REVIEW

The hepatocyte growth factor system as a regulator of female and male gonadal function

Rob Zachow and Mehmet Uzumcu¹

Department of Physiology and Biophysics, Robert Wood Johnson Medical School-UMDNJ, 675 Hoes Lane West, Piscataway, New Jersey 08854, USA

¹Department of Animal Sciences, School of Environmental and Biological Sciences, Rutgers, The State University of New Jersey, 84 Lipman Drive, New Brunswick, New Jersey 08901-8525, USA

(Correspondence should be addressed to R Zachow; Email: zachowrj@umdnj.edu)

Abstract

The hepatocyte growth factor (HGF) system comprises HGF, its receptor (the c-met tyrosine kinase), HGF activator (HGFA) protein, and HGFA inhibitor (HAI). The components of the HGF system have been identified in a plethora of tissues to include the ovary and testis. In its traditional context, the HGF system works via paracrine- and autocrine-mediated feedback in which HGF (of mesenchymal origin) binds and activates c-met (within epithelial cells); target cells then respond to HGF via any number of morphogenic and functional changes. The concomitant presence of HGFA and HAI suggests that HGF bioactivity can be locally modulated. A number of studies have collectively shown that the mammalian ovary and testis contain HGF, c-met, and HGFA; very little is currently known regarding HAI within the gonad. Within the ovary, HGF controls numerous key functions which collectively regulate the growth and differentiation of ovarian follicles; these include

cell growth, steroidogenesis, and apoptosis within theca cells and/or granulosa cells. Comparatively, less is known about the function of HGF within the testicular Leydig and Sertoli cells, but evidence is emerging that HGF may regulate somatic cell function, including Leydig cell steroidogenesis. Changes in the cellular origin of HGF and c-met during fetal and postnatal testicular development suggest that HGF, in collaboration with other growth factors, may regulate important aspects of testicular cell morphogenesis and differentiation which enable male sexual viability. Likewise, experimental evidence showing that HGF can modulate many vital processes which enable ovarian follicle growth, differentiation, and function indicate the importance of HGF in female reproduction. This review presents what is currently known regarding the expression of the HGF system and its function within the ovary and testis.

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The hepatocyte growth factor (HGF) system

HGF is an 87 kDa growth factor first identified and characterized due to its angiogenic and proliferative effects in primary epithelial cell cultures (Bussolino *et al.* 1992). This pioneered a number of studies leading to the localization of HGF within mesenchymal cell populations. As such, one key function of HGF is its action as a paracrine factor. In this context, HGF is secreted from mesenchymal cells and targets neighboring epithelial cells which express the HGF receptor, c-met (Gheradi & Stoker 1991). Thus, at the tissue level, the HGF/c-met system represents a paracrine control mechanism for the local regulation of tissue function.

HGF is synthesized as an inactive 97 kDa single chain precursor, and is processed to yield its bioactive disulfide-linked heterodimer (Miyazawa *et al.* 1989, Nakamura *et al.* 1989). Another component of the HGF system is HGF activator (HGFA) protein. This molecule is a serine protease which cleaves the immature HGF precursor molecule to form mature bioactive

HGF (Miyazawa *et al.* 1993). Thus, the concomitant presence of both HGF and HGFA within a tissue microenvironment is presumed to exert some level of control upon HGF bioactivity. Relatively little is known about the regulation of HGFA expression; however, the presence of HGFA has been established within the rat ovary (discussed below; Uzumcu *et al.* 2006).

The most recent addition of the HGF system to be identified is HGFA inhibitor protein (HAI; Shimomura *et al.* 1997). This protein is a Kunitz-type serine protease inhibitor, which, as the name implies, blocks the activation of HGFA. One study to date has demonstrated the presence of the mRNAs encoding HAI-1 and HAI-2 within the human testis (Yamauchi *et al.* 2002). The function of HAI has not been reported within the testis, and neither the expression nor functions of HAI have been described within the ovary.

The HGF receptor, identified as the p190 c-met proto-oncogene, is a member of the receptor tyrosine kinase family (Bottaro *et al.* 1991). In its mature bioactive form, plasma

membrane-bound c-met comprises disulfide-linked α and β chains; with a protein tyrosine kinase (PTK) domain (residues Y1349 and Y1356) nested within carboxyl terminus of the β chain (Gonzatti-Haces *et al.* 1988, Giordano *et al.* 1989, Peruzzi & Bottaro 2006). Upon activation by HGF, c-met PTK activity is coupled to the recruitment of several adaptor proteins including the non-receptor PTK Src, the adaptor protein SHC, growth factor receptor-bound protein 2 (Grb2), and Grb-2-associated binder (Gab1) (Peruzzi & Bottaro 2006), followed by mobilization of numerous signaling cascades. Examples of HGF-induced signaling motifs include those mediated by cyclic nucleotides, phosphoinositide-3 kinase (PI-3K)/protein kinase B (Akt), mitogen-activated protein kinases, calcium-phospholipids/protein kinase C, and Janus kinase/signal transducer and activator of transcription (Baffy *et al.* 1992, Lail-Trecker *et al.* 1998, Liu 1999, Zachow & Woolery 2002, Makondo *et al.* 2004). Pathways coupled to the regulation of cell cycle progression, morphogenesis, and cell survival are among those under the control of HGF/c-met-dependent signaling (Peruzzi & Bottaro 2006).

Ovarian function

Folliculogenesis

Folliculogenesis is a dynamic process during which follicular granulosa cells (GCs) and theca cells proliferate and differentiate to produce factors (e.g., steroid and peptide hormones and growth factors) which, through autocrine, paracrine, and endocrine pathways, support oocyte maturation and enable ovulation. In a general sense, folliculogenesis is dependent upon the pituitary gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Within preovulatory follicles, LH stimulates the production and secretion of the aromatizable androgens (androstenedione and testosterone) in theca cells. Whereas FSH targets GC, and upregulates the expression and activity of cytochrome P450 aromatase (CYP19) enabling the aromatization of theca cell androgens into estradiol-17 β (E₂). Ovarian androgens and E₂ are necessary for normal reproductive function; importantly, aberrant levels of ovarian steroids have been coupled to infertility and certain reproductive pathologies such as polycystic ovary syndrome (Erickson 1994, Ehrmann 2005). Hence, tight regulation of the production of androgens and E₂ is important for controlling both normal and pathologic intra- and extraovarian processes.

