Paracrine support of ovarian stimulation

Stephen G. Hillier

Centre for Reproductive Biology, University of Edinburgh College of Medicine and Veterinary Medicine, Chancellor’s Building, 47 Little France Crescent, Edinburgh EH16 4TJ, UK

ABSTRACT: Assisted reproductive technology has evolved on the back of blunderbuss ovarian stimulation regimes designed to maximize the number of oocytes recoverable for treatment purposes. However, oocyte ‘quality’ is finely programmed by local paracrine and autocrine signalling events during folliculogenesis and can be adversely affected by inappropriate gonadotrophic stimulation. This brief review traces the full follicular lifespan—from initiation to ovulation—to identify gonadotrophin-responsive checkpoints likely to impact oocyte quality. It is argued that these might be targeted during controlled ovarian stimulation therapy to (i) increase responsiveness to FSH through follicular priming with LH or hCG, (ii) improve follicular synchrony and oocyte quality through conditioning with FSH and (iii) promote ‘gold standard’ pre-ovulatory maturation through follicular coating with LH or hCG. It is concluded that whereas there can be no one-size-fits-all approach to ovarian stimulation, treatment regimes based on paracrine principles and tailored to personal needs will always be more likely to achieve the desired outcome.

Key words: follicle development / ovary / gonadotrophins / inhibins and activins / oocyte

Introduction

Ovarian stimulation impacts Assisted Reproductive Technology (ART) through affecting the quantity and quality of oocytes available for use during treatment. Standard stimulation regimes typically use exogenous FSH or human menopausal gonadotrophin (hMG) to drive pre-ovulatory follicular development in conjunction with a gonadotrophin-releasing hormone analogue administered chronically (GnRH-agonist) or acutely (GnRH-antagonist) to suppress endogenous gonadotrophin release (Santos et al., 2009). Despite the ready availability of pure (recombinant) FSH, LH and hCG preparations with which to customize ovarian stimulation, standardized approaches using FSH alone or formulations based on hMG with fixed FSH:LH ratios predominate with attendant limitations to individual patient success (Verberg et al., 2009).

Advances in our understanding of the developmental and cell biology of folliculogenesis are delineating the follicular physiome through which somatic cellular functions influence oocyte development and vice versa (Li et al., 2008). It is becoming increasingly clear from pre-clinical studies and in-vitro modelling that oocyte ‘quality’—in terms of maternal contribution to embryogenesis and early development—is programmed before ovulation and therefore potentially amenable to endocrine and paracrine manipulation (Merton et al., 2003; Fortier et al., 2008; Santos et al., 2009). It is therefore surprising that so little attention has been paid to translating this basic science into clinical practice. This is all the more so given the many patient scenarios that are now potentially amenable to ART.

Here the natural follicular lifespan is tracked to locate paracrine checkpoints tractable to endocrine manipulation that are likely to influence oocyte quality. Emphasis is placed on signalling by members of the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors, which contains the quintessential gonadal paracrines activin and inhibin (Hillier, 1991; Chang et al., 2002; Knight and Glister, 2006). Armed with this knowledge, it is suggested that intelligent administration of FSH and LH activity (either LH or hCG) can allow fine-tuning of ovarian stimulation on a patient-by-patient basis to improve individual responses and benefit treatment outcome.

Lessons from physiology

During the 2–3 month journey from follicular activation to ovulation, human oocytes in growing follicles are continually exposed to instructional and nutritional signals—including steroids, growth and differentiation factors and intermediary metabolites—derived from surrounding somatic cells that programme their eventual capacity to be fertilized and develop into a viable embryo (Hillier, 2009). Although initiation of follicular growth does not require stimulation by FSH or LH, as soon as granulosa cells form they possess FSH receptors (FSHR) and can respond directly to FSH (Oktay et al., 1997). Likewise, when thecal cells develop during the primary-secondary follicular transition (see below) they express LH receptors (LHR) and immediately become targets for LH (Hillier, 1994) (Fig. 1). Thus the therapeutic
window through which to influence follicular development and oocyte quality with exogenous FSH, LH or hCG potentially opens well before the follicular phase leading up to oocyte collection and may even extend to gonadotrophin-independent stages of follicular activation, as discussed below.

