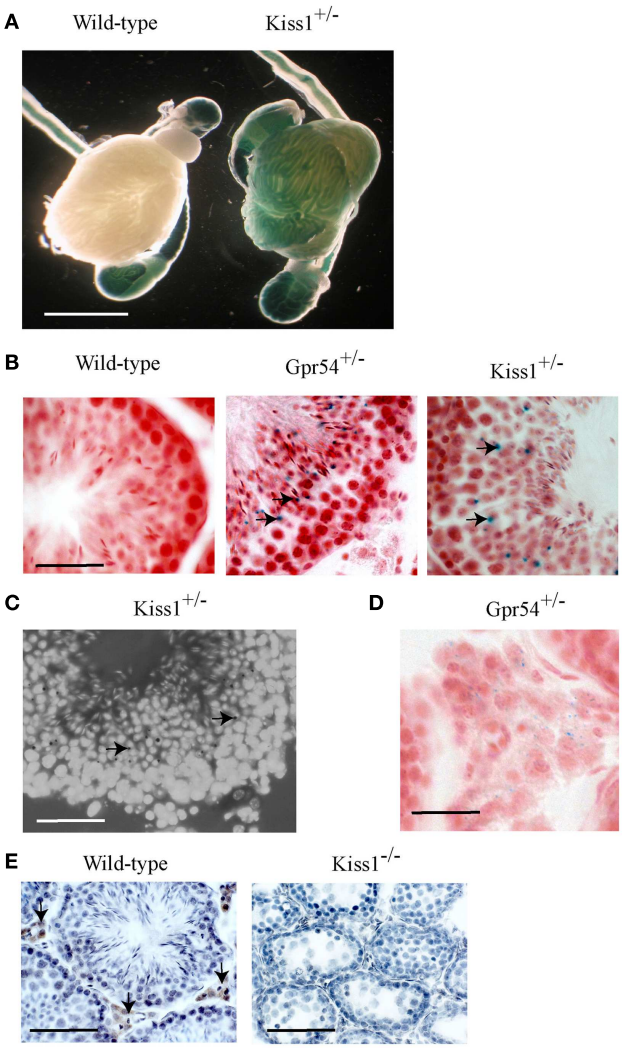

FIGURE 1 | *Kiss1* and *Gpr54* expression in the mouse testes. **(A)** Expression of *Kiss1* in seminiferous tubules of the testes visualized by X-gal staining (blue) for β-galactosidase activity. Note the non-specific staining in the epididymis and vas deferens of the wild-type testes. Scale bar = 5 mm. **(B)** Cryosections of testes from adult wild-type, *Gpr54*$^{+/-}$, and *Kiss1*$^{+/-}$ mice showing expression (arrowed) localized to spermatid cells of seminiferous tubules. Sections were stained for β-galactosidase activity (blue) and counterstained with Saffronin (red). Scale bar = 100 μm. **(C)** Testes cryosection from *Kiss1*$^{+/-}$ mice stained for β-galactosidase activity (black dots, arrowed) and counterstained with DAPI to visualize cell nuclei illustrating clearer expression in spermatid cells. Scale bar = 100 μm. **(D)** Low expression of *Gpr54* in Leydig cells visualized by X-gal staining. Scale bar = 50 μm. **(E)** Kisspeptin immunoreactivity localized to Leydig cell in wild-type mice (arrowed) but not *Kiss1* mutant mice. Scale bar = 200 μm.

Kp10 DOES NOT STIMULATE STEROIDOGENESIS IN THE LEYDIG CELL LINE, MA-10

To test whether Kp10 could stimulate testosterone release, the mouse Leydig cell line, MA-10, was used (18, 22). MA-10 cells, like normal Leydig cells, express LH receptors and respond to hCG stimulation. MA-10 cells have low expression and activity of P450c17 that is the enzyme that converts progesterone into 17-OH progesterone and finally into testosterone, thus MA-10 cells produce progesterone as the principle steroid hormone instead of testosterone (18, 22).

The MA-10 cells were examined for expression of *Kiss1* and *Gpr54* to determine whether they might be capable of responding to kisspeptins. There was a PCR product for *Gpr54* (**Figure 3A**), indicating that MA-10 cells endogenously express this gene but there was no detectable *Kiss1* expression in the MA-10 cells. The MA-10 cell also expressed GnRH and β-actin transcripts (**Figure 3A**). No products were observed when non-transcribed RNA was used as the template, indicating that the RNA was free of genomic DNA contamination. A *Kiss1* product was observed when hypothalamic cDNA was used from wild-type mice as a positive control.

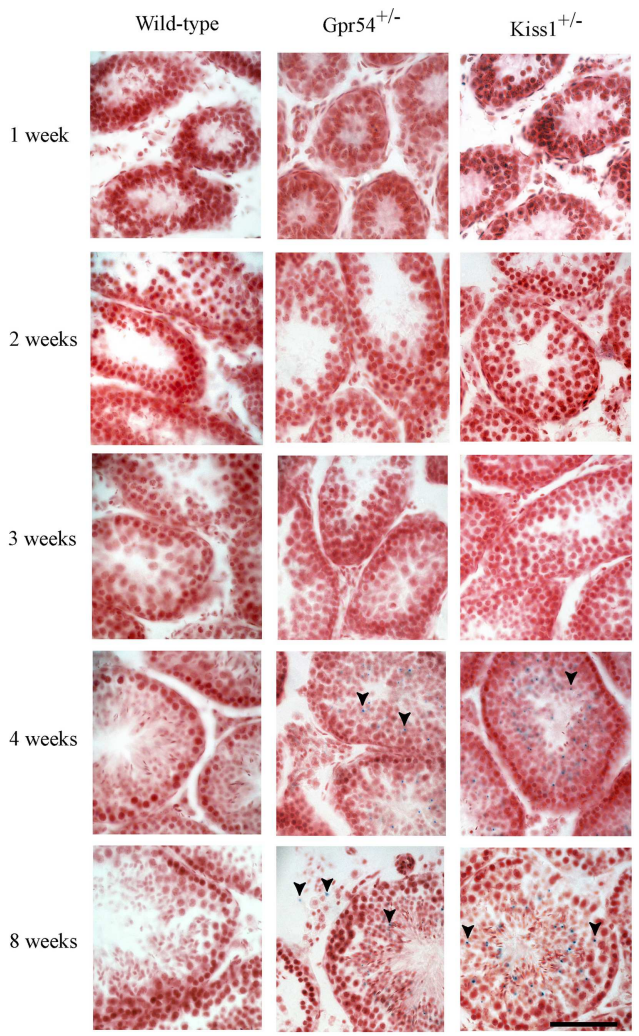

FIGURE 2 | Developmental time course of *Gpr54* and *Kiss1* expression in the mouse testes. Testes of wild-type (WT), *Kiss1*$^{+/-}$ and *Gpr54*$^{+/-}$ mice at different ages were cryosectioned, stained for β-galactosidase activity, and counterstained with Saffronin. Blue dots (arrowed) indicate expression of the *Kiss1* and *Gpr54* genes. All photographs are at the same magnification. Scale bar = 50 μm.

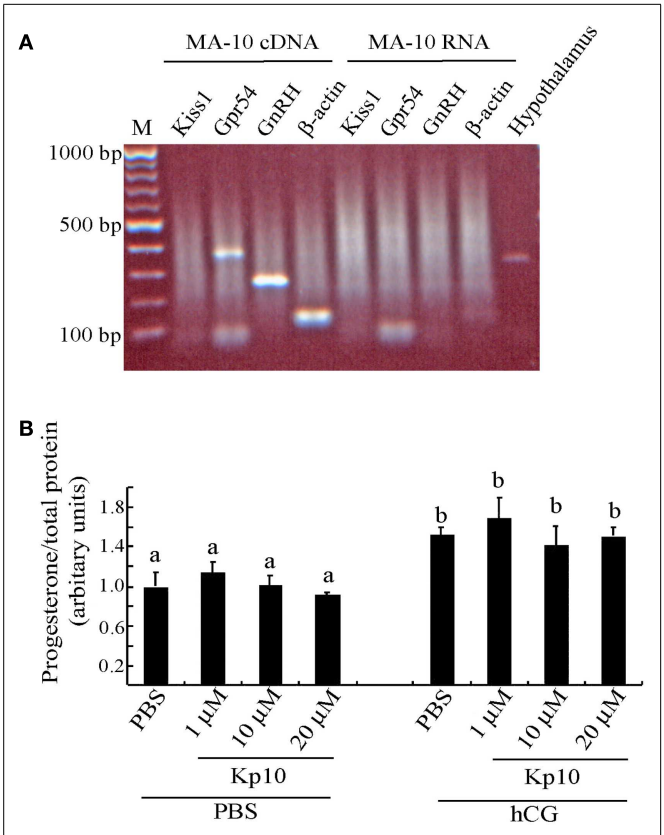

FIGURE 3 | The MA-10 Leydig cell line expresses *Gpr54* but do not respond to Kp10 treatment. **(A)** RT-PCR gene expression analysis in the MA-10 Leydig cell line. Expression of *Gpr54, GnRH*, and β-*actin* was detected in cDNA from the MA-10 cells but *Kiss1* expression was not detected. Hypothalamic cDNA was included as a positive control for *Kiss1* expression. **(B)** Effect of Kp10 on stimulating progesterone release from the MA-10 cell line. The MA-10 cells were stimulated with the hormones indicated and media collected after 4 h and tested for progesterone. Different letters (a and b) indicate statistically significant differences between groups ($P < 0.05$, one way ANOVA with a Tukey comparison post-test).