Decades of research have shown that several intraovarian growth factors work with the gonadotropins and facilitate folliculogenesis through an array of autocrine and paracrine mechanisms which control follicle growth and steroidogenesis (Webb *et al.* 2004, Barnett *et al.* 2006, Knight & Glister 2006). It is important to realize that a number of growth factors have been localized within growing follicles, and many of these work through the activation of receptor PTK, with HGF being one such factor. Some other examples of these which have been shown to regulate important aspects of follicle cell function are

insulin and insulin-like growth factor-I (IGF-I), epidermal growth factor, transforming growth factor- α (TGF- α), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF), neurotrophins, and kit ligand (reviewed in Fortune 2003, Skinner 2005). Interestingly, these receptor PTKs often induce signaling mechanisms common to one another and result in overlapping cell responses. Of course, there are many other growth factors and cytokines (e.g., TGF- β and its family members, tumor necrosis factor- α , interleukins, and others) using non-PTK mechanisms of action; each of which also mobilize some similar signaling cascades (Magoffin 1991, Terranova 1991). Such observations support the belief that an intricate web of cooperation occurs between the gonadotropins and the numerous growth factor/cytokine-dependent control mechanisms which mediate follicle cell differentiation and function. In this review, we suggest that the mesenchymal-epithelial paracrine mechanism, an example of which is mediated by HGF, is one example of the many important components which guide follicle development and ovulation.

Interestingly, of the vast number of follicles that are recruited from the non-growing ovarian follicular reserve to develop during each reproductive cycle (e.g., the menstrual cycle in primates and the estrus cycle in rodents), few (arguably <1%) of these follicles will ever fully mature and ovulate. Instead, all but the so-called selected follicles (i.e., those follicles which will ovulate) die by the process known as atresia. Apoptosis, especially within GC, has been shown to be a predominant cellular mechanism that leads to follicular atresia (Hussein 2005). Based upon a plethora of *in vivo* and *in vitro* studies using animal models, FSH is required to maintain follicle (e.g., GC) viability.

It is important to consider that, although FSH is a key folliculogenic hormone, the modulation of FSH bioactivity by intraovarian growth factors also regulates steroidogenesis, and can prevent or promote apoptosis in GC (Hsueh *et al.* 1996, Kaipia & Hsueh 1997). It seems that gonadotropin action in the ovary is primarily mediated by local growth factors (Hillier 2001, Conti *et al.* 2006). Using transgenic mice to create selective gene knockouts, it has also been shown that certain intraovarian growth factors are in fact vital for the process of folliculogenesis (Burns & Matzuk 2002, Roy & Matzuk 2006).

The HGF system and folliculogenesis

During the female reproductive lifespan, follicles are recruited from a latent resting pool of primordial follicles into a growing cohort (often referred to as the primordial to primary follicle transition). Throughout the growth phase, follicles pass through several stages of growth and differentiation which involves the exquisite temporal regulation of cell proliferation, morphogenic and functional differentiation, angiogenesis, apoptosis, and motogenesis. Ovulation of the selected follicle results in its rupture with concomitant damage to the ovarian surface epithelium; this is followed by epithelial repair and terminal differentiation of the remnant ovulated follicle into the corpus luteum. As such, the complete course of folliculogenesis (including ovulation and luteogenesis) represents a unique

example of ongoing tissue growth with angiogenesis, insult, repair, and degeneration in adult mammals. When considering this, a remarkable comparison can be made between this vital and healthy event and oncogenesis, the latter involving one or more of the aforementioned processes escaping from normal control. Many studies have documented that mutations in HGF, c-met, or overexpression of HGF and/or c-met occur in numerous cancers (e.g., breast, prostate, ovarian surface epithelial, non-small cell lung, and others; Maulik *et al.* 2002); moreover, HGF has been shown to regulate many processes involved in tumorigenesis (e.g., cell cycle control, invasion, motility, apoptosis, angiogenesis, and branching morphogenesis; Maulik *et al.* 2002). Although a description of the involvement of the HGF/c-met system in oncogenesis is beyond the scope of this review, the fact that HGF can mediate many of these mechanisms in (healthy) growing follicles, as well as tumor cells, is compelling. Future studies which reveal how the normal and pathologic processes which are regulated by HGF compare at the molecular level are likely to be beneficial for furthering our understanding of how timed cycles of growth, differentiation, injury, and repair switch from normal to an oncogenic course. The remaining discussion of ovarian function describes what is currently known about the role of the HGF/c-met system during normal follicle development.

Both HGF and c-met are expressed within mammalian ovaries as has been shown in the human, rat, pig, cow, and mouse (Liu *et al.* 1994, Parrott *et al.* 1994, Parrott & Skinner 1998a, Osuga *et al.* 1999, Shimizu *et al.* 2003). Importantly, intraovarian HGF is an autocrine/paracrine regulator of GC and theca cell growth and steroidogenesis (Zachow *et al.* 1997, 2000, Lail-Trecker *et al.* 1998, Parrott & Skinner 1998b, Zachow & Woolery 2002).

The cellular localization of HGF within the mammalian ovary was first demonstrated by Parrott *et al.* (1994). This study showed that HGF mRNA and protein were present within bovine theca cells; whereas, HGF was not detected within bovine GC. Moreover, in bovine GC *in vitro*, HGF induced cell proliferation, but did not induce theca cell growth (Parrott *et al.* 1994). This established the basic mesenchymal-epithelial cell model for HGF bioactivity (described above) within the mammalian ovary. Subsequent studies demonstrated that in addition to theca cells, HGF 1) is expressed within GC and 2) can modulate both theca cell and GC function, as described below.

