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Reproductive Biology Insights

Oocyte-Somatic Cell Interactions and Ovulation Rate: Effects on Oocyte Quality and Embryo Yield

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ABSTRACT: The use of exogenous gonadotropins to increase oocyte yields for better pregnancy outcomes remains unpredictable and inefficient. The objectives of this review are to address two questions concerning this issue: (1) are there any alternatives for improving consistency in oocyte yields? (2) is it possible to develop a molecular diagnostic test for oocytes with blastocyst potential? Studies in sheep with a heterozygous inactivating mutation in the oocyte-derived growth factor, bone morphogenetic protein 15 (BMP15), report increased oocyte yields and significantly more offspring. Moreover, partial immuno-neutralization of BMP15 bioactivity increases oocyte yield without modifying gonadotropin or ovarian steroid secretion. Therefore, it is hypothesized that the development of BMP15 antagonists may prove them to be suitable alternatives to exogenous gonadotropins. Recent evidence from analyses of candidate gene expression in human cumulus cells separated from individual oocytes before IVF indicates a potential non-invasive molecular screen for oocytes with improved blastocyst and live-birth outcomes.

KEYWORDS: gonadotropins, oocyte yield, BMP15, cumulus cells, candidate genes

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Introduction

Exogenous hormone treatments to increase yields of viable oocytes for in vitro maturation (IVM), in vitro fertilization (IVF) and/or embryo transfer in mammals have not improved substantially over the past two decades. For example, in sheep and humans, ovulation rates after super ovulation treatments continue to be highly variable (Table 1).[1,2] Moreover, when mature oocytes are subjected to IVF and multiple blastocysts result, the selection of the best of these for transfer remains an educated guess. Therefore, in terms of positive pregnancy outcomes, success rates following IVF or embryo recovery and transfer to recipients are often low (Table 1). For example, only ~5% of human oocytes recovered during assisted reproduction cycles go on to become babies.[5] The current and repetitive application of highly inefficient technologies is not only frustrating but indicates a lack of understanding of the biological processes underpinning the problem. The objective of this review is to address two questions concerning this dilemma: (1) are there any alternatives for improving the yields of quality oocytes? and (2) is it possible to develop a reliable diagnostic test for oocytes having a positive outcome? To address these questions, this review will first address some basic concepts concerning ovarian follicular development and the role of the oocyte and gonadotropins in regulating ovulation rate.[6–9] Based on this information together with knowledge of genetic mutations and manipulation of biological activities of growth factors of oocyte origin that lead to increases in ovulation rate and yields of quality oocytes,[10–13] an alternative treatment to the use of gonadotropins is proposed. To address the second question, and based on a knowledge of the hierarchical nature of ovarian follicular development,[14] and the functional interrelationships among cumulus cells

Table 1. Effects of gonadotropin treatment and ICSI on oocyte recovery, blastocyst rate and pregnancy outcomes in women (<38 y) and on ovulation rate and pregnancy outcomes after embryo transfer in sheep.

WOMEN (N = 25)		SHEEP (N = 163)	
OUTCOME	MEAN (RANGE)*	OUTCOME	MEAN (± SEM)
Oocytes	11 (3–15)	Ovulation rate	17.1 (±0.9)
Fertilised	8 (2–12)		
Blastocysts (Day 5–6)	2 (1–6)	Transferrable embryos	5.5 (±0.5)
Pregnancy (hCG)	0.76 (0–1)	Pregnancies (fetuses)	3.5 (±0.5)

*Numbers are rounded to whole numbers except for pregnancy after the transfer of a single blastocyst. Data taken from previous studies.[3,4]

(CC) and the oocyte,[15] a diagnostic test of genetic markers in CC at IVF may prove to be a reliable predictor of a positive embryological outcome.[4]

Ovarian Follicular Development: Basic Concepts

The initiation of follicular growth begins coincidently with follicular formation; and the first follicles that form are the first to grow.[16–19] However, the factors responsible for the initiation of growth remain to be determined. In a classic review, Peters et al summarized several key concepts concerning follicular development in mammals.[6] These were that: (1) follicular growth is continuous throughout infancy and adult life, irrespective of reproductive status and whether ovulation occurs or not; (2) follicles grow sequentially without rest until they die or ovulate; (3) the number that grow is dependent on the size of the small follicle pool; (4) as follicles develop, they depend increasingly on gonadotropic support and; (5) exogenous gonadotropins reduce the incidence of atresia in large follicles.