As the MA-10 cells expressed the kisspeptin receptor, they were tested to see whether Kp10 could stimulate progesterone release. The cells were divided into two experimental groups. The first group was treated with increasing concentrations of human Kp10 followed by PBS, and the second group was treated with Kp10 followed by hCG to examine possible synergistic effects. After 4 h, the media was assayed for progesterone, which was normalized to the protein content of the cell lysate to correct for variations in cell number. No significant difference in progesterone release was found between the vehicle (PBS) treatment and any of the three concentrations of Kp10 (**Figure 3B**), indicating that Kp10 cannot enhance progesterone release from the Leydig cell line even at a high concentration (20 μM). There was also no significant difference in progesterone release between the cells treated with hCG alone or those treated with hCG and Kp10 (**Figure 3B**), which suggests that Kp10 has no synergistic effect on progesterone release from MA-10 cells activated by hCG. However, there was a significant difference ($P < 0.05$) in progesterone release between the cells treated with PBS and those treated with hCG (**Figure 3B**), which indicates the functional responsiveness of the cells to hormonal stimulation.

Kp10 DOES NOT STIMULATE TESTOSTERONE RELEASE FROM TESTES TISSUE CULTURE EXPLANTS

To examine the possible action of Kp10 in a more physiological system, we tested whether Kp10 could stimulate testosterone release from primary explants of mouse testes. Pieces of adult wild-type mouse testes of similar weight (approximately 40 mg/piece) were treated with vehicle (PBS), Kp10 (1 μM), hCG (0.6 IU/ml), or a mixture of Kp10/hCG. Each condition was repeated with at least four samples. The media was collected at different time intervals for testosterone measurements. During the 0- to 4-h

time period, the testosterone released after hCG treatment was significantly higher than that with the vehicle (PBS) treatment (**Figure 4A**), indicating that the cultured testes maintained the ability to respond to hormone stimulation. However, there was no obvious stimulation of testosterone release after Kp10 treatment. Also, the testosterone released in the PBS and hCG groups was not significantly different at incubation times >4 h due to increased unstimulated testosterone release (**Figure 4A**). Therefore, a 4-h incubation time was used to test whether there was any synergy between Kp10 and hCG in stimulating testosterone release (**Figure 4B**). Once again, testosterone release after hCG treatment was significantly higher than after PBS treatment (**Figure 4B**). No difference in testosterone release was detected between the testes fragments cultured in PBS or Kp10. There was also no difference in testosterone release between testes treated with hCG only and testes treated with hCG and Kp10 together. These data indicate that Kp10 has no effect on testosterone release from adult mouse testes and it has no synergistic action on testosterone release stimulated by hCG.

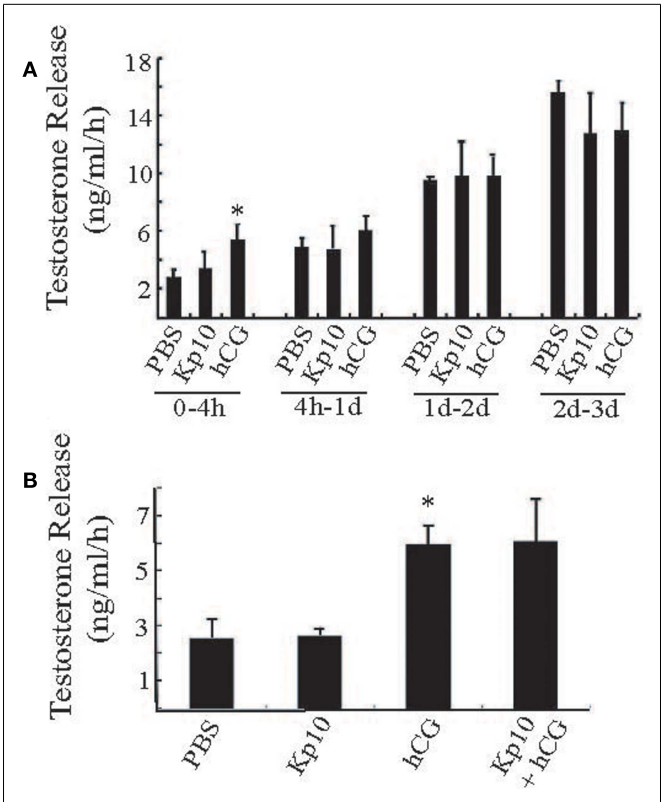

FIGURE 4 | Testing Kp10 stimulation of testosterone release from fresh testes explants. **(A)** Time course of testosterone release from explanted mouse testes tissue. Pieces of adult mouse testes were cultured in PBS, Kp10 (1 μM), or hCG (0.6 IU/ml), respectively. The media was collected at the time points indicated, and testosterone levels measured by an ELISA. **(B)** Evaluation of synergistic action of Kp10 on hCG-mediated testosterone release. Pieces of adult mouse testes were cultured in PBS, Kp10 (1 μM), hCG (0.6 IU/ml), or a mixture of Kp10 (1 μM) and hCG (0.6 IU/ml). The media was collected after 4 h incubation and testosterone levels measured by an ELISA. $n \geq 4$ for all columns. *Statistically significant difference ($P < 0.05$) between the hCG and the vehicle control group (PBS) (unpaired *t*-test with Welch's correction).

DISCUSSION

Kisspeptin neuropeptides are important central regulators of the mammalian reproductive axis with kisspeptin neurons acting upstream of GnRH neurons to stimulate GnRH release. In addition to this central role however, the expression profiles of *Kiss1* and *Gpr54* suggest that they may also have a function in peripheral tissues including the testes. We have shown using expression of a gene targeted *LacZ* reporter gene, that *Kiss1* and *Gpr54* are expressed by round spermatid cells in the mouse testes. Expression profiling during postnatal gonadal maturation confirmed this as the expression only started to emerge after 1 month of age, which is the time when the spermatids first appear. As both *Kiss1* and *Gpr54* were found to be expressed in spermatids, this raises the possibility that autocrine or paracrine kisspeptin signaling might be involved in spermiogenesis.

Round spermatid cells have just completed meiosis and will subsequently undergo the structural changes required to produce spermatozoa. During this structural remodeling, most of the cytoplasm is removed from the spermatids by the Sertoli cells, which will result in loss of β-galactosidase activity, which might explain why we do not observe staining in elongating spermatids and spermatozoa. Similarly, this cytoplasmic removal would remove any kisspeptin protein but GPR54 should be retained by virtue of its location in the plasma membrane. Indeed, GPR54 has been detected in the head region of human sperm and addition of kisspeptin can produce a modest rise in $[Ca^{2+}]_i$ and sperm motility (14).

The functional significance of *Kiss1* and *Gpr54* expression in spermatids and sperm is still not clear however. The infertility of the *Kiss1* and *Gpr54* mutant mice prevents performing functional tests with mutant sperm. It might be possible to initiate spermatogenesis in the mutant mice with pulsatile FSH and subcutaneous testosterone delivery. Although we have shown that *Kiss1* and *Gpr54* mutant mice can initiate a low level of spermatogenesis when given a chow diet containing phytoestrogens (23), the number of sperm that can be isolated from the vas deferens and epididymis is too small for functional studies. It is noteworthy, however, that several male patients with mutations in *GPR54* and hypogonadotropic hypogonadism have responded to exogenous hormone treatment and achieved fertility [for review, see Ref. (24)] suggesting that in humans, GPR54 function is not essential for sperm function.

There is an important caveat to this expression data however. Although the *LacZ* expression indicates that the *Kiss1* promoter is transcriptionally active in round spermatid cells, we could not detect kisspeptin immunoreactivity using a validated antibody capable of visualizing kisspeptin in the hypothalamus of mice (25). It is possible that the expression level of the kisspeptin protein is below the limits of detection and that X-gal staining for β-galactosidase activity is more sensitive. Alternatively, it is possible that *Kiss1* transcripts are not translated into protein in spermatid cells. Several gene transcripts encoding proteins required for late spermiogenesis are expressed in round spermatids and translationally repressed until the elongating spermatid stage (26). Translationally repressed mRNAs have unusually long poly(A) tails

of approximately 180 nt and translation is associated with shortening of these tails (27). The presence of long poly(A) tails on *Kiss1* transcripts, which are not subsequently shortened, might provide a mechanism for the proposed translational repression in spermatids.

We detected kisspeptin immunoreactivity in Leydig cells of wild-type mice similar to that reported by Anjum and colleagues (13). The specificity of this immunoreactivity was suggested by absence of staining in *Kiss1* mutant mice, which do not produce any kisspeptin protein (5). This notwithstanding, we believe that the kisspeptin immunoreactivity found in the Leydig cells may not be authentic for the following reasons. Firstly, we did not detect *Kiss1* promoter activity in Leydig cell by β-galactosidase staining in *Kiss1*$^{+/-}$ mice. Secondly, we did not detect *Kiss1* transcripts by RT-PCR in the immortalized Leydig cell line MA-10 although this may be a consequence of the cell immortalization process and the tendency for *Kiss1* expression to be suppressed during cell transformation and tumorigenesis. Finally, we failed to detect kisspeptin immunoreactivity in the Leydig cells of *Gpr54* mutant mice, which can produce kisspeptin protein. We believe that the staining pattern observed in the Leydig cells of the wild-type mice is an artifact perhaps associated the high levels of steroidogenesis in these cells, which does not occur in *Kiss1* or *Gpr54* mutant mice.

We also observed a very low level of β-galactosidase staining in Leydig cells from *Gpr54*$^{+/-}$ mice suggesting that these cells might express GPR54 protein. This was consistent with our detection of *Gpr54* transcripts in the immortalized mouse Leydig cell line MA-10. Unfortunately, there are no anti-GPR54 antibodies with sufficient specificity to confirm expression of the endogenous GPR54 protein in the Leydig cells.