Theca cells

The steroid modulatory effects of HGF were first demonstrated using primary cultures of theca-interstitial cells from sexually immature rats. In rat theca cells *in vitro*, HGF suppressed LH-dependent androstosterone secretion, while also blocking the steady-state level of CYP17 expression (Zachow *et al.* 1997). Basal and LH-induced progesterone production were stimulated in the presence of HGF; but neither the expression of CYP11A nor 3 β -hydroxysteroid dehydrogenase was affected by HGF

(Zachow *et al.* 1997). Although steroidogenesis was modulated by HGF, DNA content in rat theca cell cultures was not altered, suggesting that HGF did not stimulate proliferation of these cells. Using quantitative RT-PCR, this same study also demonstrated that, in the immature rat, HGF mRNA was expressed in both theca cells and GCs (Zachow *et al.* 1997). Therefore, in contrast to what was observed in the cow (Parrott *et al.* 1994), in the immature rat, HGF is expressed within both follicle cell populations.

The direct effect of HGF on theca cell function was questioned early on, in fact it was postulated that the HGF-correlated regulation of theca cell steroidogenesis was indirect; that is, due to actions of HGF in contaminating GC within theca cell cultures, which secreted steroid regulatory factors (Lail-Trecker *et al.* 1998). More recent data support the initial model reported by Zachow *et al.* (1997) suggesting a direct effect of HGF on theca cell function. Specifically, as shown by Uzumcu *et al.* (2006) the *in vivo* priming of immature rats with equine chorionic gonadotropin (eCG; to induce the growth of mature preovulatory follicles) resulted in elevated HGF and c-met immunoreactive proteins within the theca cells of large antral follicles. Also, c-met expression has been detected in human theca cells (Ito *et al.* 2001). Furthermore, in human chorionic gonadotropin (hCG)-primed gilts, c-met mRNA is expressed within theca cells (Shimizu *et al.* 2003). Thus, experimental evidence shows that both HGF and c-met are produced by theca cells as well as GC. Moreover, theca cells are likely respond to direct stimulation by HGF with changes in steroidogenic gene expression leading to alterations in androgen and progesterone production (Zachow *et al.* 1997).

The effects of HGF within theca cells are not restricted to the modulation of steroidogenesis. As shown by Parrott *et al.* in bovine theca cells *in vitro*, hCG stimulated the production of HGF (Parrott & Skinner 1998b). This initiated a paracrine feedback loop whereby theca-derived HGF promoted the secretion of kit ligand from GC. Kit ligand in turn 1) upregulated the production of HGF within theca cells and 2) induced theca cell proliferation (Parrott & Skinner 1998b, 2000). An essentially identical HGF-kit ligand system was also demonstrated in human theca cells and GC *in vitro* (Ito *et al.* 2001).

The presence of ovarian HGFA has been reported (Uzumcu *et al.* 2006); recall that HGFA represents a potential control mechanism for regulating local bioactive HGF concentrations. As first published by Uzumcu *et al.* (2006), in non-primed rat ovaries, a somewhat uniform HGFA signal was observed in GC within, and theca cells surrounding, preantral follicles; however, immunoreactive HGFA was not detected in oocytes. The same general pattern of HGFA was apparent within the antral follicles contained within these ovaries. In the ovaries of eCG-primed immature rats, a more intense signal for HGFA was present in theca cells when compared with that detected within GC. This pattern for HGFA was especially apparent in the theca cells associated with large antral follicles. The GC within large antral follicles showed no immunoreactive HGFA (Uzumcu *et al.* 2006).

Granulosa cells

Expression of the HGF system The proliferation and steroidogenic differentiation of GC into estrogenic and ultimately progestagenic cells is vital for reproductive function. Since the control of GC differentiation into steroidogenic cells is critical for maintaining female reproductive viability, any factor which modulates GC function is of potential importance in this context. The first studies showing an effect of HGF within the ovary were conducted by Parrott *et al.* (1994) in which HGF induced proliferation of bovine GC *in vitro*. This indicated that GC contain functional c-met, and this was indeed established shortly thereafter when the expression of c-met mRNA was identified in mouse GC (Yang & Park 1995). Subsequently, it was demonstrated that FSH and HGF each reduced c-met expression in rat GC *in vitro* (Zachow *et al.* 2000). Whereas, the cAMP analog, dibutyryl-cAMP, increased the level of c-met mRNA in cultured rat GC (Zachow *et al.* 2000). This was a compelling set of observations since FSH stimulates a profound increase in cAMP production in GC; hence, at a first approximation, these data showing changes in the level of c-met expression appeared paradoxical. One explanation for these observations is that FSH mobilizes a variety of signaling molecules, and crosstalk between cAMP-mediated and non-cAMP-mediated signaling cascades could control c-met content in GC.

Further studies investigating the regulation of ovarian c-met content were conducted by Uzumcu *et al.* (2006). As reported by these authors using ovaries harvested from immature rats, immunoreactive c-met staining was the most intense in GC within small preantral follicles; however, following eCG priming of immature rats (to induce the growth of large antral follicles), there was a shift in c-met intensity which resulted in relatively low c-met levels in GC, but relatively high c-met content in theca cells surrounding large antral follicles (Uzumcu *et al.* 2006).

The early report by Parrott *et al.* (1994) by-and-large strengthened the belief that HGF worked via the basic mesenchymal-epithelial mechanism of action that was proposed following its identification in epithelial cell cultures (Bussolino *et al.* 1992). However, subsequent studies challenged this model by showing that human, rat, and pig GC not only express c-met, but also contain HGF (Zachow *et al.* 1997, Osuga *et al.* 1999, Shimizu *et al.* 2003, Uzumcu *et al.* 2006). In the rat, eCG priming induced a reduction in immunoreactive HGF in GC, while increasing the HGF signal in theca cells of large antral follicles (Uzumcu *et al.* 2006). It is therefore important to consider that HGF is not expressed in theca cells and GC alone, but the level of ovarian HGF is subject to hormonal regulation *in vivo* (Uzumcu *et al.* 2006).