**Pre-gonadotrophic folliculogenesis**

Activation of follicular growth represents the transition of resting primordial follicles into growing primary follicles containing an enlarging oocyte surrounded by a single layer of granulosa cells and an outer basement membrane. Evidence that activation is independent of FSH is based on its persistence in hypophysectomized rodents (Hirshfield, 1985) and animals with inactivating FSH receptor (FSHR) gene knockouts (Kumar et al., 1998; Layman and McDonough, 2000). It also occurs in the ovaries of women with inactivating FSHR gene mutations although most follicles become arrested at the pre-antral stage of development beyond which stimulation by FSH is critical (Tapanainen et al., 1998) (see below).

A proportion of the total follicular stock is continuously activating from birth until the menopause (Faddy and Gosden, 1996). Paracrine communication between oocytes and surrounding somatic cells is responsible for follicular activation. The somatic factors involved include KIT ligand, which stimulates oocyte growth and survival via oocyte-expressed KIT (Liu, 2006). Conversely oocyte-derived paracrine factors are essential to granulosa cell formation and division (see reviews by Eppig et al., 1997; Chang et al., 2002; Matzuk et al., 2002), including growth differentiation factor-9 (GDF9) and bone morphogenetic protein-15 (BMP15). Follicular activation is suppressed in dormant primordial follicles by members of the FOXO subfamily of forkhead box transcription factors that inhibit PI3K/Akt signalling (Arden, 2008; John et al., 2008). De-repression of this pathway (e.g. via KIT) leads to follicle activation (Liu, 2006). It remains to be determined if activation can be influenced by gonadotrophins but it certainly seems to proceed normally in their absence.

Progression from the primary (single granulosa cell layer) to secondary (pre-antral) stage of follicular development requires further oocyte expansion, granulosa cell proliferation and investment of an LH-responsive thecal cell layer. None of which depend on FSH but all remain critically dependent on paracrine signalling by members of the TGF-β superfamily. In primary-secondary follicles, Anti-Müllerian hormone (AMH) (Visser and Themmen, 2005) and activin subunit (INHBA and INHBB) gene expression predominate (Hillier, 1991) (Fig. 2). At later stages of maturation, expression of the inhibin-α subunit (INHA) increases, accounting for increased formation of heterodimeric inhibin-A (INHA/INHBA) and inhibin-B (INHA:INHBB), respectively (Chang et al., 2002; Matzuk et al., 2002). Inhibin-B production marks the follicular cohort from which a pre-ovulatory follicle is selected, whereas the pre-ovulatory follicle itself mainly produces inhibin-A (Schneyer et al., 2000; Welt et al., 2001). AMH (AMH:AMH) has been shown to suppress early stages of follicular growth and onset of responsiveness to FSH in vitro thereby exerting a controlling influence on the rate at which follicles become available for pre-ovulatory development (Visser and Themmen, 2005). Activins (homodimers or heterodimers of INHBA and INHBB) enhance granulosa cell proliferation, promote their responsiveness to FSH and inhibit thecal androgen synthesis. Inhibins promote LH-stimulated androgen synthesis and provide a major intrafollicular drive to androgen synthesis, as discussed below (Hillier, 1991).

**Gonadotrophin dependence**

Follicular antrum formation and antral expansion are absolutely dependent on FSH (Tapanainen et al., 1998; Combelles et al., 2004). Early antral follicles (<5 mm) develop normally even in infant ovaries but pre-ovulatory (>20 mm diameter) growth depends on the adult FSH levels reached in ovulatory menstrual cycles.
Development-related requirements for FSH are at least partially explained by opposing autocrine and paracrine queues including activins and inhibins (Hillier, 1991; Hillier and Miró, 1993). Thus immature antral follicles produce activins that enhance granulosa cell sensitivity and responsiveness to FSH and simultaneously suppress LH-responsive thecal androgen synthesis (Fig. 2). Appropriate stimulation by FSH diverts the follicle to formation of inhibins, which promote androgen synthesis. Androgens in turn synergize with FSH to augment inhibin synthesis. The principle requirement for LH is to sustain androgen synthesis, without which there is no estrogen synthesis. LH is also required to finalize pre-ovulatory follicular maturation and deliver a fully competent oocyte, as discussed below. Note that although the focus here is on TGF-β superfamily members, many other polypeptide growth factors contribute to intrafollicular paracrine signalling, among which the insulin-like growth factors play particularly important pro-gonadotrophic roles (Kwintkiewicz and Giudice, 2009).