If there was co-expression of GPR54 and kisspeptin in Leydig cells, this would allow local autocrine or paracrine action within the testes. Previously published work has suggested that kisspeptins are able to enhance testosterone release after LH stimulation (17). We therefore examined whether Kp10 was able to stimulate testosterone release from the MA-10 cell line as well as testes fragments in culture. We found no evidence that Kp10 could directly stimulate testosterone release or enhance the actions of LH. This is in contrast to the recent data that kisspeptin administration significantly increased hCG-stimulated testosterone levels in acyline treated Rhesus monkeys compared to the responses with hCG treatment alone (17). As the acyline inhibits endogenous LH secretion from the pituitary, these responses suggest a direct, synergistic action of kisspeptin on the testes. The reason for the difference from our data is not known, but apart from a species difference, it might be that the enhancement by kisspeptin requires a sub-threshold level of LH stimulation and the concentration of hCG that we used was too high. It would be informative to test Kp10 responses to a lower range of hCG treatments in the testes explants. It is noteworthy, however, that Huma and colleagues have found that an intravenous injection of the kisspeptin antagonist p234 does not alter plasma testosterone levels in adult Rhesus macaques (28) suggesting that any action of kisspeptin on the testes is small. This conclusion is consistent with the observation that fertility can be restored in *Gpr54* mutant mice by expression of a *Gpr54* transgene in GnRH neurons (29) indicating that GPR54 expression in the testes is also not essential for fertility in mice.

In summary, we have shown that the *Kiss1* and *Gpr54* are both expressed in round spermatid cells of the mouse testes and *Gpr54* is expressed by Leydig cells but we have not found any supporting data that kisspeptin signaling in the testes has a major role in spermatogenesis or testosterone secretion in the mouse.

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REFERENCES

1. Oakley AE, Clifton DK, Steiner RA. Kisspeptin signaling in the brain. *Endocr Rev* (2009) **30**:713–43. doi:10.1210/er.2009-0005
2. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS Jr, Shagoury JK, et al. The GPR54 gene as a regulator of puberty. *N Engl J Med* (2003) **349**:1614–27. doi:10.1056/NEJMoa035322
3. Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, et al. The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. *Biochem Biophys Res Commun* (2003) **312**:1357–63. doi:10.1016/j.bbrc.2003.11.066
4. Lapatto R, Pallais JC, Zhang D, Chan YM, Mahan A, Cerrato F, et al. Kiss1/mice exhibit more variable hypogonadism than gpr54/mice. *Endocrinology* (2007) **148**:4927–36. doi:10.1210/en.2007-0078
5. d'Anglemont de Tassigny X, Fagg LA, Dixon JP, Day K, Leitch HG, Hendrick AG, et al. Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene. *Proc Natl Acad Sci U S A* (2007) **104**:10714–9. doi:10.1073/pnas.0704114104
6. Dungan HM, Gottsch ML, Zeng H, Gragerov A, Bergmann JE, Vassilatis DK, et al. The role of kisspeptin-GPR54 signaling in the tonic regulation and surge release of gonadotropin-releasing hormone/luteinizing hormone. *J Neurosci* (2007) **27**:12088–95. doi:10.1523/JNEUROSCI.2748-07.2007
7. Topaloglu AK, Tello JA, Kotan LD, Ozbek MN, Yilmaz MB, Erdogan S, et al. Inactivating KISS1 mutation and hypogonadotropic hypogonadism. *N Engl J Med* (2012) **366**:629–35. doi:10.1056/NEJMoa1111184
8. Teles MG, Bianco SD, Brito VN, Trarbach EB, Kuohung W, Xu S, et al. A GPR54-activating mutation in a patient with central precocious puberty. *N Engl J Med* (2008) **358**:709–15. doi:10.1056/NEJMoa073443
9. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, et al. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J Biol Chem* (2001) **276**:34631–6. doi:10.1074/jbc.M104847200
10. Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, et al. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* (2001) **411**:613–7. doi:10.1038/35079135
11. Terao Y, Kumano S, Takatsu Y, Hattori M, Nishimura A, Ohtaki T, et al. Expression of KiSS-1, a metastasis suppressor gene, in trophoblast giant cells of the rat placenta. *Biochim Biophys Acta* (2004) **1678**:102–10. doi:10.1016/j.bbaexp.2004.02.005
12. Chianese R, Ciaramella V, Fasano S, Pierantoni R, Meccariello R. Kisspeptin receptor, GPR54, as a candidate for the regulation of testicular activity in the frog *Rana esculenta*. *Biol Reprod* (2013) **88**:73. doi:10.1095/biolreprod.112.103515
13. Anjum S, Krishna A, Sridaran R, Tsutsui K. Localization of gonadotropin-releasing hormone (GnRH), gonadotropin-inhibitory hormone (GnIH), kisspeptin and GnRH receptor and their possible roles in testicular activities from birth to senescence in mice. *J Exp Zool A Ecol Genet Physiol* (2012) **317**:630–44. doi:10.1002/jez.1765
14. Pinto FM, Cejudo-Roman A, Ravina CG, Fernandez-Sanchez M, Martin-Lozano D, Illanes M, et al. Characterization of the kisspeptin system in human spermatozoa. *Int J Androl* (2012) **35**:63–73. doi:10.1111/j.1365-2605.2011.01177.x
15. Thompson EL, Murphy KG, Patterson M, Bewick GA, Stamp GW, Curtis AE, et al. Chronic subcutaneous administration of kisspeptin-54 causes

testicular degeneration in adult male rats. *Am J Physiol Endocrinol Metab* (2006) **291**:E1074–82. doi:10.1152/ajpendo.00040.2006
16. Ramaswamy S, Seminara SB, Pohl CR, DiPietro MJ, Crowley WF Jr, Plant TM. Effect of continuous intravenous administration of human metastin 45-54 on the neuroendocrine activity of the hypothalamic-pituitary-testicular axis in the adult male rhesus monkey (*Macaca mulatta*). *Endocrinology* (2007) **148**:3364–70. doi:10.1210/en.2007-0207
17. Irfan S, Ehmcke J, Wahab F, Shahab M, Schlatt S. Intratesticular action of kisspeptin in rhesus monkey (*Macaca mulatta*). *Andrologia* (2013). doi:10.1111/and.12121
18. Ascoli M. Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses. *Endocrinology* (1981) **108**:88–95. doi:10.1210/endo-108-1-88
19. Desroziers E, Mikkelsen J, Simonneaux V, Keller M, Tillet Y, Caraty A, et al. Mapping of kisspeptin fibres in the brain of the pro-oestrous rat. *J Neuroendocrinol* (2010) **22**:1101–12. doi:10.1111/j.1365-2826.2010.02053.x
20. Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A. Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. *Neurosci Lett* (2006) **401**:225–30. doi:10.1016/j.neulet.2006.03.039
21. Clarkson J, d'Anglemont de Tassigny X, Colledge WH, Caraty A, Herbison AE. Distribution of kisspeptin neurones in the adult female mouse brain. *J Neuroendocrinol* (2009) **21**:673–82. doi:10.1111/j.1365-2826.2009.01892.x
22. Rommerts FF, King SR, Span PN. Implications of progesterone metabolism in MA-10 cells for accurate measurement of the rate of steroidogenesis. *Endocrinology* (2001) **142**:5236–42. doi:10.1210/en.142.12.5236
23. Mei H, Walters C, Carter R, Colledge WH. Gpr54-/- mice show more pronounced defects in spermatogenesis than Kiss1-/- mice and improved spermatogenesis with age when exposed to dietary phytoestrogens. *Reproduction* (2011) **141**:357–66. doi:10.1530/REP-10-0432
24. Wahab F, Quinton R, Seminara SB. The kisspeptin signaling pathway and its role in human isolated GnRH deficiency. *Mol Cell Endocrinol* (2011) **346**:29–36. doi:10.1016/j.mce.2011.05.043
25. Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE. Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *J Neurosci* (2008) **28**:8691–7. doi:10.1523/JNEUROSCI.1775-08.2008
26. Kleene KC. Patterns, mechanisms, and functions of translation regulation in mammalian spermatogenic cells. *Cytogenet Genome Res* (2003) **103**:217–24. doi:10.1159/000076807
27. Kleene KC. Poly(A) shortening accompanies the activation of translation of five mRNAs during spermiogenesis in the mouse. *Development* (1989) **106**:367–73.
28. Huma T, Ullah F, Hanif F, Rizak JD, Shahab M. Peripheral administration of kisspeptin antagonist does not alter basal plasma testosterone but decreases plasma adiponectin levels in adult male rhesus macaques. *Eur J Sci Res* (2013) **109**:668–77.
29. Kirilov M, Clarkson J, Liu X, Roa J, Campos P, Porteous R, et al. Dependence of fertility on kisspeptin-Gpr54 signaling at the GnRH neuron. *Nat Commun* (2013) **4**:2492. doi:10.1038/ncomms3492

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Hypothalamic-pituitary-gonadal endocrine system in the hagfish

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The hypothalamic-pituitary system is considered to be a seminal event that emerged prior to or during the differentiation of the ancestral agnathans (jawless vertebrates). Hagfishes as one of the only two extant members of the class of agnathans are considered the most primitive vertebrates known, living or extinct. Accordingly, studies on their reproduction are important for understanding the evolution and phylogenetic aspects of the vertebrate reproductive endocrine system. In gnathostomes (jawed vertebrates), the hormones of the hypothalamus and pituitary have been extensively studied and shown to have well-defined roles in the control of reproduction. In hagfish, it was thought that they did not have the same neuroendocrine control of reproduction as gnathostomes, since it was not clear whether the hagfish pituitary gland contained tropic hormones of any kind. This review highlights the recent findings of the hypothalamic-pituitary-gonadal endocrine system in the hagfish. In contrast to gnathostomes that have two gonadotropins (GTH: luteinizing hormone and follicle-stimulating hormone), only one pituitary GTH has been identified in the hagfish. Immunohistochemical and functional studies confirmed that this hagfish GTH was significantly correlated with the developmental stages of the gonads and showed the presence of a steroid (estradiol) feedback system at the hypothalamic-pituitary levels. Moreover, while the identity of hypothalamic gonadotropin-releasing hormone (GnRH) has not been determined, immunoreactive (ir) GnRH has been shown in the hagfish brain including seasonal changes of ir-GnRH corresponding to gonadal reproductive stages. In addition, a hagfish PQRFamide peptide was identified and shown to stimulate the expression of hagfish GTHβ mRNA in the hagfish pituitary. These findings provide evidence that there are neuroendocrine-pituitary hormones that share common structure and functional features compared to later evolved vertebrates.