HGF and GC steroidogenesis Since a HGF system has been localized within the ovary, it is not surprising that HGF affects several key aspects of GC function as has been shown by numerous *in vitro* studies. In GC obtained from human *in vitro* fertilization (IVF) patients, HGF stimulated progesterone

production (Ito *et al.* 2001). In contrast, in the rat HGF did not alter FSH-stimulated progesterone production, nor that induced by combined treatment with FSH and IGF-I in GC cultures (Zachow & Woolery 2002). Whereas in bovine GC, HGF suppressed hCG-supported progesterone synthesis (Parrott & Skinner 1998a). Therefore, depending upon the species, and perhaps the state of follicle differentiation, HGF can cause changes in progesterone production, and may thus exert control over luteogenesis and/or corpus luteum function.

One hallmark feature of folliculogenesis is the steroidogenic differentiation of theca cells and GC which appears to occur sometime during late the preantral or early antral phases of follicle growth. Within GC, theca cell androgens are aromatized into estrone (E_1), which is subsequently converted into E_2 by 17 β -HSD type 1. Through this cooperation, the GCs within preovulatory follicles account for the majority of the preovulatory rise in serum E_2 ; and these changes in serum E_2 lead to the pattern of gonadotropin secretion which enables ovulation. Although the steroidogenic differentiation process is held in check in small preantral follicles *in vivo*, GC harvested from preantral follicles will produce copious amounts of E_2 when challenged with FSH or cAMP analogs (in the presence of aromatizable androgens) *in vitro* (Erickson 1983). Likewise, theca cells obtained from sexually immature rat ovaries will secrete substantial quantities of androgen in the presence of LH *in vitro* (Erickson 1983). Thus, it appears that the GC and theca cells in immature follicles, which do not produce appreciable amounts of androgens and E_2 *in vivo*, have an abundant steroidogenic capacity when removed from the *in vivo* milieu. These observations have led to the development of a model suggesting that one or more factors within the follicular microenvironment most likely restrain E_2 production in less immature (preantral) GC by directly impairing CYP19 and/or blocking the production of theca cell androgens. Although some of these factors, such as growth differentiation factor-9 and bone morphogenetic protein-15 are produced and secreted by oocytes (Vitt *et al.* 2000, Matzuk *et al.* 2002, McNatty *et al.* 2005), somatic cell-derived factors, including HGF, regulate follicular cell proliferation, differentiation, and steroidogenesis. This notion is supported by the fact that many intraovarian growth factors have been identified, including HGF, which modulate both FSH-directed E_2 synthesis in GC and LH-supported androgen production in theca cells (Magoffin 1991, Terranova 1991).

As shown by Zachow *et al.* in immature rat GC *in vitro*, HGF blocked E_2 production that was stimulated by FSH and FSH in the presence of IGF-I (Zachow & Woolery 2002). Interestingly, while HGF did not suppress FSH-stimulated E_1 production in rat GC, HGF did block the aromatization of androstenedione into E_1 which is promoted by FSH and IGF-I (Zachow & Woolery 2002). These effects were correlated to changes in CYP19 expression and activity, as well as modulation of the level of 17 β -HSD type 1 mRNA; indicating that, dependent upon the hormonal milieu, HGF can block the metabolism of androstenedione at one or more loci within the estrogenic pathway (Parrott & Skinner 1998a, Zachow *et al.* 2000).

A recent and interesting observation supported the above evidence that HGF suppresses E_2 production in immature follicles. In GC harvested from human follicles from non-IVF patients, HGF impaired E_2 production in GC isolated from small diameter preantral follicles. Whereas, HGF did not significantly impair FSH-dependent E_2 production in GC harvested from the larger, antral human follicles (Zachow unpublished observations; Fig. 1). This indicates that the suppressive effects of HGF are sensitive to the state of GC maturation since different effects were detected in GC from small versus large diameter follicles. In addition, in the GC that were harvested from younger follicles, HGF did not impair E_2 production in the presence of the highest dose of FSH tested (Fig. 1). One interpretation of these observations is that HGF dampens E_2 synthesis in immature GC (i.e., those harvested from immature follicles); however, as GC mature (i.e., those harvested from large diameter follicles), and as FSH concentrations rise, yet-to-be defined mechanisms impair the ability of HGF to attenuate FSH-directed E_2 production in GC. One plausible candidate for this downregulatory effect is at level of c-met content in GC, since FSH, HGF, and eCG each down-modulated c-met mRNA or protein *in vivo* and in GC cultures as described above (Zachow *et al.* 2000, Uzumcu *et al.* 2006). These observations further support the hypothesis that a developmental switch, perhaps at the level of c-met expression, occurs in GC which overcomes or prevents the suppressive effect of HGF on E_2 secretion. This concept also fits within the physiologic context of what occurs during folliculogenesis *in vivo* (i.e., a switch from non-estrogenic to estrogenic GC); and the identification of HGF as a modulator of this intricate and tightly regulated process is compelling.

HGF signaling mechanisms in GC Relatively few studies have attempted to reveal the intracellular mechanisms of action used by HGF in GC. As reported by Zachow & Woolery (2002), the HGF-induced block in FSH-supported E_2 synthesis was prevented by dibutyryl-cAMP or the cAMP phosphodiesterase inhibitor, isobutylmethylxanthine. Thus, it appeared that HGF down-modulated the FSH-directed cAMP signaling cascade upstream from cAMP production or by promoting the degradation of cAMP. Further investigations revealed that in rat GC, cAMP production was indeed impaired by HGF; whereas, cGMP production was elevated by HGF. The latter effect is interesting since the induction of cGMP signaling impairs FSH-stimulated E_2 production in rat GC (LaPolta & Hong 1995); although a direct link between HGF-induced cGMP production and its inhibitory effect of E_2 synthesis has not been reported. In addition, the activities of FSH-dependent cAMP and cGMP phosphodiesterases were each reduced by HGF in a time-dependent manner (Zachow & Woolery 2002). The best interpretation of these data is that a complex array of effects leading to cyclic nucleotide production and degradation in GC are modulated by HGF; however, further research is required to couple these mechanisms with the observed HGF-stimulated changes in steroidogenesis.