Early follicular phase
As each menstrual cycle begins, the intercyclic FSH rise recruits intermediately mature (2–5 mm diameter) follicles to enter initial stages of pre-ovulatory development. A spectrum of FSH sensitivity exists at this time and each follicle within the cohort possesses its own ‘threshold’ requirement for further stimulation FSH if development is to proceed (Brown, 1978; McNatty et al., 1983; Schipper et al., 1998). Autocrine and paracrine signalling are integral to the establishment of individual FSH threshold requirements, which differ by as little as 10–30%. Crucially, the follicle with the lowest FSH threshold is most likely to be selected to ovulate, as discussed below.

Late follicular phase
Usually only one of several follicles recruited by FSH into pre-ovulatory development actually survives to ovulate. This ‘dominant’ follicle is able to develop in the face of the falling mid-late follicular phase serum FSH level because—as a direct consequence of previous stimulation by FSH—its granulosa cells are not only sensitized to FSH but now express LHR and therefore respond directly to LH as well as FSH (Zeleznik and Hillier, 1984; Hillier, 1994) (Fig. 1). During its final week of maturation, the pre-ovulatory follicle increasingly produces estradiol, driven by endocrine (LH) and paracrine (inhibin-A) signalling (Hillier, 1991). LH-dependent stages of oocyte and cumulus cell maturation remain critically dependent upon paracrine signalling, most notably involving GDF9 and BMP15 (Russell and Robker, 2007). Induction of epidermal growth factor (EGF)-like growth factors and transactivation of EGF receptor signalling are also integral to LH-induced ovulation (Panigone et al., 2008). Similar to follicular activation approximately 3 months earlier, resumption of meiosis in the pre-ovulatory follicle occurs via a molecular de-repression mechanism, this time due to the withdrawal or
inactivation of second messenger cyclic AMP induced by LH (Vaccari et al., 2008).

**Gonadotrophic manipulation of paracrine signalling**

Paracrine signalling explains how gonadotrophins are potentially able to benefit oocyte function and by extension how inappropriate use of exogenous gonadotrophins might be deleterious (Sirard et al., 2007). Oocytes do not possess functional gonadotrophin receptors but depend on follicular somatic cells to relay gonadotrophic cues via cumulus-derived microvilli that project through the zona pellucida into the ooplasm (Eppig et al., 1997). Low molecular weight molecules produced by thecal cells diffuse across the lamina basalis to enter mural granulosa cells. These last interconnect among themselves and cumulus cells via gap junctions, formation of which is stimulated by FSH. LH stimulated paracrine signals are thereby able to impact oocytes even in follicles whose granulosa cells do not express LHR. However, once granulosa cells acquire LHR in response to FSH, direct paracrine signalling to the oocyte via LH-stimulated mural and cumulus cells becomes possible, which ultimately explains the pre-ovulatory resumption of meiosis in response to the spontaneous LH surge or injection of hCG (Fig. 1). Gap junctions also link cumulus cells to the oocyte and their closure due to Erk1/Erk2 mediated phosphorylation is thought to be part of the mechanism through which LH surge re-initiates meiosis and oocyte maturation at the time of ovulation (Fan et al., 2009).

Although ovarian stimulation with gonadotrophins has the obvious benefit of increasing the number of oocytes available for treatment, it can also compromise oocyte quality leading to chromosomal anomalies and interference with imprinting during pre-implantation development (Fortier et al., 2008; Santos et al., 2009). 'Superstimulation' is also costly, time-consuming and potentially unhealthy to patients due to complications such as ovarian hyperstimulation syndrome and possible long-term health risks such as ovarian cancer (Verberg et al., 2009). Hence, there is increasing interest in the use of more gentle approaches that more closely mimic physiology and the emphasis is on achieving oocyte quality over oocyte quantity. Towards achieving these goals, more effective controlled ovarian stimulation regimes might be reverse engineered from the natural follicular life cycle. To which end, I posit three tactics for manipulating the ovarian paracrine system.