Hierarchical Follicular Development

In all mammals examined thus far, the evidence supports the notion that follicular growth continues unabated during infancy, pregnancy and during other anovulatory conditions such as anestrus.[20–24] From examinations of individual antral follicles in ovaries of sheep and humans, the responsiveness of granulosa cells (GC) to gonadotropins in vitro, together with steroid concentrations in follicular fluid, confirms the notion that follicles develop in a hierarchical, and thus sequential, manner.[14,25,26] In sheep, cows and humans, most follicles appear to survive the transit through the preantral stages of growth, but after antrum formation, the prevalence of atresia, as assessed from pyknosis in GC, is widespread. This infers that there is a high level of synchrony and co-ordination between maturation of the oocyte and follicular somatic cells during preantral follicular development. However, after antrum formation and as the follicular responsiveness to gonadotropins increases, the consequence of coordinating the proliferation and maturation of the follicular somatic cell population in synchrony with that of the oocyte becomes increasingly challenging. For example, in ovaries of women aged 25–49 years, ≤2 antral follicles (≥4 mm diameter) from the population of all antral follicles (ie 14–50%) contain a full complement of viable GC.[27,28] After exogenous gonadotropin treatments to enhance oocyte yields in sheep or humans, measures of GC functions (ie cAMP synthesis *in vitro*) or gene expression profiles of CC from oocytes before IVF, suggest that the hierarchical pattern of follicular development is not overcome.[4,14] This suggests that most of these exogenously-treated follicles that yield oocytes were probably rescued from atresia. Thus, even though exogenous gonadotropins may result in more follicles ovulating, most oocytes are derived from different biochemical microenvironments whereby the signaling exchanges with their adjacent CC and GC are also diverse. Therefore, it is perhaps not surprising, given the failure of exogenous gonadotropin treatments to overcome the hierarchical nature of follicular development, that these treatment regimens will yield unpredictable numbers of viable oocytes.

The Antral Follicle Population and Exogenous Gonadotropins

Another significant variable to oocyte yield is related to the number of small (non-growing) follicles,[29] as this ultimately determines the number of antral follicles.[30] In sheep, the numbers of primordial follicles formed during fetal life (ie by 120 days of gestation) is highly variable between individual ewes (~23000–350000).[31] In cattle, between 14 and 33 months of age, there is a 7-fold variation in number of antral follicles ≥4 mm diameter between animals, but within individual animals, this number is highly repeatable (0.95 where 1 = fully repeatable).[29] Similarly in women (aged 25–46 years), there is a ≥10-fold variation between individuals in the number of antral follicles between 2–10 mm diameter.[32] The evidence from studies in sheep suggests that exogenous gonadotropins do not increase the numbers of antral follicles at least in the short-term. For example, a continuous infusion of exogenous FSH for 24–28 days led to plasma FSH concentrations 7-fold above normal and an 11-fold increase in ovulation-rate (15.3 cf 1.4). However, there was no change in the population of antral follicles nor in the proportion of non-atretic follicles as assessed by their GC populations.[33] Moreover, there was a mean of 16 follicles between 4.5 and 6 mm in diameter in the treated animals (cf ≤3 in the controls) and all follicles responded differently to LH with respect to cAMP production by GC *in vitro*. Thus, in mammals with a low ovulation-rate phenotype (eg sheep, cattle, human), it is only the extant population of antral follicles from which oocytes are recruited for ovulation following exogenous gonadotropin treatment. Moreover, since this extant population is variable both in number and GC composition, these factors are likely to contribute to the unpredictable yield of viable oocytes. Peters et al proposed that exogenous gonadotropins reduced the incidence

of atresia in large follicles.[6] This is supported by evidence that indicates the yields of blastocysts or transferrable embryos are increased after gonadotropin treatment (Table 1). However, the large discrepancy between oocyte recovery and the number that subsequently result in positive pregnancy outcomes suggests that the benefits of exogenous gonadotropin treatment are modest.