In addition to its modulatory actions on E_2 and progesterone production, HGF blocks apoptosis in cultured rat follicles and isolated immature rat GC (Uzumcu *et al.* 2006). Although not linked within the same study, it was discovered that HGF caused biphasic increases in the steady-state level of Akt1 mRNA, and as described above, HGF increased cGMP content in GC (Zachow *et al.* 2000,

Figure 1 A model for HGF in promoting folliculogenesis. Upon recruitment into a growing cohort, follicles undergo an orderly course of morphogenesis that involves cell proliferation and functional differentiation into a steroidogenic tissue. Follicles destined for ovulation are selected from the growing cohorts; whereas, apoptosis within non-selected follicles leads to atresia. Data show that HGF blocks gonadotropin-stimulated androgen and E_2 production in TC and GC respectively. When combined with the anti-apoptotic effects of HGF that have been shown in immature rat GC, this concomitant mechanism would support the viability of younger follicles; it is proposed that this control system is functional in follicles destined for selection. FSH has been shown to reduce c-met expression in GC, and eCG lowers c-met content in the GC within large antral follicles *in vivo* as described in this review. It follows that a downregulation in c-met content would diminish HGF bioactivity in the dominant follicle; thereby releasing the production of androgens, CYP19 activity, and the secretion of E_2 which is a vital feedback vehicle for triggering ovulation. TC, theca cells; GC, granulosa cells; and O, oocyte.

Zachow & Woolery 2002). This is potentially significant in that the activation of PI-3K/Akt- and cGMP-dependent signaling mechanisms block apoptosis in GC (Westfall *et al.* 2001, Peluso & Pappalardo 2004). Collectively, these findings imply that the anti-apoptotic effect of HGF within GC may be mediated by the induction of PI-3K/Akt and/or cGMP-dependent signal transduction. A review of some signaling mechanisms that are used by HGF is presented in Fig. 2.

With these data assembled, it is apparent that HGF can control every major component of folliculogenesis: 1) theca cell and GC steroidogenesis, 2) theca cell and GC growth and differentiation, and 3) GC apoptosis (reviewed in Table 1). Collectively, these effects strengthen the credibility for an *in vivo* function of HGF in supporting the progression of folliculogenesis from preantral growth to selection of the dominant follicle(s) and ovulation. One attractive model that is supported by the available data centers around shifting levels of c-met and HGF between theca cells and GC as folliculogenesis proceeds. As shown in Fig. 3, during early follicle development, the steroid modulatory effects of HGF would keep the intraovarian concentrations of androgens and E_2 at bay. In more mature antral follicles, a gonadotropin-directed reduction in c-met content in GC would alleviate the anti-estrogenic effect of HGF, and allow FSH-dependent E_2 production to proceed unimpeded. Since HGF blocked LH-stimulated androgen production in theca cells, yet theca cells of large antral follicles contain c-met and HGF, yet-to-be identified mechanisms must arrest the reported HGF-dependent block in androgen synthesis during advanced

folliculogenesis. Of interest would be the identification of intrafollicular growth factors and/or further elaboration of the intraovarian HGFA and HAI mechanisms which could block the suppressive effect of HGF on theca cell androgen production.

Since an expanding body of experimental evidence indicates that HGF is a key regulatory factor of ovarian function, it is plausible that a loss of intraovarian HGF responsiveness would severely disrupt follicle development and thereby induce female reproductive failure. When all of these data are considered, one glaring question remains: is an intraovarian HGF system required for normal ovarian function to occur *in vivo*? This question cannot be definitively answered using *in vitro* methods; however, knockout transgenics provide a promising technology to address this issue. Importantly, homozygous null mutant mice, which lack either the endogenous production of HGF or c-met, die during gestation due to placental and developmental defects (Uehara *et al.* 1995). Thus, in order to define the intraovarian function of HGF *in vivo*, a cell-specific, conditional knockout approach is a plausible and attractive method which could address these questions.

The testis

Many parallel mechanisms exist when comparing ovarian and testicular function. Like the ovary, the testis comprises distinct cell populations which work in concert to control fertility. Testicular cells include germ cells, as well as Leydig and Sertoli

Figure 2 A summary of HGF-dependent signal transduction. Shown are some of the signaling cascades known to be activated by HGF. The co-localization of HGFA (as detected in growing rat follicles) and HAI proteins in tissues concomitantly expressing HGF and c-met suggests a mechanism for the local modulation of HGF bioactivity. The initial steps of HGF-directed signal transduction involve HGF binding to c-met and the subsequent autophosphorylation of residues Y1349 and Y1356 within the c-met carboxyl region. Shaded arrows show cascades affected by HGF within GC. HGF modulates cAMP and cGMP content and cyclic nucleotide phosphodiesterase (PDE) activity in GC. These effects occur during the time course for HGF-induced suppression of FSH- and FSH + IGF-I-stimulated E_2 synthesis. Also, HGF increases the steady-state level of Akt1 mRNA in GC; this effect, taken together with increased cGMP content, may mediate the observed anti-apoptotic effect of HGF in GC, although this link has not been confirmed. In non-ovarian cells, HGF mobilizes many signaling molecules including the Ras-MAPK and Ca^{2+} -phospholipid-dependent signaling cascades which have been linked with HGF-orchestrated changes in apoptosis, cell cycle progression, and proliferation. The recruitment of adaptor proteins (Gab1, Ras, Rac, and PI-3K) to the multifunction docking site within the c-met PTK domain has been coupled to HGF-dependent morphogenesis.

