Interplay Between mTOR and Hippo Signaling in the Ovary: Clinical Choice Guidance Between Different Gonadotropin Preparations for Better IVF

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One of the most widely used types of assisted reproduction technology is the *in vitro* fertilization (IVF), in which women undergo controlled ovarian stimulation through the administration of the appropriate hormones to produce as many mature follicles, as possible. The most common hormone combination is the co-administration of gonadotropin-releasing hormone (GnRH) analogues with recombinant or urinary-derived follicle-stimulating hormone (FSH). In the last few years, scientists have begun to explore the effect that different gonadotropin preparations have on granulosa cells' maturation and apoptosis, aiming to identify new predictive markers of oocyte quality and successful fertilization. Two major pathways that control the ovarian development, as well as the oocyte–granulosa cell communication and the follicular growth, are the PI3K/Akt/mTOR and the Hippo signaling. The purpose of this article is to briefly review the current knowledge about the effects that the different gonadotropins, used for ovulation induction, may exert in the biology of granulosa cells, focusing on the importance of these two pathways, which are crucial for follicular maturation. We believe that a better understanding of the influence that the various ovarian stimulation protocols have on these critical molecular cascades will be invaluable in choosing the best approach for a given patient, thereby avoiding cancelled cycles, reducing frustration and potential treatment-related complications, and increasing the pregnancy rate. Moreover, individualizing the treatment plan will help clinicians to better coordinate assisted reproductive technology (ART) programs, discuss the specific options with the couples undergoing IVF, and alleviate stress, thus making the IVF experience easier.

Keywords: granulosa cells, r-hFSH, r-hLH, HP-hMG, ovarian stimulation, PI3K/mTOR/Akt, Hippo

INTRODUCTION

The theory that FSH and luteinizing hormone (LH) are both required for the complete stimulation of follicular maturation and steroidogenesis was put forward 60 years ago from the Swedish scientist Bengt Falck (1). This idea was the basis for the stimulation of both hormonal systems for optimal follicular growth and maturation in IVF programs. Nowadays, ovarian stimulation during IVF includes the co-administration of GnRH analogues with the gonadotropins FSH, LH, and human chorionic gonadotropin (hCG).

A major drawback in IVF approaches is that the percentage of successful pregnancies is still low – approximately 27% pregnancies per IVF treatment in Europe (2, 3). Moreover, there is still a need for interventions to improve the initial recruitment and later survival of follicles to ensure good quality oocytes in healthy women, as well as in patients with poor ovarian response (POR), primary ovarian insufficiency (POI), or polycystic ovary syndrome (PCOS). Many studies compare the effects of different FSH-containing gonadotropin preparations in ovarian stimulation and IVF cycle outcomes, namely highly purified urinary human menopausal gonadotropin (HP-hMG) containing both FSH and LH activity, and recombinant human FSH (r-hFSH) alone or in combination with recombinant human LH (r-hLH). However, in most cases, the results are contradictory and inconclusive, and have led to controversial interpretations regarding the effectiveness of these gonadotropin regimens on follicular growth, antral follicle count, total oocytes retrieved, 2 pronuclear stage (2PN) oocytes, number of embryos, clinical pregnancy, and live birth rates in IVF (4–9). A pioneering study, a few years ago, demonstrated that the r-hFSH/r-hLH combination was more effective compared to HP-hMG, when the number of retrieved oocytes was high, also with regard to pregnancy rate per embryo transfer (10). Importantly however, a critical component of the stimulation regimens in IVF is the administration of a GnRH analogue, either agonist or antagonist to control the premature LH surge (11). Accordingly, an increasing number of studies reveal that the efficacy and the clinical outcomes of the different gonadotropin regimens appear to be dependent also, on the GnRH protocol used (9, 12–18). It is well known that the GnRH analogues can activate specific signal transduction pathways leading to distinct biological responses (19). Apparently, these treatments can alter the hormonal milieu, thereby favoring or hindering embryo quality and pregnancy rate (20). It is pertinent to note that FSH through binding to its cognate receptor FSHR (21), regulates the proliferation and differentiation of granulosa cells and prepares them to respond to gonadotropins and other endocrine signals, in order to undergo their final maturation. FSH is a glycoprotein, and it was recently shown that the hypo-glycosylated forms might be more efficient in promoting follicular growth and supporting granulosa cell survival *in vivo*, possibly by increasing serum estradiol levels (22). Interestingly, young women express partially glycosylated FSH whereas postmenopausal women express mainly the fully glycosylated form (23, 24), and this might influence both the biochemical properties and the efficacy

of the various FSH preparations (25). This issue has been thoroughly discussed in a Delphi Consensus study recently (26).