Oocyte-specific Growth Factors and Gonadotropins

While it has been known for almost 40 years that gonadotropins do not initiate follicular growth, it is well-established that follicles become increasingly dependent upon gonadotropins as they develop towards ovulation.[6,9] Whilst the molecular regulators that initiate follicular growth remain to be resolved, a key advance in our understanding was the discovery of the oocyte-derived growth factor, growth differentiation factor 9 (GDF9). It was shown that GDF9 was essential for follicular development in mice, once growth initiation had occurred.[34] Subsequently, this was also shown to be true in sheep,[11,35] and in women where abnormal GDF9 concentrations have been linked to premature ovarian failure,[36] polycystic ovarian syndrome (PCOS),[37–39] diminished ovarian reserve,[40] and dizygotic twinning.[41] Interestingly, in sheep, but not mice, inactivating mutations in another oocyte-derived growth factor, bone morphogenetic protein 15 (BMP15) were also found to inhibit ovarian follicular development after growth initiation.[10,13] Indeed in sheep, both GDF9 and BMP15 were deemed important throughout follicular development including the preovulatory phase.[35] The passive transfer of GDF9- or BMP15-specific antibodies during the follicular phase in sheep either blocked ovulation or disrupted normal corpus luteum (CL) function. In addition to playing a key role throughout follicular development, sheep with heterozygous mutations in GDF9 or BMP15 also had increased ovulation rates. Significantly, those with heterozygous mutations in both GDF9 and BMP15 had ovulation rates that were greater than additive for each mutation alone.[42] Depending on the location of the eight known mutations in BMP15, the increase in ovulation rate in heterozygous ewes was between 16 and 100% above that of their respective wild-type counter parts.[42,43] For GDF9, the data from the heterozygous Belclare/Cambridge mutant indicates an ovulation rate increase of 87% above that of the wild-type. Based on modeling predictions, all the aforementioned heterozygous mutations increase ovulation rate either by reducing the concentration of growth factor, by disrupting the binding of the growth factor to either a Type II or I receptor or possibly by disrupting dimerization or hetero-dimerization between BMP15 and GDF9.[13,43,44] From an analysis of individual BMP15 heterozygous mutant and wild-type ewes (Inverdale ewes; N ≥ 140 per genotype) over several years, the repeatability of ovulation rate per animal was twice as high as that in wild-types (0.37 ± 0.02 cf 0.16 ± 0.04).[45]

Therefore ovulation rate, and thus oocyte yield, is critically dependent upon both the oocyte-derived growth factors (BMP15 and/or GDF9) as well as the pituitary hormones (LH and FSH) (Fig. 1). Follicular growth in sheep, cattle and humans continues uninterrupted in a hierarchical