Table 1 Expression and effects of the hepatocyte growth factor (HGF) system within theca and granulosa cells

Cell	Presence of HGF, c-met, and HGF	Effect of HGF on steroid gene expression	Effect of HGF on steroidogenesis	Effect of HGF on proliferation	Effect of HGF on apoptosis	HGF-dependent signaling
Theca	Rat: temporal/eCG-dependent changes in HGF, c-met, HGFα proteins (<i>in vivo</i>); c-met mRNA detected. Human: c-met, Pig: c-met mRNA detected.	Rat: (–) CYP17 mRNA; NE on CYP11A and 3β-HSD	Rat: (–) LH-stimulated androstosterone, (+) basal and LH-stimulated P ₄	Cow: (+) Rat: NE	Not reported	Not reported
Granulosa	Rat: temporal/eCG-dependent changes in HGF, c-met, HGFα proteins <i>in vivo</i> ; c-met mRNA (–) by HGF and FSH, c-met mRNA (+) by db-cAMP. Human, cow, rat, mouse: c-met mRNA detected.	Rat: (–) FSH-dependent CYP19 and 17β-HSD1 expression	Rat: (–) FSH- and FSH + IGF-I-stimulated E ₂ , NE on P ₄ . Human: stimulated E ₂ P ₄ , (–) FSH-stimulated E ₂ in small follicles, NE in large follicles. Cow: (–) hCG-stimulated P ₄	Cow: (+) Rat: NE	Rat: (–)	Rat: (–) FSH-stimulated cAMP content; (+) cGMP content; (–) cyclic nucleotide PDE activity; (+) Akt1 expression

(+), stimulated, increased; (–), blocked, reduced; NE, no effect; db-cAMP, dibutyl-*l*-cAMP; PDE, phosphodiesterase; eCG, equine chorionic gonadotropin; hCG, human CG.

cells. Leydig and Sertoli cells respond to LH and FSH respectively, to produce androgens (testosterone), E₂, several growth factors, and cytokines which control testicular differentiation and function (Bornstein *et al.* 2004, Haider 2004, Rao 2005, Itman *et al.* 2006, Mackay & Smith 2007).

The precise function of testicular E₂ remains debatable, and has been thoroughly reviewed elsewhere (Sierens *et al.* 2005, Carreau *et al.* 2006). However, through the collective production of testosterone, several growth factors (including HGF), and other proteins, Leydig and Sertoli cells (like ovarian theca cells and GC) cooperate to support germ cell maturation, and are thus vital for male reproductive health.

In addition to Sertoli and Leydig cells, peritubular myoid cells represent another somatic cell type which is involved in epithelial–mesenchymal interactions within the testis. During embryonic gonadal development, peritubular myoid cells differentiate from cells that migrate from neighboring mesonephros, which is essential for normal testicular cord formation (Tilman & Capel 1999). During postnatal life, peritubular myoid cells provide normal tubule morphology and contractility, processes that are critical for spermatogenesis (Zhang *et al.* 2006). Peritubular myoid cells and Sertoli cells interact with each other through growth factor-mediated feedback; these cells collaborate to produce extracellular matrix components which comprise the basement membrane that separates these two cell types (Skinner *et al.* 1985, Skinner 1991).

The ontogeny of HGF and c-met expression during testicular development

As with the ovary, the testis contains an endogenous HGF system, with a pattern of expression for HGF and c-met which is species specific. Although there are no published reports to date showing the ontogeny of HGF and/or c-met expression during ovarian development, changes in HGF and c-met expression during embryonic and postnatal testicular development have been described (Catizone *et al.* 1999, 2005, Ricci *et al.* 1999, 2002, 2006).

HGF expression starts in the mesonephros and coelomic epithelium of the undifferentiated male urogenital ridge as early as 11.5 day post-coitum (dpc) in the mouse (Ricci *et al.* 2002), and remains within the interstitium (presumably in peritubular myoid cells). Although c-met is first expressed inside the cords starting on 12.5 dpc, it is not clear at this time whether c-met is expressed by Sertoli or germ cells within cords. However, based on the classical paracrine model proposed for the HGF/c-met system (suggesting that HGF is produced by mesenchymal cells and acts on epithelial cells), Sertoli cells are the likely target for HGF within the cords. Ricci *et al.* (2006) reported that in the mouse at 13.5 and 15.5 dpc, c-met was mainly present within the testicular cords, and by 18.5 dpc, c-met was more localized to the interstitium (presumably fetal Leydig cells) and is no longer expressed in the cords (Table 2).

In the rat, however, c-met mRNA and immunoreactive protein were detected in the postnatal testis on day 10, with expression appearing in Sertoli cells on day 25, and increasing

Figure 3 Effect of HGF on E_2 production in GC from small (A) versus large diameter (B) human follicles. Follicles were isolated from normal human ovaries following removal for non-ovarian pathology. GCs were harvested by follicle puncture and incubated for 48 h in the presence of testosterone with and without FSH + HGF. Specific RIA was used to measure the level of E_2 in conditioned media. Values shown are the mean $\pm$ S.E.M. from triplicate treatments from two independent experiments. Significantly different ($P \leq 0.05$) levels of E_2 (HGF versus control) were determined using one-way ANOVA followed by Tukey's test and are indicated by an asterisk.

at day 35 (Catizone *et al.* 2001). In another study using 15.5 and 18.5 dpc mice, immunoreactive HGF was detected within the tunica albuginea, interstitium, and peritubular myoid cells, but was not found within Leydig cells (Ricci *et al.* 2006). In the postnatal rat testis, HGF is produced by myoid cells, and intratesticular HGF was purported to stimulate c-met expression in Sertoli cells (Catizone *et al.* 2005).

In contrast to the diverse palette of regulatory functions which have been shown for HGF within ovarian follicle cells, within the testis, HGF bioactivity appears to be relatively more specific to critical morphogenic effects during embryonic and postnatal development (Catizone *et al.* 2001, 2002, 2005, 2006, Ricci *et al.* 2006). Noteworthy is the observation that germ cells express c-met in human and rat testes (Depuydt *et al.* 1996, Catizone *et al.* 2006). Interestingly, using the adenoviral-mediated overexpression and a postnatal testis tissue culture system, HGF was shown to block apoptosis and stimulate proliferation of germ cells in the rat testis (Goda *et al.* 2004, Catizone *et al.* 2006). These findings show similarities with the proposed anti-apoptotic function of HGF within ovarian GC described above (Uzumcu *et al.* 2006). If such an effect occurs under normal conditions *in vivo*, this would indicate an essential role of the intratesticular HGF system in controlling male fertility by affecting fetal testicular development and adult testicular function.