THE DIFFERENTIAL EFFECTS OF GONADOTROPIN TREATMENTS ON OOCYTE – GRANULOSA CELL COMMUNICATION AND FOLLICULAR MATURATION

Considering the vital role of granulosa cells in oocyte and follicle maturation, scientists have sought to investigate the influence of gonadotropin treatment on granulosa gene expression profiles. For example, the administration of r-hFSH, in comparison to HP-hMG (27) has been associated with higher expression of LH receptors and enzymes involved in the biosynthesis of steroids, and with lower mRNA levels of the FSH receptors in the granulosa cells (28). The presence of the FSH ligand (in cultured rat and bovine granulosa cells) leads to follicular activation and steroidogenesis, through the action of the highly conserved phosphoinositide-3 kinase (PI3K)/Akt/mammalian (or mechanistic) target of rapamycin (mTOR) and Hippo signaling pathways (29–31). The dysregulation of these pathways leads to increased apoptosis in ovarian cells (32, 33). Importantly, the incidence of apoptosis in granulosa cells has been linked to the quality of the oocytes and to the pregnancy outcome (34–36). There is some evidence indicating that the administration of HP-hMG increases the apoptosis of cumulus cells compared to r-hFSH or urinary FSH (37), and a recent study showed that high doses of r-hFSH suppress the apoptosis of granulosa cells in patients with endometriosis undergoing IVF (38). Therefore, researchers are currently exploring the consequences of the different protocols of gonadotropin ovarian stimulation on the apoptosis rate of granulosa cells (35, 39). However, in the ART clinical setting, more upstream effectors need to be considered since follicular growth is a dynamic and continuous process, characterized by a tightly regulated equilibrium between apoptosis and cell proliferation. For example, recently, it was elegantly shown that the FSH receptor synergizes with the G protein-coupled estrogen receptor (GPER), hence reprogramming FSH-induced death signals to proliferative stimuli that are important for nourishing oocyte survival (40). Heterodimerization of GPER with FSHR in granulosa cells switches the signaling mode from cAMP to pAKT activation, thereby positively affecting follicle maturation, and appears to correlate with the FSH responsiveness of patients undergoing IVF. This is particularly interesting, in light of evidence showing that estrogen can regulate Hippo signaling *via* GPER in breast tissue (41, 42). Accordingly, it might be more insightful to investigate the effects of the different gonadotropin preparations on the maturation of granulosa cells and the oocyte quality by monitoring the activity of the PI3K/Akt/mTOR and Hippo signaling cascades.

Although there are no studies yet comparing the effect of different gonadotropins on the Hippo pathway, there are data showing that r-hFSH and HP-hMG can differentially modulate the activities of the PI3K/Akt/mTOR signaling. For example, Ji et al., 2020 (43) using a GnRH antagonist protocol, observed that

HP-hMG resulted in significantly higher insulin-like growth factor-1 (IGF-1) levels compared to r-hFSH on the day of oocyte retrieval, an effect that has been associated with better oocyte quality and pregnancy rate (44, 45). Interestingly, this was not the case in earlier studies when a GnRH agonist protocol had been employed (20, 46). The insulin/IGF-1 signaling pathway regulates the PI3K/mTOR/p70S6K cascade which as mentioned above plays an essential role in the FSH-mediated development of granulosa cells (30, 47, 48). This is important, also in light of recent findings showing that the hypo-glycosylated form of FSH, which is less abundant in the pituitary of postmenopausal women, activates more efficiently the PI3K/mTOR/p70S6K signaling (22).