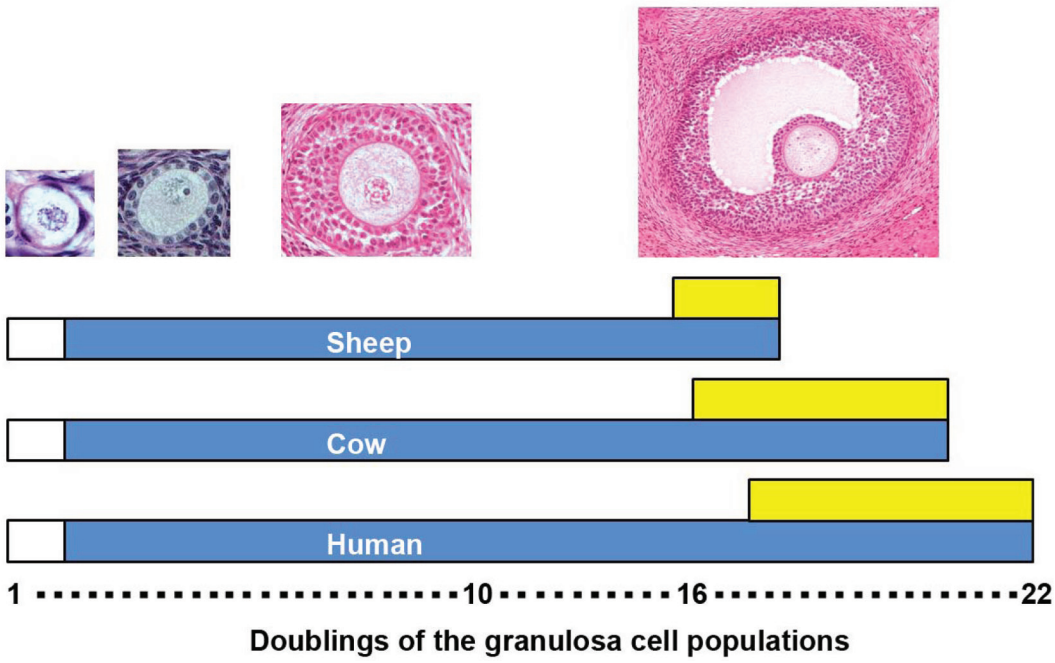

Figure 1. An outline of ovarian follicular development in the sheep, cow and human, relative to the doublings of the granulosa cell (GC) populations and stage-dependency upon gonadotropins (in yellow). The blue rectangles refer to the stages where follicles are suggested to be critically-dependent upon the oocyte growth factors, GDF9 and/or BMP15. The white rectangles refer to the primordial follicles where the mean number of GC cells is ~16 and the transition to the growth phase is both GDF9/BMP15 and gonadotropin-independent. Antral follicle formation occurs around the 10th doubling of the GC population.[46]

manner even in the absence (ie, after hypophysectomy) or presence of very low concentrations of LH and FSH (ie, the luteal phase in women),[28,47] until the gonadotropin-dependent phase is reached. In sheep, cattle and humans, the gonadotropin-dependent phase corresponds to follicles reaching 3, 4 and 4 mm in diameter, respectively. These gonadotropin-independent and -responsive phases of growth correspond to follicles having undergone between 16–18 doublings of their GC populations. Thereafter, for the final 2–4 doublings of the GC populations in putative preovulatory follicles, which correspond to growth during the follicular phase, the pattern of gonadotropin secretion is critical to the yield of 1–3 viable oocytes.

Are There Alternatives to Gonadotropins for Increasing Yields of Viable Oocytes?

One desirable outcome is a predictable and modest yield of viable oocytes (2–4) that is closely linked to ovulation rate without any substantial increase in ovarian estradiol secretion. A modest yield of viable oocytes is considered to be a reasonable goal, as extensive studies in gonadotropin-treated sheep and women reveals that as ovulation rate increases, the individual chance of any one ova resulting in a positive pregnancy outcome decreases.[5,48] A suitable animal model indicative of an alternative to exogenous gonadotropins is the heterozygous BMP15-mutant sheep. The best studied of the eight known BMP15-mutant sheep models is the Inverdale ($FecX^{I}$). These animals have a valine to aspartic acid substitution at residue position 31 of the mature peptide.[10] The ovarian phenotype for the heterozygous Inverdale (I+) is identical to that for another heterozygous BMP15-mutant sheep (Hanna; $FecX^{H}$), in which a stop codon has been introduced into the mature BMP15 protein eliminating expression of the protein.[10] Collectively, these mutants indicate that a reduction in BMP15 protein secretion in sheep results in an increase in ovulation rate,[8] that is accompanied with a corresponding increase in litter size (Table 2).[49,50] Moreover, based on the predictive effects of the heterozygous GDF9 mutation ($FecG^{H}$) leading to a reduction in bioactivity, this also leads to an increase in ovulation rate.[11] The effects on litter size are less well understood although the animals were originally identified from sheep flocks of mixed genetic backgrounds albeit with major effects on lamb yields.[51] Of considerable significance is the finding that the effects of heterozygous mutations in GDF9 and BMP15 have at least additive, if not multiplicative, effects on ovulation rate as do heterozygous mutations in the type I receptor for BMP15 (activin-like kinase 6; ALK6).[42] The latter double mutant (ie. heterozygous for a mutation in BMP15 and ALK6) leads to an even greater level of precocious follicular maturation with ovulation rates of 8–10.[52] It's also noteworthy that the ALK6-mutant sheep express lower levels of BMP15 mRNA.[53] Evidence that a partial reduction in BMP15 or GDF9 bioactivity is directly responsible for the significant increases in ovulation rate was demonstrated by immuno-neutralization experiments[12]: the increase was due to significantly more animals having two or more ovulations. In a large follow-up study involving more than 400 ewes, BMP15 immuno-neutralization significantly increased pregnancy-rates without affecting embryo or fetal survival.[13]