**Tactic 1: follicular priming with LH or hCG**

There is considerable pre-clinical evidence that androgens directly stimulate early stages of follicular growth (Weil et al., 1999) and increase granulosa responsiveness to FSH (Hillier and Tetsuka, 1997; Walters et al., 2008). Granulosa cells express androgen receptor (AR) through which theca-derived testosterone amplifies FSH-stimulated PKA signalling (Sirard et al., 2007; Escamilla-Hernandez et al., 2008). The capacity for androgen to sensitize granulosa cell response to FSH declines with follicular maturity, at least partly due to a development-related loss of AR (Hillier et al., 1997). Accordingly, LH-driven thecal androgen production may be a component of the natural mechanism through which antral follicles initially acquire sensitivity to FSH.

To test this hypothesis Durnerin et al. (2008) evaluated sequential ovarian stimulation with LH and FSH in IVF patients receiving a depo GnRH agonist formulation to induce profound suppression of endogenous gonadotrophins. The aim was to determine if prior exposure to LH would modify the spectrum of follicles available to respond to FSH, and whether there would be a corresponding change in the profile of follicular development determined by ultrasound or oocyte yield, or an influence on oocyte maturity leading to an increased yield of viable embryos. Results revealed increased development of small antral follicles due to LH priming as well as a statistically significant increase in the number of normally fertilized embryos obtained after subsequent FSH treatment (Fig. 3). It should, however, be stressed that no clinically important outcomes were significantly altered in the LH group versus controls.

Additional support for the LH-priming concept comes from the study by Balasch et al. (2009), reporting follicular development and serum hormone concentrations in eight hypogonadotrophic-hypogonadism women who underwent ovarian stimulation with recombinant human FSH preceded or not by recombinant LH or hCG. Results showed that pre-treatment with LH or hCG significantly decreased by 24% the effective daily FSH dose required to generate an estrogen secreting pre-ovulatory follicle. Taken together, these studies are sufficiently encouraging to warrant further clinical evaluation of follicular priming using LH/hCG. In this regard, the meta-analysis by Mochtar et al. (2007) points to a particular role for LH supplementation in women with poor response to FSH.

Note that the mechanism of action through which aromatase inhibitors promote ovarian stimulation might also involve paracrine androgen action (Casper and Mitwally, 2006). These compounds increase

![Figure 3](https://academic.oup.com/molehr/article-abstract/15/12/843/1049045/1049045)
intrafollicular androgen accumulation through selectively inhibiting the conversion of androgen to estrogen in granulosa cells (Garcia-Velasco et al., 2005). Again, any clinically important benefit of adding aromatase inhibitors to ovarian stimulation in the context of ART remains to be established (Verberg et al., 2009).