Keywords: hagfish, agnathan, cyclostomes, HPG axis, pituitary gland, gonadotropin, GnRH, estradiol

INTRODUCTION

Reproduction in gnathostomes (jawed vertebrates) is controlled by a hierarchically organized endocrine system called the hypothalamic-pituitary-gonadal (HPG) axis (1). In spite of the diverged patterns of reproductive strategies and behaviors within this taxon, this endocrine network is remarkably conserved throughout gnathostomes. In response to hypothalamic gonadotropin-releasing hormone (GnRH), gonadotropins (GTHs) are secreted from the pituitary and stimulate the gonads, where they induce the synthesis and release of sex steroid hormones, which in turn elicit growth and maturation of the gonads (**Figure 1**).

The pituitary gland is present in all vertebrates from agnathans (jawless fishes) to mammals and consists of the same two principal elements, the neurohypophysis and adenohypophysis. The neurohypophysis develops from the floor of the diencephalon as an infundibular extension, whereas the adenohypophysis develops from the epithelium that comes in contact with this infundibulum. The enigma of the pituitary gland is that evolution of a composite organ with such a complex double developmental origin must have been associated with some functionally adaptive value. Yet demonstration of this adaptive value in the agnathans themselves remains elusive. Most surprising facts are that not only the pituitary gland but also all major adenohypophysial hormones such as GTHs, growth hormone (GH), prolactin, and adrenocorticotropin (ACTH) and their receptors are also considered to be vertebrate novelties (2). Thus, the hypothalamic-pituitary system, which is specific to vertebrates, is considered to be a seminal event that emerged prior to or during the differentiation of the ancestral agnathans. Such an evolutionary innovation is one of the key elements leading to physiological divergence, including reproduction, growth, metabolism, stress, and osmoregulation in subsequent evolution of gnathostomes.

Lampreys and hagfish are the only two extant representatives of agnathans. Paleontological analysis of extinct agnathans had suggested that lampreys were more closely related to gnathostomes than either group is to the hagfishes (3, 4). However, both recent molecular phylogenetic analyses (5–7) and developmental study on the craniofacial pattern of the hagfish (8) strongly support the monophyly of the cyclostomes (lampreys and hagfishes as closest relatives). Therefore, studies on reproduction of the cyclostomes are important for understanding the evolution of the HPG axis

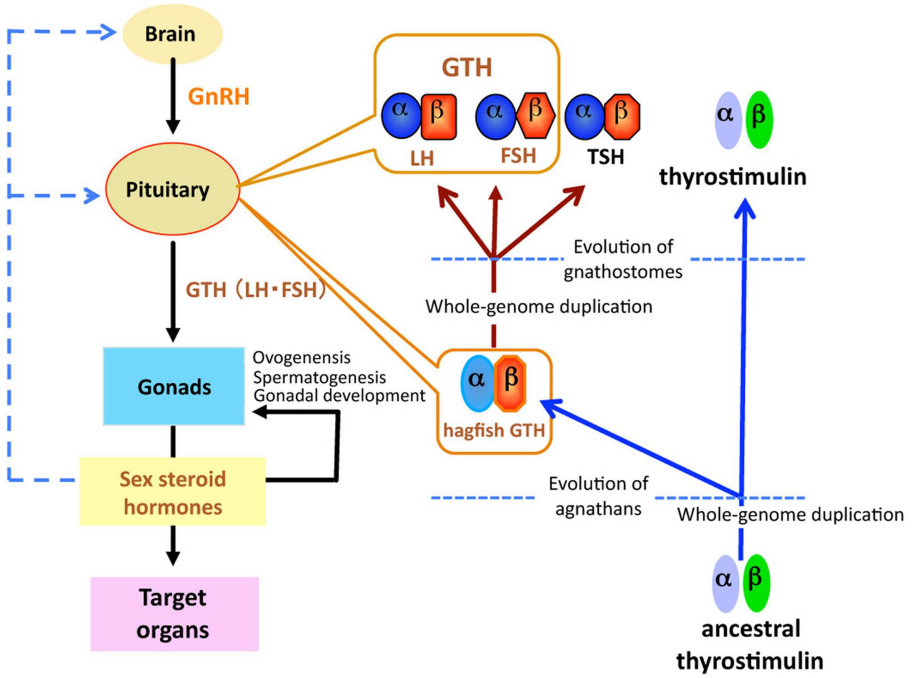

FIGURE 1 | Schematic diagram of the evolution of glycoprotein hormones in the hypothalamic-pituitary-gonadal axis. Ancestral thyrostimulin (α and β) existed before divergence of vertebrates. An ancestral thyrostimulin (α and β) diverged into GTH (α and β) and thyrostimulin (α and β) during the early phase of agnathan divergence. The GTH (α and β) formed a heterodimer in the pituitary and acted as the first adenohypophysial gonadotropic hormone during the evolution of agnathan species. This GTH dimer further diverged into three functional units of adenohypophysis, LH and FSH as two gonadotropins, and TSH as a thyrotropin, in the lineage to gnathostomes.

related to vertebrate reproduction. Findings from many molecular, biochemical, physiological, and morphological studies indicate that the HPG axis is present in the lamprey [for review, see Ref. (1)]. In contrast, endocrine regulation of reproduction in the hagfish is poorly understood [for reviews, see Ref. (9, 10)]. For example, until the recent identification of functional GTH in the brown hagfish, *Paramyxine atami* (11), it was not established whether the hagfish pituitary gland contains tropic hormones of any kind. Herein, this report summarizes the recent findings of the HPG endocrine system involved in reproduction in hagfish.

HAGFISH PITUITARY GLAND

The hagfish is considered the most primitive vertebrate known, living or extinct (3) (**Figure 2**). In addition to their primitive external body features, hagfish possess the most primitive hypothalamic-pituitary system among the vertebrates (12). The neurohypophysis is a flattened sac-like structure, whereas the adenohypophysis consists of a mass of clusters of cells embedded in connective tissue below the neurohypophysis (12, 13) (**Figures 3A,B**). The adenohypophysis and the neurohypophysis are completely separated by a layer of connective tissue, and there is no or little anatomical relationship between them (14, 15) (**Figure 3B**). In addition, there is no clear cytological differentiation between the pars distalis and the pars intermedia (12, 13) (**Figure 3B**). The question arises whether the simplicity of the hagfish pituitary gland is a primitive or a degenerate feature. For example, some authors have claimed that the pars intermedia seems to have been lost via a secondary degenerative process (13, 16). Moreover, until recent identification of a functional GTH in the hagfish pituitary (11), it had not been established whether the hagfish pituitary gland contained adenohypophysial hormones of any kind (9). Because of the simplicity and primitiveness of the pituitary morphology and equivocal data on the adenohypophysial hormones in the hagfish, many researchers had questioned whether there were any functional adenohypophysial hormones (9, 17). On the other hand, arginine vasotocin (AVT), as a single neurohypophysial hormone, was identified in the hagfish (18). In addition, the presence of GnRH has been suggested in the hagfish hypothalamus by both radioimmunoassay (RIA) and immunohistochemistry (19–22) (**Figure 4**). Thus, the hagfish appears to have neurohypophysial and hypothalamic hormones similar to those of other vertebrates.

At present, the adenohypophysis of the hagfish is the least understood of all the vertebrates. However, our immunohistochemical studies provided the first clear-cut evidence for the presence of GTH and ACTH in the hagfish (23–25). Although not conclusive, our data also suggested the presence of GH in the hagfish (23). In addition, these three adenohypophysial hormones were suggested to be the ancestral adenohypophysial hormones that have maintained their original functions throughout vertebrate evolution. On the other hand, the later derived hormones, such as prolactin and thyroid-stimulating hormone (TSH), may have contributed to the expansion of vertebrates into new environments, as suggested by Kawauchi et al. (26) and Kawauchi and Sower (27). Moreover, our study further revealed that GTH

cells, ACTH cells, and unidentified cells which were assumed to include both undifferentiated cells and GH cells, were packed together in the same cell cluster of the hagfish adenohypophysis, and thus each cluster appeared to serve as a separate functional unit (10, 24) (**Figures 3C–E**). If the absence of the pars intermedia is the most ancestral vertebrate pituitary gland, melanophore-stimulating hormone (MSH) activity seems to be gained secondarily together with the differentiation of the pars intermedia. Further studies are needed to clarify this possibility.

FIGURE 2 | Brown hagfish, *Paramyxine atami*.

GLYCOPROTEIN HORMONE FAMILY

Gonadotropins, in response to hypothalamic GnRH, are released from the pituitary and act on the gonads to regulate steroidogenesis and gametogenesis. Two GTHs, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), together with TSH form a family of pituitary hormones (**Figure 1**). They are heterodimeric glycoproteins consisting of two subunits, an α-subunit and a unique β-subunit. These glycoprotein hormones (GPH) are believed to have evolved from a common ancestral molecule through duplication of β-subunit genes and subsequent divergence (27, 28). Two GTHs have been identified in all taxonomic groups of gnathostomes, including actinopterygians (29, 30), sarcopterygians (31), and chondrichthyans (32), but not in agnathans.

A single β-subunit of GTH was identified from the sea lamprey pituitary gland after extensive and exhaustive research for over 20 years (27, 33). However, the α-subunit of lamprey GTH is not found even in the lamprey genome (34). This is very strange fact, since a huge amount of physiological and morphological evidence has suggested the presence of GTH in the lamprey (33, 35–38). The lack of α-subunit of lamprey GTH makes difficulty to study the HPG axis in relation to GTH functions in the lamprey. The second form of β-subunit of pituitary GPHs as a candidate for TSHβ is not found in the lamprey genome (34), and thus the lamprey does not have TSH.