Adding to the complexity of these interactions is the fact that there are many other signaling cues converging on both pathways. For example, other growth factors in addition to insulin/IGF-1, such as EGF, PDGF or VEGF are potent regulators of the PI3k/Akt/mTOR signaling in the follicles (49). Moreover, steroid hormones, like androgens which are the precursors for estrogen production, and known to stimulate granulosa and theca cell proliferation and to promote early antral follicle growth, can also regulate the expression of both FSH and IGF-1 receptor genes (50, 51). Furthermore, complex disorders such as the PCOS syndrome can affect the activation of both mTOR and Hippo signaling pathways. The development of PCOS has been associated with Hippo disruption and YAP overactivation leading to multiple early antral follicles and theca hyperplasia (49, 52). In addition, the expression of mTOR is elevated in a DHEA-treated PCOS animal model that could lead to insulin resistance, which is a characteristic of the PCOS phenotype (53). Other pathological conditions, such as endometriosis and ovarian cancer can exert an impact on the mTOR pathway by altering the expression of its targets (54). Scientists have also noticed increased expression of YAP protein in mouse models with endometriosis whereas in mice treated with YAP inhibitors the endometriotic lesions were significantly decreased (55). Notably, the activation of mTOR pathway plays a fundamental role in the development of many autoimmune disorders (56), whereas Hippo signaling prevents autoimmunity and tissue damage (57, 58). In addition, vitamin D deficiency decreases mTOR activation in rat models (59) and human uterine fibroid cells (60). These are conditions that can influence the IVF outcomes (61–64). Future studies addressing the effects of the various gonadotropin combinations on the PI3k/Akt/mTOR and Hippo pathways in physiological conditions (including ageing) and disease states, are expected to increase our understanding of follicle development and develop personalized treatment plans that will help clinician's decision and improve the success rate of IVF.

THE INTERPLAY BETWEEN PI3K/AKT/mTOR AXIS AND HIPPO PATHWAY IN FOLLICULAR DEVELOPMENT

The PI3K/Akt/mTOR axis is a key regulator of survival that fosters the processes of proliferation and differentiation, and

inhibits apoptosis and autophagy (65, 66). The activation of this pathway is crucial for granulosa cell proliferation and follicular growth, especially during the primordial follicle development (67). Recent work from our lab revealed that the controlled pharmacological inhibition of the mTOR pathway in a rat experimental model can increase the number of competent primordial follicles while reducing atresia. Specifically, we showed that the follicles preserve their competence to resume growth two weeks after mTOR reactivation (68). Consistent with this, factors like Tsc1/2 and PTEN, which negatively regulate mTORC1, are capable to maintain the dormancy state of primordial follicles (69). Deregulation of these inhibitors leads to overactivation of the mTOR pathway that is linked to pathological situations where the entire pool of primordial follicles matures simultaneously resulting in an accelerated loss of primordial follicles and premature ovarian failure (POF) (70, 71). Over-activation of the mTOR pathway has been also associated with the emergence of PCOS and ovarian cancer (72). Importantly, however, there are no studies yet comparing the activation of mTOR pathway on granulosa cells obtained from IVF patients undergoing different protocols of gonadotropin stimulation.

Recent studies indicate that the Hippo signaling plays an instrumental role in the regulation of follicular growth. This pathway responds to mechanotransduction signals in order to maintain organ size through regulating cell proliferation and apoptosis (73, 74). The central components of the Hippo pathway are the kinases Mst1/2 and Lats1/2 which lead to the inactivation of its key downstream effectors Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) (75). When Hippo signaling is disrupted, YAP and TAZ translocate into the nucleus where they bind to the TEA Domain Transcription Factors (TEADs) promoting the expression of growth factors and apoptosis inhibitors (73, 76, 77). It has been reported that the development of primordial follicles is accompanied by an inhibition of the Hippo pathway (78, 79), while its overstimulation leads to a reduction in follicular proliferation and estrogen production in granulosa cells, both *in vivo*, and *in vitro* (80, 81). Before ovulation, oocyte-secreted factors contribute to the activation of YAP protein in granulosa cells stimulating their proliferation, whereas after ovulation, the Hippo pathway is transiently activated leading to YAP degradation, which allows the differentiation of granulosa cells into luteal cells and the production of progesterone (79).