**Tactic 2: follicular conditioning with FSH**

The present trend is towards single or at most double embryo replacement in ART procedures but several high-quality oocytes are desirable to allow for repeat treatment. Where natural ovarian cycles are not suppressed with a GnRH-(ant)agonist, the tactic should be to increase or supplement endogenous FSH and LH levels to override the selection process whereby only one follicle becomes fully mature and ovulates. Before the application of GnRH-analogues to suppress endogenous gonadotrophins, it was common to give anti-estrogenic clomiphene citrate (50–100 mg/day) for 5 days followed by a period (at least 2 days) of treatment with hMG; typically 150 IU LH and 150 IU FSH/day. Clomiphene was usually started on Day 5 of the menstrual cycle aiming to prolong the intercyclic FSH rise and present multiple follicles for pre-ovulatory development in response to hMG. This presupposes that the onset of menses (i.e. treatment cycle Day 1) reliably reflects the status of the intercyclic FSH surge, which in fact it does not. FSH measurements on daily serum samples collected from cyclic women reveal that in some women the intercyclic FSH rise can occur as much as 3–4 days before the first day of menses (S.G. Hillier, unpublished observation), in which case the onset of menses does not report the ovarian cycle with sufficient accuracy to time the initiation of clomiphene therapy. To obviate this problem, a preliminary course of FSH was proposed (150 IU/day for 5 days) to generate a synchronem for routine initiation of clomiphene on Day 5. The case-study presented in Fig. 4 reports a patient in whom FSH conditioning led to the development of four pre-ovulatory follicles from which four oocytes were collected; leading to the development of four 4-cell embryos, which upon uterine replacement resulted in a quadruplicate maternity. Whereas not ideal from obstetric and paediatric perspectives, this outcome unequivocally confirms that the oocytes obtained were synchronous and of the highest quality (Hillier et al., 1985a). Similar tactics have succeeded in the cattle breeding industry where it is well established that follicular wave dynamics induced by endogenous or exogenous FSH affect ovarian responses to ovulation induction (Bó et al., 2008). There is also experimental evidence that FSH priming in vivo can benefit primate oocyte maturation in vitro (Schramm and Bavister, 1994).

The paracrine case for follicular conditioning with FSH is that the small antral follicles from which pre-ovulatory follicles emerge in natural menstrual cycles become increasingly responsive to FSH during the preceding luteal phase (McNatty et al., 1983). Granulosa cells in such follicles abundantly express INHBA and INHBB genes encoding dimeric activins (Schwall et al., 1990). Activins in concert with other growth factors such as insulin-like growth factors augment multiple actions of FSH on immature granulosa cells including up-regulation of FSHR and increased formation of inhibin-a subunit. The increased inhibin-a diverts granulosa cells from activin to inhibit biosynthesis and creates the potential for thecal androgen production to be stimulated by inhibin (Hillier, 1991; Schneyer et al., 2000).

![Figure 4](https://academic.oup.com/molehr/article-abstract/15/12/843/1049045)

**Figure 4** Evidence for follicular conditioning by FSH: an IVF case study reproduced with permission from Hillier et al. (1985a). ‘The 29-year-old patient had an average cycle length of 28 days. ‘Pure’ FSH (Metrodin, Serono Laboratories UK Ltd) was given from Days 1 to 5 (150 IU/day) to act as a “synchronem” for subsequent clomiphene/hMG therapy (Hillier et al., 1984a, b). Clomiphene (100 mg/day for 5 days) was started on Day 5; HMG 150 IU/day was started on Day 8. The last dose of HMG was given on Day 12 since on Day 13 four follicles between 14 and 17 mm diameter were seen by ultrasound: serum estradiol on the morning of Day 13 was 4.4 nmol/l and still rising. HCG (5000 IU) was injected at 03.00 h on Day 14; since a spontaneous LH surge had not started, laparoscopy was done at 15.30 h on Day 15 (arrows). Four follicles giving between 4.5 and 7.0 ml follicular fluid and a fifth yielding only 1.0 ml were aspirated: each of the four large ones gave up an egg. The eggs were inseminated with the husband’s sperm at 21.20 h on Day 15. At 13.50 h on Day 17 (40.5 h after insemination) four 4-cell embryos had developed and all were replaced transcervically to the uterus. A quadruplet pregnancy resulted’.

(Fig. 2). Experimental studies confirm that FSH up-regulates thecal Cyp17 gene expression and hence androgen synthesis via inhibin-mediated paracrine signalling in rodent ovary (Smyth et al., 1993, 1994, 1995). Androgen so produced is able to synergise with FSH in promoting granulosa cell mitosis and carbohydrate metabolism, including the increased formation of lactate required for energy production by the maturing oocyte (Hillier et al., 1985b).