Table 2. Ovulation rate and litter size in heterozygous Inverdale (I+) mutant sheep and their wild-type (++) litter mates with respect to age.

TRAIT	AGE (y)	GENOTYPE		SED
		I+	++	
Ovulation rate	1.5	2.54	1.74	0.10
	2.5	2.85	1.83	0.15
	3.5	3.18	1.94	0.28
Litter size	2.0	2.12	1.63	0.12
	3.0	2.40	1.59	0.15
	4.0	2.47	1.57	0.26

Data taken from a previous study.[50]

The increased ovulation rate in the I+ mutants occurs without any observable changes in plasma FSH and LH concentrations,[54] numbers of primordial or developing follicles,[55,56] responsiveness of follicles to FSH *in vitro*,[56] or aromatase activity of GC *in vitro* when expressed on a per cell basis.[57] Moreover, notwithstanding a difference in ovulation rate between I+ and wild-type (++) ewes (2.6 vs. 1.8; $P < 0.01$), the mean ovarian secretion rates of inhibin, estradiol and progesterone during the luteal phase were not different.[54] However, what was noticeably different between I+ and ++ animals was that despite more CL being present in I+ ewes, the total mass of CL tissue between the genotypes was not different. Also, as assessed by the concentrations of estradiol in follicular fluid and the ability of GC to produce cAMP in response to LH *in vitro*, the sum of the total responsiveness and secretory output of GC in these putative ovulatory follicles was not different between the genotypes.[54,57] The latter finding is due to significant interactive effects of follicular diameter and genotype where the GC populations were often lower in follicles >1.0 mm in I+ ewes than in ++ ewes.[53] Moreover, from detailed analyses of individual follicles, the higher ovulation rate in I+ ewes was due to a greater proportion of follicles acquiring LH-responsive GC at smaller diameters, compared to that in ++ animals.[57] Thus, the ovulation rate in I+ ewes occurs because follicles of different diameters respond to LH and ovulate with a total number of GC, and subsequent CL cells, that are not different from that in ++ animals. Consequently, the pituitary gland secretions of LH and FSH remain similar between genotypes. This phenotypic characteristic of I+ ewes was replicated, in part, by immunization of ewes against BMP15 where it was shown that a higher proportion of smaller diameter follicles have GC that are responsive to LH.[57] A summary of these events is illustrated in Figure 2. One surprising finding from the partial BMP15 immuno-neutralization experiments designed to