There is an intrinsic mechanism that orchestrates the function of the mTOR and Hippo pathways through YAP and indirectly controls the granulosa cell–oocyte interactions. Interestingly, recent studies show that the communication of the Hippo pathway with the PI3K/Akt/mTOR axis and their coordinated regulation play a key role in follicular size and primordial maturity, through YAP and SMAD2/3 complex (48, 82, 83). Activation of the Akt/mTOR pathway using Akt stimulators in combination with inhibition of Hippo through ovarian fragmentation appears to increase the number of mature follicles in mouse models, but also in patients with POI or PCOS,

adjusting follicular growth and ovulation, thereby leading to successful fertilization and pregnancy (49, 52, 84, 85). Cytoskeleton remodeling is one of the key factors regulating Hippo signaling and promoting the nuclear localization of YAP/TAZ complex (86). Importantly, recent findings in mouse models show that hMG administration leads to activation of the mTOR pathway (87), and GnRH induces cytoskeleton reorganization (a key process for the synthesis and secretion of gonadotropins) by activation of the mTOR kinase (88). Actin cytoskeleton dynamics mediates vital roles, also, for oocyte meiotic cell divisions through Hippo and mTOR signaling (89–91). In early stage oocytes (germinal vesicle) YAP is predominantly located in the cytoplasm, whereas during the subsequent stages of oocyte development (metaphase I), YAP becomes activated and translocates into the nucleus, suggesting a role of Hippo signaling in oocyte maturation (92). In addition, the mTOR pathway plays fundamental role on oocyte meiotic maturation through the activation of translation of specific mRNAs involved in spindle morphology and chromosomal alignment (93, 94). Consistently, disruption of mTOR signaling inhibits spindle migration and asymmetric division in mouse oocytes (95).

Thus, it becomes evident from the above that a better understanding of the way that the different gonadotropin regimens affect the PI3K and Hippo pathways within the follicular environment in women with reduced ovarian reserve, polycystic ovary syndrome or advanced maternal age will allow their use as potential benchmarks for guidance of physicians regarding more efficient strategies for IVF (**Figure 1**).

CONCLUDING REMARKS

It is clear, that further randomized controlled studies are needed to investigate the effects of the different gonadotropin preparations in the IVF outcome, and importantly, to combine both clinical and molecular attributes in order to appreciate the ovarian biological underpinnings of the various treatments. A better knowledge of the effects of the various gonadotropin preparations on the activation of follicles will allow the elaboration of appropriate biomarkers which in turn will render it possible to evaluate the efficacy of the different stimulation protocols in *in vitro* fertilization in different groups of patients. Current evidence reveals the presence of an active
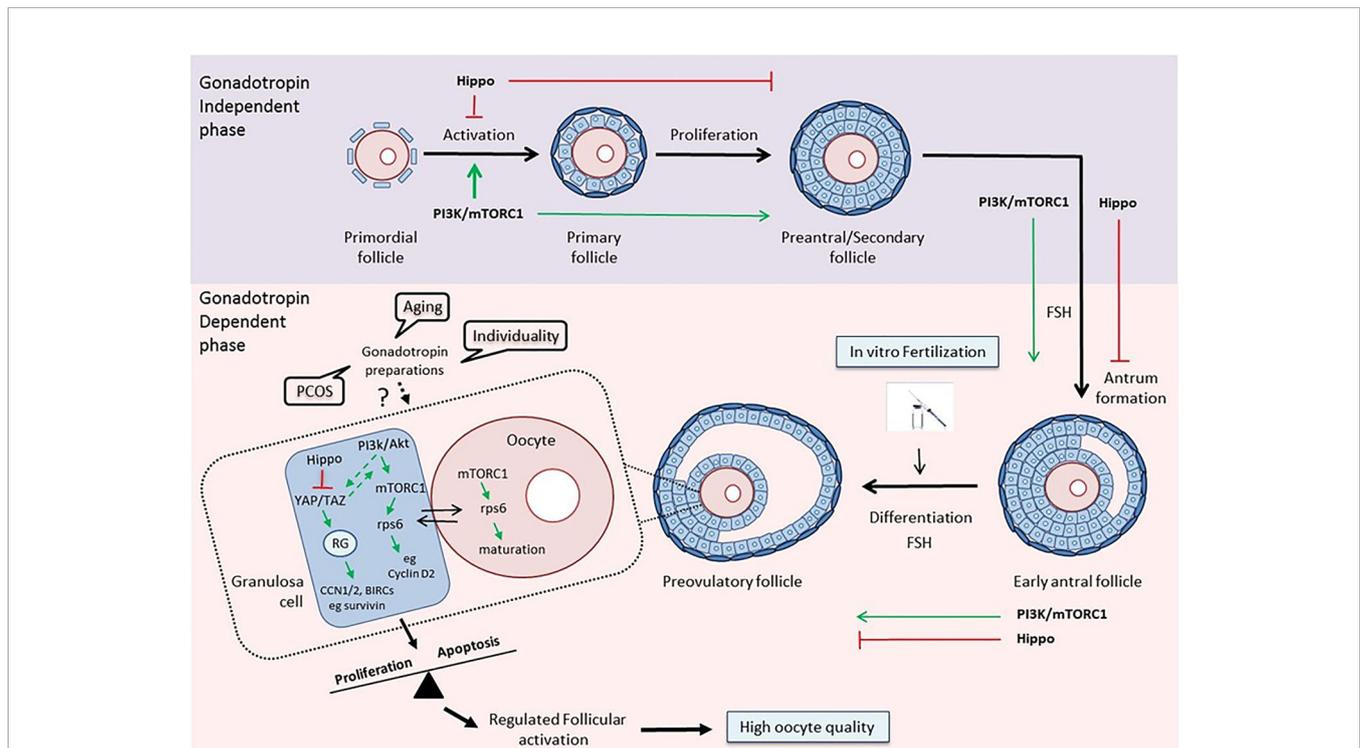
FIGURE 1 | The PI3K/mTOR/Hippo pathways as guidance for clinical decision-making. Top: The PI3K/Akt/mTOR and Hippo pathways exert opposite effects on follicular development during the gonadotropin-independent phase. Activation of the PI3K pathway is crucial for each growing stage of the follicle, especially at the primordial and primary stages (30, 94). The Hippo pathway acts in a coordinated manner with PI3K in order to accelerate primordial follicle activation and promote follicular development (48). Bottom: The two pathways maintain their concerted action on follicular development during the gonadotropin-dependent phase of follicular growth, and especially on the maturation of granulosa cells and oocytes in the preovulatory follicles, thereby assuring regulated follicular activation and high oocyte quality (79, 96). Various disease states, aging, and the uniqueness of each woman, by influencing this balance, may affect the response to different gonadotropin preparations, and consequently, the outcome of the IVF. The activation status of key components of the PI3K and Hippo pathways may serve as a prognostic or predictive biomarker that can help clinicians guide treatment planning. (RG, Regulatory Genes).