Recently, a fourth heterodimeric GPH has been discovered in the human genome and termed "thyrostimulin" due to its thyroid-stimulating activity (39). The thyrostimulin α-subunit, called glycoprotein α-subunit 2 (GPA2), is homologous but not identical to the common α-subunit (GPHα or GPA1). With the

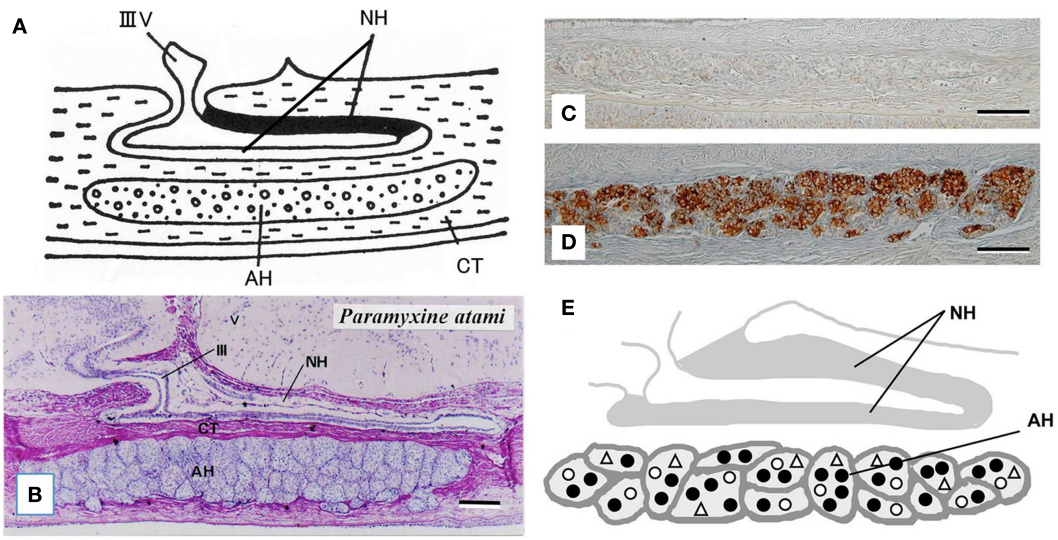

FIGURE 3 | (A) Diagrammatically sagittal section of the hagfish pituitary gland. Dark area of the neurohypophysis (NH) shows posterior part of the dorsal wall, where ir-GnRH nerve fibers and AVT nerve fibers are densely accumulated [for AVT, see Ref. (82)]. **(B)** Nearly mid-sagittal section of the pituitary gland of the brown hagfish, stained with hematoxylin and eosin. **(C,D)** GTHβ-like immunoreaction in the adenohypophysis of the juvenile **(C)** and sexually mature **(D)** brown hagfish stained with anti-hagfish GTHβ. Note that GTH-positive cells are almost absent in **(C)**, whereas they are abundant in **(D)**. **(E)**, Diagrammatically sagittal section of the hagfish pituitary gland showing the topographic distribution of adenohypophysial cells. Closed circle, GTH cell; open circle, ACTH cell; open triangle, undifferentiated cell and possible GH cell. AH, adenohypophysis; CT, connective tissue; IIIV, third ventricle. Scale bars: 100 μm.

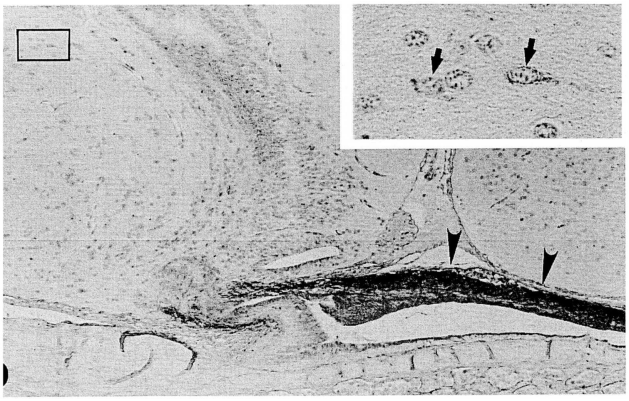

FIGURE 4 | A nearly mid-sagittal section through the neurohypophysis of the Atlantic hagfish, *Myxine glutinosa*, showing the accumulation of ir-GnRH in the dorsal wall of the neurohypophysis (arrowheads). This section was stained with anti-salmon GnRH. Inset, an enlargement of the rectangular area showing GnRH-positive neuronal cells. Arrows show GnRH-positive cell bodies. Scale bars: 100 μm; inset, 20 μm. From Oshima et al. (21).

discovery of GPA2 and glycoprotein β-subunit 5 (GPB5, thyrostimulin beta) homologs not only in other vertebrates but invertebrates including fly, nematode, and sea urchin (40, 41), it is proposed that ancestral glycoprotein existed before the divergence of vertebrates/invertebrates, and that later gene duplication events in vertebrates produced the thyrostimulin (GPA2 and GPB5) and GTH/TSH [GPHα and GPHβ (LHβ/FSHβ/TSHβ)] (40) (**Figure 1**). The basal lineage of chordates such as tunicates and amphioxus contains GPA2 and GPA5 in their genome but not GPHα or GPHβ (2, 42–45). Lamprey also has GPA2 and GPB5 genes in addition to the canonical GTHβ (1, 33, 34, 42). At present, no information is available as to the presence of GPA2/GPB5 in the hagfish.

IDENTIFICATION OF HAGFISH GTH

A single GPH, which comprises α- and β-subunits, was recently identified in the pituitary of the brown hagfish, *P. atami*, one of the Pacific hagfish (11) (**Figure 2**). Both subunits of GPH are produced in the same cells of the adenohypophysis, providing definitive evidence for the presence of a heterodimeric GPH in the hagfish. GPH increases at both the gene and protein levels corresponding to the reproductive stages of the hagfish (**Figures 3C,D**). Moreover, purified native GPH induces sex steroid release (estradiol-17β and testosterone) from cultured testis in a dose-dependent manner. From the phylogenetic analysis, the hagfish GPHα forms a clade with the gnathostome GPHαs. The hagfish GPHβ forms a clade with the TSHβs, however the bootstrap values are low and hagfishes evolved prior to the gnathostomes. The sea lamprey GTHβ also groups with the GPHβs but appears to be one of the outgroups of the LHβs. These results clearly show that the GPH identified in the hagfish acts as a functional gonadotropin, and hereafter it is referred as to GTH. Hagfish GTH is the earliest evolved pituitary GPH that has been identified in a basal vertebrate leading to the gnathostome GTH and TSH lineages.

FEEDBACK REGULATION OF HAGFISH GTH SYNTHESIS AND SECRETION

Gonadal steroid hormones and hypothalamic hormones play major roles in controlling the synthesis and release of LH and FSH in gnathostomes. Both positive and negative feedback effects of gonadal steroids have been demonstrated in teleosts, depending on modes of administration and reproductive stages of animals. In general, in sexually mature fish, sex steroids are considered to regulate gonadal maturation and recrudesce, whereas in juvenile fish, sex steroids are considered to regulate puberty. Thus, negative feedback effects of estradiol and testosterone are evident during the latter stages of gonadal development; specifically, it has been shown that gonadal removal increases LH secretion in salmon (46), goldfish (47), and African catfish (48). The observed increases in LH levels can be suppressed by treatment with estradiol, testosterone, or both. FSH is also controlled by steroid-dependent negative feedback loops in rainbow trout (49), salmon (50), and goldfish (51). The negative feedback effects of steroids may be mediated primarily at the levels of the hypothalamic GnRH neurons (52–54), because both *in vivo* and *in vitro* studies have shown that the expression of LHβ mRNA or FSHβ mRNA is often unchanged or increases following exposure to estradiol, testosterone, or both (49, 53, 55). However, in sexually immature teleosts, sex steroids appear to exert primarily a positive feedback effect that acts directly at the level of the pituitary and via effects on the GnRH system (55, 56). LH content and LH mRNA levels of the pituitary in juvenile fish increase in response to estrogens and aromatizable androgens (49, 57).

Estradiol treatment in the juvenile brown hagfish resulted in the marked accumulation of both immunoreactive (ir)-GTHα and ir-GTHβ in the pituitary (58). However, mRNA levels of GTHα and GTHβ in the pituitary were not, or only transiently, increased by the estradiol treatment (58). The latter results suggest that syntheses of both α- and β-subunits of GTH were not, or only transiently, affected by the estradiol treatment. Accordingly, the marked accumulation of both ir-GTH subunits could be attributed to the suppression of GTH secretion from the pituitary. From that study, the feedback effects of estradiol appeared to be inhibitory rather than stimulatory, and mediated by the possible suppression of the secretion of GTH from the pituitary in these juvenile hagfish. These conditions in juvenile hagfish resembled to those in adults, but not in juveniles, of teleosts (49, 53, 55). Such suppression of GTH secretion in the hagfish is probably regulated by the hypothalamic factors including GnRH, as mentioned below.

On the other hand, testosterone treatment in the juvenile brown hagfish had no effect on the staining intensities of the ir-GTHα and ir-GTHβ in the pituitary (58). Nevertheless, testosterone treatment resulted in the suppression of mRNA expressions of both GTHα and GTHβ in the pituitary (58). Therefore, testosterone probably acts to suppress both the synthesis and the secretion of GTH. This conclusion follows from the reasoning that if the secretion of GTH was not suppressed, the intensities of immunoreactions of both GTHα and GTHβ would have decreased due to decreased levels of mRNA expressions of both GTH subunits. Thus, it seems likely that estradiol and testosterone differ with regard to their roles in the regulation of synthesis and secretion of GTH in the pituitary of the hagfish.