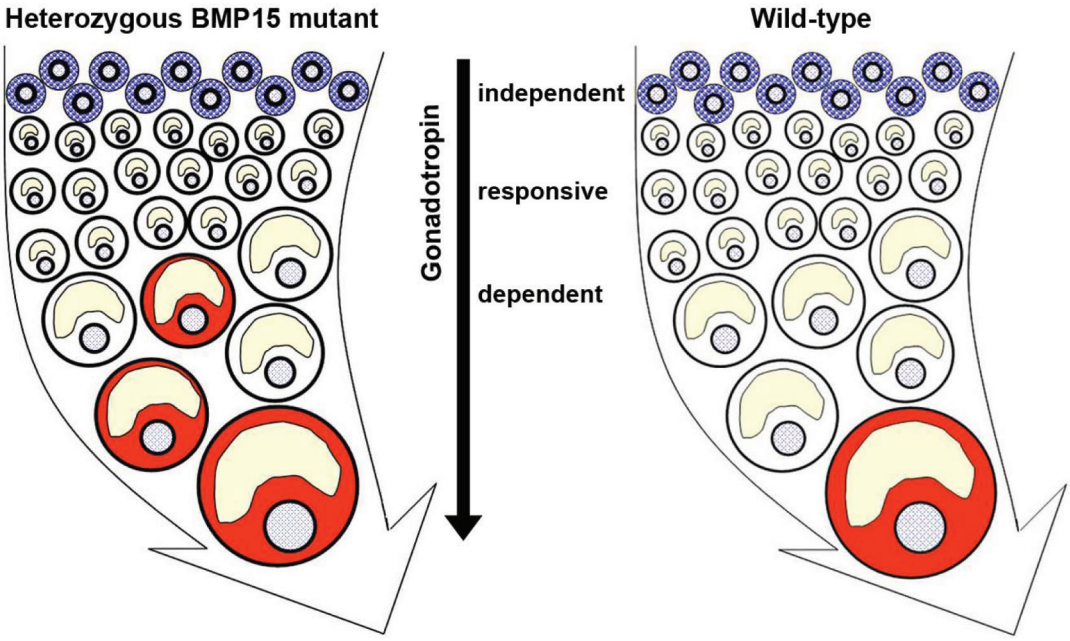

Figure 2. Intra-follicular BMP15 concentrations affect the timing of LH receptor acquisition in granulosa cells. Schematic outline of follicular development in heterozygous BMP15 mutant and wild-type sheep. In the heterozygote, two or more differently-sized follicles acquire LH receptors in granulosa cells (GC; shown in red); whereas in the wild-type, only one or sometimes two follicles have LH receptors in GC. Overall, the total population of LH-receptive GC in the follicles in the BMP15 mutant is similar to that in the wild-type follicle(s). Follicles with LH-receptive GC are the putative ovulatory follicles,[59] and are the dominant ovarian sources of estradiol and inhibin.[54] Since the major sources of these hormones are the GC, the total ovarian secretions do not differ between these genotypes and consequently the ovulation rate differences occur without any differences in the plasma concentrations of pituitary gonadotropins.[54] The conceptual outline of this figure was based on a figure taken from another study,[60] published with permission from CSIRO publishing.

increase ovulation rate was that the additional treatment of supplementary gonadotropins in these animals failed to further augment the ovulatory response,[58] as has previously been observed in Inverdale ewes.[3] One interpretation of this result was that the BMP15 immunization by itself induced precocious follicular maturation and increased responsiveness of GC to LH so that supplementary gonadotropin treatment induced premature luteinization, thereby negating the effects of BMP15 immuno-neutralization. Overall, the evidence from these studies of heterozygous BMP-15 mutant sheep raises the possibility of an alternative approach to increasing oocyte yield without the use of gonadotropins. The application of a primary and booster BMP15 vaccination procedure, notwithstanding the unpredictable antibody response, was remarkably effective in causing a modest increase in ovulation rate.[12,13]

Humans, like sheep, are critically dependent on BMP15 for ovarian follicular development.[61,62] For these reasons, the development of an orally-active, long-acting BMP15 antagonist would be worth exploring as an alternative method for yielding modest and consistent increases in oocyte yield. This does not imply that GDF9 antagonists or a combined BMP15-GDF9 antagonist are not appropriate, but a better understanding of the key roles of GDF9 in species other that sheep are needed before these options are considered. There are several potential benefits for such an approach using BMP15 antagonists. There would be: (1) no major changes in ovarian steroid secretion; (2) no changes in gonadotropin secretion and; (3) no anticipated side-effects since long-term studies of heterozygous mutant Inverdale sheep showed no increased prevalence of tissue pathologies relative to wild-type ewes, and there was no influence on life-expectancy.