We propose that another similarity may exist between the roles of the HGF system in the ovary and the testis if it is found that HGF controls prepubertal E_2 production in Sertoli cells. To elaborate, HGF and c-met are not expressed in Sertoli cells on postnatal day 10 (Catizone *et al.* 1999, 2001) when Sertoli cells are capable of producing a greater quantity

of E_2 (Le Margueresse & Jegou 1988). Whereas on postnatal day 25, Sertoli cells first show the expression of HGF and c-met (Catizone *et al.* 2001, 2005); interestingly, this is the time when E_2 production by Sertoli cells is reduced (Le Margueresse & Jegou 1988). Since the HGF-dependent suppression of E_2 was observed in rodent and human GC, it is plausible that HGF may control this aspect of the Sertoli differentiation; a novel mechanism which is worth further investigation.

HGF and Sertoli cells

In response to FSH, testosterone, and growth factors, Sertoli cells maintain a microenvironment that is conducive for spermatogenesis (Griswold 1998, Huleihel & Lunenfeld 2004, Walker & Cheng 2005). During embryonic life, sex-determining region of Y chromosome (Sry) expression initiates Sertoli cell differentiation leading to testis development (Sinclair *et al.* 1990). Paracrine growth factors expressed in the differentiating testis mediate the action of Sry. For example, FGF-9 exerts a significant role in critical cellular events during testis differentiation, such as mesonephric cell migration, Sertoli cell proliferation, and ultimately cord formation (Schmahl *et al.* 2004). In the absence of FGF-9, testicular abnormalities ranging from testicular hypoplasia to sex reversal occur in XY (i.e., genetically male) embryos (Colvin *et al.* 2001). Platelet-derived growth factors (PDGFs; Colvin *et al.* 2001, Uzumcu *et al.* 2002b, Brennan *et al.* 2003) constitute another group of paracrine growth factors which function in Leydig cell differentiation as well as other cellular events downstream from Sry activation (Brennan *et al.* 2003).

Table 2 Ontogeny and functions of c-met and hepatocyte growth factor (HGF) in the prenatal (mouse) and postnatal (rat) testis

	Fetal (dpc)				Post-natal (d)				
	12.5	13.5	15.5	18.5	10	20	25	30–35	60
Cells									
Sertoli	c-met, (C) (Ricci <i>et al.</i> 1999)	c-met, (C) (Ricci <i>et al.</i> 1999)	c-met, (C) (Ricci <i>et al.</i> 2006)	(–) (Ricci <i>et al.</i> 2006)	(–) (Catizone <i>et al.</i> 2001)	(–) (Catizone <i>et al.</i> 1999, 2001)	c-met (Catizone <i>et al.</i> 2001) HGF (Catizone <i>et al.</i> 2005)	c-met (Catizone <i>et al.</i> 2001) HGF (Catizone <i>et al.</i> 2005)	
Germ	c-met, (C) (Ricci <i>et al.</i> 1999)	c-met, (C) (Ricci <i>et al.</i> 1999)	c-met, (C) (Ricci <i>et al.</i> 2006)	(–) (Ricci <i>et al.</i> 2006)	c-met (Catizone <i>et al.</i> 2006)	c-met (Catizone <i>et al.</i> 2006)		c-met (Catizone <i>et al.</i> 2006)	c-met (Catizone <i>et al.</i> 2002)
Myoid	(–) (Ricci <i>et al.</i> 1999)	(–) (Ricci <i>et al.</i> 1999) HGF, (I) (Ricci <i>et al.</i> 2002)	(–) (Ricci <i>et al.</i> 2006) HGF, (I) (Ricci <i>et al.</i> 2002)	(–) (Ricci <i>et al.</i> 2006) HGF, (I) (Ricci <i>et al.</i> 2006)	c-met (Catizone <i>et al.</i> 2001) HGF (Catizone <i>et al.</i> 2005)	c-met (Catizone <i>et al.</i> 1999) HGF (Catizone <i>et al.</i> 2005)		c-met (Catizone <i>et al.</i> 2001) HGF (Catizone <i>et al.</i> 2005)	
Leydig	(–) (Ricci <i>et al.</i> 1999)	(–) (Ricci <i>et al.</i> 1999)	(–) (Ricci <i>et al.</i> 2006)	c-met (Ricci <i>et al.</i> 2006)	c-met, (I) (Catizone <i>et al.</i> 2001)	c-met, (I) (Catizone <i>et al.</i> 1999)		c-met (Catizone <i>et al.</i> 2001)	
Shown or proposed functions	Cord formation and development (Ricci <i>et al.</i> 1999)			Fetal Leydig cell steroidogenesis (Ricci <i>et al.</i> 2006)	Germ cell survival, proliferation, and motility (Catizone <i>et al.</i> 2002, 2006, Naz <i>et al.</i> 1994) Seminiferous tubule formation & Sertoli cell differentiation (Catizone <i>et al.</i> 2001, 2005, van der Wee & Hofmann 1999) Leydig cell survival and steroidogenesis (Del Bravo <i>et al.</i> 2007)				

C, cords; I, interstitial cells; (–), c-met not detected; dpc, day post-coitum.

The inhibition of PDGF bioactivity or deletion of PDGFR- α causes abnormal cord formation (Uzumcu *et al.* 2002b, Brennan *et al.* 2003).

Additional paracrine factors with important functions in the developing testis are neurotrophins (Cupp *et al.* 2000, Levine *et al.* 2000), TGF- β (Cupp *et al.* 1999), VEGF (Bott *et al.* 2006), kit ligand (Vincent *et al.* 1998), and glial cell line-derived neurotrophic factor (GDNF; Meng *et al.* 2000). The roles of these growth factors in promoting testicular differentiation and development have been extensively reviewed elsewhere (Brennan & Capel 2004, Mackay & Smith 2007).

HGF is somewhat unique among growth factors. Unlike the growth factors mentioned above that are mainly products of Sertoli cells (an epithelial cell type) and act on mesenchymal cells, HGF is primarily produced by myoid cells (a mesenchymal cell type), especially during embryonic development. Thus, when considering communication between Sertoli and myoid cells, HGF represents an interesting growth factor because it mostly acts in the mesenchymal-to-epithelial direction. The identification of c-met within Sertoli cells enforces the validity of this model.