PLASMA LEVELS OF SEX STEROID HORMONES IN THE HAGFISH

Only a few studies exist regarding sex steroid hormonal profiles in relation to gonadal function in hagfish. Matty et al. (17) reported that estradiol and testosterone were measurable in the plasma of *Eptatretus stouti* using RIA; however, the observed levels of these steroids were near the lower limit of RIA sensitivity. Schützinger et al. (59) found using a more sensitive RIA that plasma estradiol content increased in relation to the stages of ovarian development in female Atlantic hagfish, *Myxine glutinosa*. Powell et al. (60, 61) also reported using *in vitro* organ cultured ovaries that the number of females with large eggs increased following estradiol peaks in January in *M. glutinosa*. Thus, estrogen seems to be involved in the ovarian development.

Plasma concentrations of estradiol, testosterone, and progesterone were examined with respect to gonadal development, sexual differences, and possible function of atretic follicles in the brown hagfish, *P. atami*, using a time-resolved fluoroimmunoassay (62). In females, plasma estradiol levels showed a significant positive correlation with ovarian development, while plasma testosterone and progesterone levels were highest in non-vitellogenic adults (62). Thus, our data on plasma estradiol levels in female *P. atami* were consistent with the results of Schützinger et al. (59) and Powell et al. (60). In another study, Yu et al. (63) demonstrated that the synthesis of hepatic vitellogenin was inducible by estrogens, estradiol, and estrone, in *E. stouti*. Based on these results, estrogenic control of ovarian development and hepatic vitellogenesis seemed to have arisen early in vertebrate evolution.

In males, no clear relationships were observed between plasma estradiol or testosterone concentrations and testicular development, while plasma progesterone concentrations showed a significant inverse relationship with testicular development (62). However, in that study data on sexually mature males with high incidences of spermatids or spermatozoa were lacking, since they were very few in our populations (62). Therefore, it is still possible to consider that estradiol and testosterone are involved in the regulation of male reproduction in hagfish. In support of this possibility, it is reported that purified native hagfish GTH induced secretion of estradiol and testosterone from cultured hagfish testes (11). Moreover, intraperitoneal administration of these steroids in juvenile hagfish affected on the GTH functions as mentioned above.

On the other hand, in relation to our failure to correlate plasma concentrations of estradiol or testosterone to testicular development, recent studies in the lamprey have emphasized the importance of non-classical steroids, such as androstenedione and 15α-hydroxylated sex steroids (15α-hydroxytestosterone and 15α-hydroxyprogesterone) in serving as functional androgens (64–67). Indeed, evidence demonstrating testosterone functionality in lampreys was scarce [see Ref. (68)], while androstenedione was found in substantial amounts within the testicular tissue of lampreys, and plasma and tissue levels of the hormone increased significantly in prespermiating male sea lampreys after injection of GnRH (66). In addition, prespermiating males implanted with androstenedione reached maturation significantly faster and exhibited larger secondary sex characteristics than placebo or non-implanted males (66). A receptor for androstenedione was recently described by Bryan et al. (66). 15α-Hydroxylated steroids are also suggested to be involved in the regulation of lamprey reproduction (67). Since hagfish gonads also produce substantial amounts of unusual androgens, such as 6β-hydroxy testosterone and 5α-androstane-3β, 7α, 17β-triol, as well as androstenedione (69–71), some of these steroids may act as functional androgens in the hagfish.

HYPOTHALAMIC FACTORS REGULATING THE GONADOTROPIC FUNCTION OF HAGFISH

The synthesis and secretion of GnRH is the key neuroendocrine function in the hypothalamic regulation of the HPG axis. To date, two to three isoforms have been identified in representative species of all classes of gnathostomes and lampreys (1). GnRHs are also identified in tunicates (72), and several invertebrates belonging to lophotrochozoans [mollusk and annelid; (73, 74)], but not in the ecdysozoan lineages. On the other hand, adipokinetic hormone (AKH) has been identified as the ligand of the GnRH receptor of the insects, *Drosophila* and *Bombyx* (75). An AKH-GnRH-like neuropeptide has been identified in the nematode *C. elegans* (76). A comparative and phylogenetic approach shows that the ecdysozoan AKHs, lophotrochozoan GnRHs, and chordate GnRHs are structurally related and suggested that they all originate from a common ancestor (77).

In the hagfish, GnRH has not yet been identified, but previous chromatographic and immunohistochemical studies have suggested the presence of a GnRH-like molecule in the hypothalamic-neurohypophysial area (19, 20). Kavanaugh et al. (22) reported the seasonal changes in hypothalamic ir-GnRH contents in relation gonadal reproductive stages in the Atlantic hagfish (*M. glutinosa*). In *M. glutinosa*, a dense accumulation of GnRH-like immunoreaction was observed in the dorsal wall of the neurohypophysis with the use of antisera against chicken GnRH-II, salmon GnRH, lamprey GnRH-I, and lamprey GnRH-III (19, 21) (**Figure 4**). Neuronal cells containing ir-GnRH were found in the preoptic nucleus and the dorsal hypothalamic nucleus (20, 21). In another study, Osugi et al. (78) identified several PQRFamide peptides in the brain of the brown hagfish (*P. atami*). Based on *in situ* hybridization and immunohistochemistry, hagfish PQRFamide peptide precursor mRNA and its translated peptides were localized in the infundibular nucleus of the hypothalamus. Dense ir fibers were found in the infundibular nucleus and some of them were terminated on blood vessels within the infundibular nucleus. They further showed that one of the hagfish PQRFamide peptides significantly stimulated the expression of GTHβ mRNA in the cultured hagfish pituitary. The latter result clearly indicates that GTH functions of the hagfish pituitary are controlled by the hypothalamic factors.

Puzzling aspect of the hagfish hypothalamic-pituitary system is that there is no or little anatomical relationship between them. It is generally considered that the hypothalamic factors, such as GnRH, reach the adenohypophysis simply by diffusion (79, 80). However, the dorsal wall of the hagfish neurohypophysis, where ir-GnRH nerve fibers are terminated (**Figure 4**), is far from the adenohypophysis by the presence of the neurohypophysis itself. On the other hand, the blood vessels are richly distributed on the surface of the dorsal wall, and make the posterior hypophysial vascular

plexus (14, 15). Although most blood in the posterior hypophysial vascular plexus enter the posterior hypophysial vein of the anterior cardinal system, several small vessels proceed from the dorsal wall to the adenohypophysis in *Eptatretus burgeri* (15). These small vessels may contribute the regulation of the adenohypophysial functions. A pair of small blood vessels from the hypothalamus also enters the posterior hypophysial vascular plexus (14). Together with the fact that some PQRFamide neuronal fibers terminated on the blood vessels within the hypothalamus (78), further studies are needed to understand the hypothalamic-pituitary system of the hagfish.

CONCLUSION

Not only the pituitary gland but also all major adenohypophysial hormones and their receptors are considered to be vertebrate novelties. Since hagfish represent the most basal and primitive vertebrate that diverged over 550 millions years ago (81), they are of particular importance in understanding the evolution of the HPG axis related to vertebrate reproduction. Our data clearly show that the hagfish has a functional HPG axis similar to that of more advanced gnathostomes. It is strongly expected that the functional GTH found in hagfish pituitary helps to delineate the evolution of the complex HPG axis of reproduction in vertebrates. Furthermore, this HPG system likely evolved from an ancestral, pre-vertebrate exclusively neuroendocrine mechanism by gradual emergence of components of a new control level, the pituitary gland.