cross-talk between the PI3K/Akt/mTOR and the Hippo pathways, which is instrumentally involved in the regulated activation of primordial follicles, as well as, in follicular and oocyte growth. Consequently, a deeper understanding of the influence of the various ovarian stimulation protocols might exert on this interplay could help scientists to translate the emerging novel knowledge into clinical success and contribute to more efficient management of assisted reproduction methods. However, this is not an easy task. Despite the substantial progress in understanding ovarian follicular physiology, ART remains an inefficient process (97, 98). While the success rates of IVF/ART programs initially displayed an upward trend, the pregnancy and birth rates are declining in recent years (3). This issue has been thoroughly discussed by Norbert Gleicher and co-workers (99). Apparently, there are several causes, including potentially harmful add-ons to IVF practice, the woman's age that dramatically influences the responses to exogenous gonadotropin stimulation (100–102) but also an evolving industrialization and commoditization of IVF (99). Considering the heterogeneity of the infertile population, understanding the best gonadotropin regimen for a particular patient necessitates two prerequisites. On the one hand, a personalized tailored approach (103, 104) which implies that we need to understand the mechanisms by which the same protocol results in different outcomes in different women, for example by monitoring gene expression profiles (105–108). On the other hand, the international cooperation between fertility societies such as ESHRE (European Society of Human Reproduction and Embryology), ASRM (American Society for Reproductive Medicine), or IFFS (International Federation of Fertility Societies) as well as Delphi Consensus statements, which by continuing to periodically update progress in basic research and reinforcing the dissemination of evidence-based information can facilitate and foster the translation of basic research into clinical practice.

In the long term, the elaboration of more straightforward and simple testing procedures based on key signaling cascades governing granulosa cell biology will help clinicians to prevent their patients from unnecessary treatment, and hopefully, will lead to more effective and individualized treatment protocols to improve birth rates.

AUTHOR CONTRIBUTIONS

KP and TM contributed to text conception. TM wrote the manuscript and KP has generated the figure. All authors contributed to the article and approved the submitted version.

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