The pattern of c-met expression within the cords during embryonic life is not exactly known, but beginning sometime between postnatal days 20–25 (in the rat), Sertoli cells contain functional c-met protein (Catizone *et al.* 2001). In dissociated rat Sertoli cells *in vitro*, the administration of exogenous HGF induces the formation of structures resembling testicular seminiferous cords (Catizone *et al.* 2001). This experimental observation led to the notion that HGF may be instrumental in testicular lumen formation in mature animals (Catizone *et al.* 2001). It is also imperative to consider that testicular cord formation is a major morphologic event during embryonic gonad development in the male (Uzumcu *et al.* 2002a,b). This process requires Sertoli cell growth and differentiation, as well as mesonephric cell migration (reviewed in Yao *et al.* 2002), changes which may be mediated by HGF (Ricci *et al.* 1999). Overall, the available evidence then suggests that HGF is one of several growth factors (including TGF- β , FGF-9, PDGF, neurotrophins, GDNF, VEGF and others) with an important role in development and function of the testis including cord formation and Sertoli cell differentiation.

HGF and myoid cells

It seems that HGF has two different modes of action depending upon the stage of development. Initially, during the embryonic developmental stage, the mode of action used by HGF is essentially paracrine. Thus, HGF from myoid cells targets cells inside the seminiferous cords (i.e., Sertoli cells and/or germ cells) between 12.5 and 15.5 dpc, and then acts on fetal Leydig cells on 18.5 dpc (Ricci *et al.* 1999, 2002, 2006). However, a switch occurs during postnatal development, when HGF activity becomes primarily autocrine, with some paracrine function still operational.

Both HGF and c-met are expressed in myoid cells prior to postnatal day 25. After day 25, the expression of HGF and

c-met appears in Sertoli cells, while the expression of HGF and c-met in myoid cells is relatively subsided (Catizone *et al.* 1999, 2001, 2006). In addition to myoid and Sertoli cells, c-met mRNA is also present in germ cells between postnatal days 10 and 60 (Catizone *et al.* 2002, 2006), and in Leydig cells in adolescent rats (days 30–32; Del Bravo *et al.* 2007).

HGF and Leydig cells

The testicular correlate of the ovarian theca cell is the Leydig cell. As such, Leydig cells do not express FSH receptors, but do express LH receptors, and are stimulated by LH to produce testosterone as the predominant steroid. Beginning around fetal day 18.5, c-met is expressed within the mouse testicular interstitium; and organ culture of fetal day 18.5 testes revealed that HGF stimulated basal testosterone secretion (Ricci *et al.* 2006). Postnatal (days 30–32) rat Leydig cells also express immunoreactive c-met, and HGF stimulates basal testosterone secretion and inhibits apoptosis in rat Leydig cells as well as testicular tissue explants (Del Bravo *et al.* 2007).

Proposed regulatory function for HGF in the testis

What is apparent is that shifts in the expression of c-met and HGF between Sertoli cells, germ cells, Leydig cells, and myoid cells during fetal and postnatal testicular growth and differentiation suggest that HGF guides changes in testicular morphogenesis and function (including steroidogenesis) in a developmentally regulated manner. This type of model overlaps with that proposed for HGF during folliculogenesis. One hypothesis which can be derived from these data from the testis is that HGF promotes Sertoli cell differentiation and cord formation during *in utero* development, but then HGF control switches to the regulation of Leydig cell steroidogenic differentiation during the last stages of fetal development and preceding sexual maturity. Beginning around the time of puberty (i.e., day 25 in the rodent), Sertoli cells resume the expression of c-met (Table 2), and once again gain HGF responsiveness. It is possible that, in the prepubertal male, HGF controls Sertoli cell function at multiple levels, to potentially include the modulation of FSH-stimulated E₂ production and cell growth; this, however, remains uncertain pending future studies. By regulating Sertoli cell function, HGF would indirectly control sperm growth and maturation, in addition to the direct effects of HGF on sperm function.

The stimulatory effect of HGF on steroid production in postnatal Leydig cells has recently been reported (Del Bravo *et al.* 2007). Based upon the most current data, HGF directly stimulates rat Leydig cell steroidogenesis, although mature human Leydig cells do not express c-met (Depuydt *et al.* 1996, Catizone *et al.* 2001). Considering that any effects of HGF are plausibly species specific, as has been documented with the ovarian HGF system, this difference is not surprising. What has been substantiated is that, collectively, the intratesticular HGF system has several potentially vital roles, those being the regulation of 1) seminiferous cord formation and development,

2) Leydig cell steroidogenesis and survival, 3) Sertoli cell proliferation and terminal differentiation, and 4) germ cell function and/or survival. To date, the ability of HGF to control testicular function *in vivo* (i.e., male reproductive viability) has not been reported. Considering that the either HGF or c-met null mutations are embryonic lethal (as described above), a tissue-specific conditional knockout approach is needed to establish the importance of the HGF system in regulating testis development and function *in vivo*.

Concluding thoughts

Data from several laboratories have shown that HGF controls many key functions within the ovary and testis; these range from modulation of both steroidogenesis (Parrott *et al.* 1994, Zachow *et al.* 1997, 2000, Ito *et al.* 2001, Ricci *et al.* 2006, Del Bravo *et al.* 2007) and apoptosis (Goda *et al.* 2004, Uzumcu *et al.* 2006, Del Bravo *et al.* 2007), to guiding mitosis and morphogenesis (Parrott & Skinner 1998b, Catizone *et al.* 2001, 2006, Ricci *et al.* 2002, 1999). The breadth of the HGF-mediated control mechanisms strongly indicates that an intact HGF system is required for proper gonad development and/or function; this would of course make HGF a key regulator of female and male fertility. The next step in determining this is to blend *in vitro*, molecular mechanism studies with an elucidation of the role of HGF in regulating reproductive function *in vivo*.

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