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REFERENCES

1. Sower SA, Freamat M, Kavanaugh SI. The origins of the vertebrate hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) endocrine systems: new insights from lampreys. *Gen Comp Endocrinol* (2009) **161**:20–9. doi:10.1016/j.ygcen.2008.11.023
2. Holland LZ, Albalat R, Azumi K, Benito-Gutiérrez È, Blow MJ, Bronner-Fraser M, et al. The amphioxus genome illustrates vertebrate origins and cephalochordate biology. *Genome Res* (2008) **18**:1100–11. doi:10.1101/gr.073676.107
3. Forey P, Janvier P. Agnathans and the origin of jawed vertebrates. *Nature* (1993) **361**:129–34. doi:10.1038/361129a0
4. Forey P, Janvier P. Evolution of the early vertebrates. *Am Sci* (1994) **82**:554–66.
5. Delarbre C, Gallut C, Barriel V, Janvier P, Gachelin G. Complete mitochondrial DNA of the hagfish, *Eptatretus burgeri*: the comparative analysis of mitochondrial DNA sequences strongly supports the cyclostome monophyly. *Mol Phylogenet Evol* (2002) **22**:184–92. doi:10.1006/mpev.2001.1045
6. Takezaki N, Figueroa F, Zaleska-Rutczynska Z, Klein J. Molecular phylogeny of early vertebrates: monophyly of the agnathans as revealed by sequences of 35 genes. *Mol Biol Evol* (2003) **20**:287–92. doi:10.1093/molbev/msg040
7. Heimberg AM, Cowper-Sal-Iari R, Semon M, Donoghue P, Peterson KJ. microRNAs reveal the interrelationships of hagfish, lampreys, and gnathostomes and the nature of the ancestral vertebrate. *Proc Natl Acad Sci USA* (2010) **107**:19379–83. doi:10.1073/pnas.1010350107
8. Oisi Y, Ota KG, Kuraku S, Fujimoto S, Kuratani S. Craniofacial development of hagfishes and the evolution of vertebrates. *Nature* (2013) **493**:175–80. doi:10.1038/nature11794
9. Gorbman A. Reproduction in cyclostome fishes and its regulation. In: Hoar WS, Randall DJ editors. *Fish Physiology*. (Vol. IXA), New York: Academic Press (1983). p. 1–28.
10. Nozaki M. The hagfish pituitary gland and its putative adenohypophysial hormones. *Zoolog Sci* (2008) **25**:1028–36. doi:10.2108/zsj.25.1028
11. Uchida K, Moriyama S, Chiba H, Shimotani T, Honda K, Miki M, et al. Evolutionary origin of a functional gonadotropin in the pituitary of the most primitive vertebrate, hagfish. *Proc Natl Acad Sci U S A* (2010) **107**:15832–7. doi:10.1073/pnas.1002208107
12. Holmes RL, Ball JN. *The Pituitary Gland, A Comparative Account*. London: Cambridge University Press (1974).
13. Hardisty MW. *Biology of Cyclostomes*. London: Chapman and Hall (1979).
14. Gorbman A, Kobayashi H, Uemura H. The vascularisation of the hypophysial structure of the hagfish. *Gen Comp Endocrinol* (1963) **3**:505–14. doi:10.1016/0016-6480(63)90083-2
15. Kobayashi H, Uemura H. The neurohypophysis of the hagfish, *Eptatretus burgeri* (Girard). *Gen Comp Endocrinol* (1972) **3**:114–24. doi:10.1016/0016-6480(72)90139-6
16. Gorbman A, Dickhoff WW, Vigna SR, Clark NB, Ralph CL. *Comparative Endocrinology*. New York: John Wiley & Sons (1983).
17. Matty AJ, Tsuneki K, Dickhoff WW, Gorbman A. Thyroid and gonadal function in hypophysectomized hagfish, *Eptatretus stouti*. *Gen Comp Endocrinol* (1976) **30**:500–16. doi:10.1016/0016-6480(76)90120-9
18. Suzuki M, Kubokawa M, Nagasawa K, Urano A. Sequence analysis of vasotocin cDNAs of the lamprey, *Lampetra japonica* and the hagfish *Eptatretus burgeri* – evolution of cyclostome vasotocin precursors. *J Mol Endocrinol* (1995) **14**:67–77. doi:10.1677/jme.0.0140067
19. Sower SA, Nozaki M, Knox CJ, Gorbman A. The occurrence and distribution of GnRH in the brain of Atlantic hagfish, an Agnathan, determined by chromatography and immunocytochemistry. *Gen Comp Endocrinol* (1995) **97**:300–7. doi:10.1006/gcen.1995.1030
20. Braun CB, Wicht H, Northcutt RG. Distribution of gonadotropin-releasing hormone immunoreactivity in the brain of the Pacific hagfish *Eptatretus stouti* (Craniata, Myxinoidea). *J Comp Neurol* (1995) **353**:464–76. doi:10.1002/cne.903530313
21. Oshima Y, Ominato K, Nozaki M. Distribution of GnRH-like immunoreactivity in the brain of lampreys and hagfish. *Annual Activity Reports of the Sado Marine Biological Station, Niigata University, No. 31*. Sado: Daiichi Press (2001). p. 4–5.
22. Kavanaugh SI, Powell ML, Sower SA. Seasonal changes of gonadotropin-releasing hormone in the Atlantic hagfish *Myxine glutinosa*. *Gen Comp Endocrinol* (2005) **140**:136–43. doi:10.1016/j.ygcen.2004.10.015
23. Nozaki M, Oshima Y, Miki M, Shimotani T, Kawauchi H, Sower SA. Distribution of immunoreactive adenohypophysial cell types in the pituitaries of the Atlantic and the Pacific hagfish, *Myxine glutinosa* and *Eptatretus burgeri*. *Gen Comp Endocrinol* (2005) **143**:142–50. doi:10.1016/j.ygcen.2005.03.002
24. Nozaki M, Shimotani T, Uchida K. Gonadotropin-like and adrenocorticotropin-like cells in the pituitary gland of hagfish, *Paramyxine atami*: immunohistochemistry in combination with lectin histochemistry. *Cell Tissue Res* (2007) **328**:563–72. doi:10.1007/s00441-006-0349-3
25. Miki M, Shimotani T, Uchida K, Hirano S, Nozaki M. Immunohistochemical detection of gonadotropin-like material in the pituitary of brown hagfish (*Paramyxine atami*) correlated with their gonadal functions and effect of estrogen treatment. *Gen Comp Endocrinol* (2006) **148**:15–21. doi:10.1016/j.ygcen.2006.01.018
26. Kawauchi H, Suzuki K, Yamazaki T, Moriyama S, Nozaki M, Yamaguchi K, et al. Identification of growth hormone in the sea lamprey, an extant representative of a group of the most ancient vertebrates. *Endocrinology* (2002) **143**:4916–21. doi:10.1210/en.2002-220810
27. Kawauchi H, Sower SA. The dawn and evolution of hormones in the adenohypophysis. *Gen Comp Endocrinol* (2006) **148**:3–14. doi:10.1016/j.ygcen.2005.10.011
28. Dayhoff MO. *Atlas of Protein Sequence and Structure*. Silver Springs, MD: National Biomedical Research Foundation (1976).
29. Kawauchi H, Suzuki K, Itoh H, Swanson P, Naito N, Nagahama Y, et al. The duality of teleost gonadotropins. *Fish Physiol Biochem* (1989) **7**:29–38. doi:10.1007/BF00004687
30. Quérat B, Sellouk A, Salmon C. Phylogenetic analysis of the vertebrate glycoprotein hormone family including new sequences of sturgeon *(Acipenser baeri)*