**Tactic 3: follicular coasting with LH activity**

Once granulosa cells have acquired LHR in response to stimulation by FSH, follicular maturation falls under direct LH control. In the spontaneous cycle, a single ‘gold standard’ follicle coasts through the late follicular phase sustained by basal LH in the face of declining FSH.
Thus, each follicle recruited by FSH acquires tolerance to LH defined by the minimal (‘threshold’) and maximal (‘ceiling’) levels of stimulation by LH (or hCG) required to promote paracrine signalling between somatic cells and the oocyte (Hillier, 1994). Estrogen secretion is sustained through dual LH support of granulosa cell aromatase activity and thecal androgen synthesis, augmented by paracrine inhibin (Hillier, 1991, 1994). Oocyte quality is assured by LH-driven nutritional and informational signals emanating in cumulus cells (Assou et al., 1991, 1994). Conversely oocyte-derived factors such as GDF9 and BMP15 influence granulosa cell function such as cumulus expansion, apoptosis, carbohydrate metabolism and steroidogenesis (Russell and Robker, 2007; Li et al., 2009).

Proof-of-concept that LH alone can sustain the final stages of pre-ovulatory follicular maturation was provided by Sullivan et al. (1999) from a study of women undergoing ovulation induction following down-regulation of pituitary gonadotrophin secretion with GnRH agonist. Follicular growth was stimulated with recombinant FSH until a 14-mm follicle was identified by ultrasound. Patients were then randomized for a 2-day period to continued FSH treatment alone, LH treatment alone or saline. Results showed that serum estradiol concentrations continued to rise in women receiving either FSH or LH compared with those in the saline-treated group, which declined. A similar study of hypogonadotrophic-hypogonadism patients undergoing ovulation induction confirmed that LH alone can conclude pre-ovulatory maturation and foster the emergence of a single dominant follicle more effectively than FSH given alone or in combination with LH (Fig. 5) (Loumaye et al., 2003).

Flicori et al. (2005) successfully applied coasting with hCG (LH surrogate) to an ART (ICSI) setting. They compared clinical outcomes in GnRH-agonist-suppressed women receiving FSH or hMG to stimulate multiple estrogen secreting follicles. Then one group continued to receive FSH/hMG whereas the other was switched to low-dose (200 IU/d) hCG for the final 3–4 days. This led to a significant (54%) increase in fertilization rates. Thus, biphasic stimulation regimes using FSH followed by LH activity to target the gold standard oocyte and its nearest neighbours (in developmental terms) could have potential clinical merit.

Translational implications

The current emphasis on oocyte quality over quantity in ART procedures calls for a more intelligent approach to ovarian stimulation based on sound physiological principles. Follicular responses in standard FSH/hMG alone regimes are inevitably asynchronous, yielding many oocytes that do not fertilize or are unable undergo normal embryonic development. Here, I have highlighted paracrine pathways emanating in thecal and granulosa cells that potentially impact oocytes and which can be manipulated by sequential and/or combined use of FSH and LH (or hCG). The tactics offered are designed to increase follicular responsiveness to FSH, synchronize oocyte competence and maximize oocyte quality. There can be no one-size-fits-all approach to ovarian stimulation. Factors such as age, genotype and general health will always affect individual responses. However, treatment tailored to personal needs based on paracrine principles will always be more likely to achieve the desired outcome.

Funding

Work in the author’s laboratory leading to this review was funded by the Medical Research Council.

References

Arden KC. FOXO animal models reveal a variety of diverse roles for FOXO transcription factors. Oncogene 2008;27:2345–2350.

*Figure 5* Evidence that follicular coating with LH can promote mono-ovulation: a double-blind, placebo-controlled, multicentre pilot study in patients undergoing ovulation induction. WHO I anovulatory patients received treatment with recombinant FSH and LH. When at least one follicle reached a mean diameter of 10–13 mm, patients were randomised using a computer-generated randomization list (stratified by centre) to FSH and LH (225 IU/day) (n=8) or LH alone (n=6) or FSH alone (n=6). Histogram shows the mean number (±SEM) of follicles ≥11 mm on day of hCG administration (or on the last day of treatment if hCG was not administered). Results of this pilot study suggest that treatment during the late follicular phase with rLH alone is superior to FSH alone or LH and FSH combined in promoting the development of a single preovulatory follicle. Reproduced with permission from Loumaye et al. (2003).


Submitted on September 4, 2009; resubmitted on September 18, 2009; accepted on September 30, 2009