beta subunits of the two gonadotropins and the thyroid-stimulating hormone. *Biol Reprod* (2000) **63**:222–8. doi:10.1095/biolreprod63.1.222
31. Quérat B, Arai Y, Henry A, Akama Y, Longhurst TJ, Joss JM. Pituitary glycoprotein hormone beta subunits in the Australian lungfish and estimation of the relative evolution rate of these subunits within vertebrates. *Biol Reprod* (2004) **70**:356–63. doi:10.1095/biolreprod.103.022004
32. Quérat B, Tonnerre-Doricarli C, Géniès F, Salmon C. Duality of gonadotropins in gnathostomes. *Gen Comp Endocrinol* (2001) **124**:308–14. doi:10.1006/gcen.2001.7715
33. Sower SA, Moriyama S, Kasahara M, Takahashi A, Nozaki M, Uchida K, et al. Identification of sea lamprey GTHbeta-like cDNA and its evolutionary implications. *Gen Comp Endocrinol* (2006) **148**:22–32. doi:10.1016/j.ygcen.2005.11.009
34. Decatur WA, Hall JA, Smith JJ, Li W, Sower SA. Insight from the lamprey genome: glimpsing early vertebrate development via neuroendocrine-associated genes and shared synteny of gonadotropin-releasing hormone (GnRH). *Gen Comp Endocrinol* (2013) **192**:237–45. doi:10.1016/j.ygcen.2013.05.020
35. Larsen LO, Rothwell B. Adenohypophysis. In: Hardisty MW, Potter IC editors. *The Biology of Lampreys.* (Vol. 2), London: Academic Press (1972). p. 1–67.
36. Hardisty MW, Baker BI. Endocrinology of lampreys. In: Hardisty MW, Potter IC editors. *The Biology of Lampreys.* London: Academic Press (1982). p. 1–115.
37. Sower SA. Brain and pituitary hormones of lampreys, recent findings and their evolutionary significance. *Am Zool* (1998) **38**:15–38.
38. Nozaki M, Ominato K, Shimotani T, Kawauchi H, Youson JH, Sower SA. Identity and distribution of immunoreactive adenohypophysial cells in the pituitary during the life cycle of sea lampreys, *Petromyzon marinus*. *Gen Comp Endocrinol* (2008) **155**:403–12. doi:10.1016/j.ygcen.2007.07.012
39. Nakabayashi K, Matsumi H, Bhalla A, Bae J, Mosselman S, Hsu SY, et al. Thyrostimulin, a heterodimer of two new human glycoprotein hormone subunits, activates the thyroid-stimulating hormone receptor. *J Clin Invest* (2002) **109**:1445–52. doi:10.1172/JCI14340
40. Sudo S, Kuwabara Y, Park JI, Hsu SY, Hsueh AJ. Heterodimeric fly glycoprotein hormone-alpha2 (GPA2) and glycoprotein hormone-beta5 (GPB5) activate fly leucine-rich repeat-containing G protein-coupled receptor-1 (DLGR1) and stimulation of human thyrotropin receptors by chimeric fly GPA2 and human GPB5. *Endocrinology* (2005) **146**:3596–604. doi:10.1210/en.2005-0317
41. Park J-II, Semeyonov J, Cheng CL, Hsu SYT. Conservation of the heterodimeric glycoprotein hormone subunit family proteins and the LGR signaling system from nematodes to humans. *Endocrine* (2005) **26**:267–76. doi:10.1385/ENDO:26:3:267
42. Dos Santos S, Bardet C, Bertrand S, Escriva H, Habert D, Querat B. Distinct expression patterns of glycoprotein hormone-α2 (GPA2) and -β5 (GPB5) in a basal chordate suggest independent developmental functions. *Endocrinology* (2009) **150**:3815–22. doi:10.1210/en.2008-1743
43. Dos Santos S, Mazan S, Venkatesh B, Cohen-Tannoudji J, Querat B. Emergence and evolution of the glycoprotein hormone and neurotrophin gene families in vertebrates. *BMC Evol Biol* (2011) **11**:332. doi:10.1186/1471-2148-11-332
44. Tando Y, Kubokawa K. Expression of the gene for ancestral glycoprotein hormone beta subunit in the nerve cord of amphioxus. *Gen Comp Endocrinol* (2009) **162**:329–39. doi:10.1016/j.ygcen.2009.04.015
45. Tando Y, Kubokawa K. A homolog of the vertebrate thyrostimulin glycoprotein hormone a subunit (GPA2) is expressed in amphioxus neurons. *Zoolog Sci* (2009) **26**:409–14. doi:10.2108/zsj.26.409
46. Larsen D, Swanson P. Effects of gonadectomy on plasma gonadotropins I and II in coho salmon, *Oncorhynchus kisutch*. *Gen Comp Endocrinol* (1997) **108**:152–60. doi:10.1006/gcen.1997.6958
47. Kobayashi M, Stacey NE. Effects of ovariectomy and steroid hormone implantation on serum gonadotropin levels in female goldfish. *Zoolog Sci* (1990) **7**:715–21.
48. Habibi HR, de Leeuw R, Nahorniak CS, Goos HJ, Peter RE. Pituitary gonadotropin-releasing hormone (GnRH) receptor activity in goldfish and catfish: seasonal and gonadal effects. *Fish Physiol Biochem* (1989) **7**:109–18. doi:10.1007/BF00004696
49. Saligaut C, Linard B, Mananos EL, Kah O, Breton B, Govoroun M. Release of pituitary gonadotropins GtHI and GtHII in the rainbow trout (*Oncorhynchus mykiss*): modulation by estradiol and catecholamines. *Gen Comp Endocrinol* (1998) **109**:302–9. doi:10.1006/gcen.1997.7033
50. Dickey JT, Swanson P. Effects of sex steroids on gonadotropin (FSH and LH) regulation in coho salmon (*Oncorhynchus kisutch*). *J Mol Endocrinol* (1998) **21**:291–306. doi:10.1677/jme.0.0210291
51. Kobayashi M, Sohn YC, Yoshiura Y, Aida K. Effects of sex steroids on the mRNA levels of gonadotropin subunits in juvenile and ovariectomized goldfish *Carassius auratus*. *Fish Sci* (2000) **66**:223–31. doi:10.1046/j.1444-2906.2000.00038.x
52. Vacher C, Ferrière F, Marmignon MH, Pellegrini E, Saligaut C. Dopamine D2 receptors and secretion of FSH and LH: role of sexual steroids on the pituitary of the female rainbow trout. *Gen Comp Endocrinol* (2002) **127**:198–206. doi:10.1016/S0016-6480(02)00046-1
53. Levavi-Sivan B, Biran J, Fireman E. Sex steroids are involved in the regulation of gonadotropin-releasing hormone and dopamine D2 receptors in female tilapia pituitary. *Biol Reprod* (2006) **75**:642–50. doi:10.1095/biolreprod.106.051540
54. Banerjee A, Khan I. Molecular cloning of FSH and LH β subunits and their regulation of estrogen in Atlantic croaker. *Gen Comp Endocrinol* (2008) **155**:827–37. doi:10.1016/j.ygcen.2007.09.016
55. Huggard-Nelson DL, Nathwani PS, Kermouni A, Habibi HR. Molecular characterization of LH-beta and FSH-beta subunits and their regulation by estrogen in the goldfish pituitary. *Mol Cell Endocrinol* (2002) **188**:171–93. doi:10.1016/S0303-7207(01)00716-X
56. Aroura S, Weltzien F-A, Belle NL, Dufour S. Development of real-time RT-PCR assays for eel gonadotropins and their application to the comparison of *in vivo* and *in vitro* effects of sex steroids. *Gen Comp Endocrinol* (2007) **153**:333–43. doi:10.1016/j.ygcen.2007.02.027
57. Huggard D, Khakoo Z, Kassam G, Mahmoud SS, Habibi HR. Effect of testosterone on maturational gonadotropin subunit messenger ribonucleic acid levels in the goldfish pituitary. *Biol Reprod* (1996) **54**:1184–91. doi:10.1095/biolreprod54.6.1184
58. Nozaki M, Uchida K, Honda K, Shimotani T, Nishiyama M. Effects of Estradiol or testosterone treatment on expression of gonadotropin subunit mRNAs and proteins in the pituitary of juvenile brown hagfish, *Paramyxine atami*. *Gen Comp Endocrinol* (2013) **189**:142–50. doi:10.1016/j.ygcen.2013.04.034
59. Schützinger S, Choi HS, Patzner RA, Adam H. Estrogens in plasma of the hagfish, *Myxine glutinosa* (Cyclostomata). *Acta Zool (Stockh)* (1987) **68**:263–6. doi:10.1111/j.1463-6395.1987.tb00893.x
60. Powell ML, Kavanaugh S, Sower SA. Seasonal concentrations of reproductive steroids in the gonads of the Atlantic hagfish, *Myxine glutinosa*. *J Exp Zool* (2004) **301A**:352–60. doi:10.1002/jez.a.20043
61. Powell ML, Kavanaugh SI, Sower SA. Current knowledge of hagfish reproduction: implications for fisheries management. *Integr Comp Biol* (2005) **45**:158–65. doi:10.1093/icb/45.1.158
62. Nishiyama M, Chiba H, Uchida K, Shimotani T, Nozaki M. Relationships between plasma concentrations of sex steroid hormones and gonadal development in the brown hagfish, *Paramyxine atami*. *Zoolog Sci* (2013) **30**:967–74. doi:10.2108/zsj.30.967
63. Yu JYL, Dickhoff WW, Swanson P, Gorbman A. Vitellogenesis and its hormonal regulation in the Pacific hagfish, *Eptatretus stouti* L. *Gen Comp Endocrinol* (1981) **43**:492–502. doi:10.1016/0016-6480(81)90234-3
64. Lowartz S, Petkam R, Renaud R, Beamish FWH, Kime DE, Raeside J, et al. Blood steroid profile and in vitro steroidogenesis by ovarian follicles and testis fragments of adult sea lamprey, *Petromyzon marinus*. *Comp Biochem Physiol A Mol Integr Physiol* (2003) **134**:365–76. doi:10.1016/S1095-6433(02)00285-4
65. Young BA, Bryan MB, Glenn JR, Yun SS, Scott AP, Li W. Dose-response relationship of 15α-hydroxylated sex steroids to gonadotropin-releasing hormones and pituitary extract in male sea lampreys (*Petromyzon marinus*). *Gen Comp Endocrinol* (2007) **151**:108–15. doi:10.1016/j.ygcen.2006.12.005
66. Bryan MB, Scott AP, Li W. The sea lamprey (*Petromyzon marinus*) has a receptor for androstenedione. *Biol Reprod* (2007) **77**:688–96. doi:10.1095/biolreprod.107.061093
67. Bryan MB, Scott AP, Li W. Sex steroids and their receptors in lampreys. *Steroids* (2008) **73**:1–12. doi:10.1016/j.steroids.2007.08.011
68. Young BA, Bryan MB, Sower SA, Scott AP, Li W. 15α-Hydroxytestosterone induction by GnRH-I and GnRH-III in Atlantic and Great Lakes sea lamprey (*Petromyzon marinus* L.). *Gen Comp Endocrinol* (2004) **136**:276–81. doi:10.1016/j.ygcen.2003.12.022
69. Hirose K, Tamaoki B, Fernholm B, Kobayashi H. In vitro bioconversions of steroids in the mature ovary of the hagfish, *Eptatretus burgeri*. *Comp Biochem Physiol* (1975) **51B**:403–8.
70. Kime DE, Hews EA, Gafter J. Steroid biosynthesis by testes of the hagfish *Myxine glutinosa*. *Gen Comp Endocrinol* (1980) **41**:8–13. doi:10.1016/0016-6480(80)90026-X

71. Kime DE, Hews EA. Steroid biosynthesis by the ovary of the hagfish *Myxine glutinosa*. *Gen Comp Endocrinol* (1980) **42**:71–5. doi:10.1016/0016-6480(80)90258-0
72. Adams BA, Tello JA, Erchegyi J, Warby C, Hong DJ, Akinsanya KO, et al. Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, *Ciona intestinalis*. *Endocrinology* (2003) **114**:1907–19. doi:10.1210/en.2002-0216
73. Tsai PS, Zhang L. The emergence and loss of gonadotropin-releasing hormone in protostomes: orthology, phylogeny, structure, and function. *Biol Reprod* (2008) **79**:798–805. doi:10.1095/biolreprod.108.070185
74. Zhang L, Tello JA, Zhang W, Tsai PS. Molecular cloning, expression pattern, and immunocytochemical localization of a gonadotropin-releasing hormone-like molecule in the gastropod mollusk, *Aplysia californica*. *Gen Comp Endocrinol* (2008) **156**:201–9. doi:10.1016/j.ygcen.2007.11.015
75. Staubli F, Jorgensen TJ, Cazzamali G, Williamson M, Lenz C, Sondergarrd L, et al. Molecular identification of the insect adipokinetic hormone receptors. *Proc Natl Acad Sci USA* (2002) **99**:3446–51. doi:10.1073/pnas.052556499
76. Lindemans M, Liu F, Janssen T, Husson SJ, Mertens I, Gade G, et al. Adipokinetic hormone signaling through the gonadotropin-releasing hormone receptor modulates egg-laying in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* (2009) **106**:1642–7. doi:10.1073/pnas.0809881106
77. Lindemans M, Jannsen T, Beets I, Temmerman L, Meelkop E, Schoofs L. Gonadotropin-releasing hormone and adipokinetic hormone signaling systems share a common evolutionary origin. *Front Endocrinol* (2011) **12**:16. doi:10.3389/fendo.2011.00016
78. Osugi T, Uchida K, Nozaki M, Tsutsui K. Characterization of novel RFamide peptides in the central nervous system of the brow hagfish: isolation, localization, and functional analysis. *Endocrinology* (2011) **152**:4252–64. doi:10.1210/en.2011-1375
79. Tsukahara T, Gorbman A, Kobayashi H. Median eminence equivalence of the neurohypophysis of the hagfish, *Eptatretus burgeri*. *Gen Comp Endocrinol* (1986) **61**:348–54. doi:10.1016/0016-6480(86)90220-0
80. Gorbman A. Olfactory origins and evolution of the brain-pituitary endocrine system: facts and speculation. *Gen Comp Endocrinol* (1995) **97**:171–8. doi:10.1006/gcen.1995.1016
81. Janvier P. *Early Vertebrates*. Oxford: Clarendon Press (1996).
82. Nozaki M, Gorbman A. Immunocytochemical localization of somatostatin and vasotocin in the brain of the Pacific hagfish, *Eptatretus stouti*. *Cell Tissue Res* (1983) **229**:541–50.

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