Can Gene Expression Profiles in Cumulus Cells (CC) Isolated From MII Oocytes Undergoing IVF be Used to Predict Pregnancy Outcomes?

A significant challenge following the recovery of multiple cumulus-oocyte-complexes (COC) is selecting from a pool of COC, one or more with the highest probability of blastocyst development and a live birth. Recently, significant advances have been made with non-invasive imaging of human and bovine embryos using time-lapse monitoring.[63–65] Success rates approaching 80% with respect to positive pregnancy outcomes have been achieved. An alternative, non-invasive approach has been used to analyze gene expression profiles in CC that are removed from oocytes before IVF. In recent years, advances using this approach have increasingly shown promise for predicting oocyte quality.[66–68] One of the challenges in using CC mRNA data is handling the considerable variability in expression values for individual candidate genes both within and between patients.[66] Recently, a multiplex quantitative polymerase chain reaction (QPCR) study using human CC identified four candidate genes that were associated with a significant improvement in predicting viable blastocysts and live birth outcomes relative to random selection of related meta phase II (MII) oocytes before intra cytoplasmic

sperm injection (ICSI).[4] The key to interpreting the data from this study was first an acknowledgement that ovarian follicular development, after exogenous gonadotropin treatment, was hierarchical with few, if any, follicles within individuals being functionally identical. Secondly, that the variability was not due to differences in the viability of the isolated CC used for analyses: in this study CC viability exceeded 85%. Thirdly, it was important to establish that the expression levels of each of the candidate genes was standardized to a reference gene, 60s ribosomal protein L19 (*RPL19*), which was directly correlated to CC number ($R^2 = 0.822$). Based on the aforementioned criteria, each of the informative candidate genes from each CC preparation isolated from MII oocytes for individual patients was ranked with the lowest expression value equaling 1, the next lowest equaling 2 and so on. On the occasions where the relative expression levels of any individual gene were not different between two follicles, they were assigned an equal rank. Thereafter, the ranks for each of the four CC expressed genes were summed from individual CC from each woman separately. Subsequently, the relationships between the ranked characteristics were related to blastocyst and pregnancy outcome. Of the eight originally selected genes, the four most informative were hyaluronan synthase 2 (*HAS2*), follicle stimulating hormone receptor (*FSHR*), versican (*VCAN*) and progesterone receptor (*PR*) (Fig. 3). Based on the total sum of these ranks, the chances of successfully selecting a single MII oocyte for blastocyst formation from (i) the collected pool of MII oocytes was 52% compared with 23% following random selection ($P < 0.01$), (ii) the three highest-ranked MII oocytes was 80% compared with 52% following random selection ($P < 0.01$). The latter predictor was equivalent to that of using all oocytes available after recovery (80% versus 96%; $P = 0.085$). It is worth noting that these findings were based on only 25 patients (<38 y); all of whom were treated with a standard gonadotropin treatment regimen, and all recovered MII oocytes were subjected to ICSI.[4] While these results require confirmation from a much larger prospective study, the high predictive values for positive blastocyst and pregnancy outcomes were achieved with just 4 candidate genes. With recent advances in multiplex QPCR technologies, up to 30 informative genes could easily be screened from individual CC preparations in a single multiplex QPCR assay (eg, using a GeXP system; Beckman Coulter, USA). This is also made possible by the fact that >20000 CC can easily be recovered from each human oocyte so that the amount of cDNA available for analysis is not a limiting factor. Moreover, it would be feasible for the QPCR assays and ranking values to be available within 24 h after ICSI, namely when the fertilized oocytes were at the 2 pronuclear stage of development.

Conclusions

Ovarian follicular development is one of the most intensely studied subjects in reproductive biology. Most *in vitro* studies have involved the pooling of cell-types or tissues to elucidate mechanisms of action. However, it has been known for over 20 years in species with a low ovulation rate phenotype

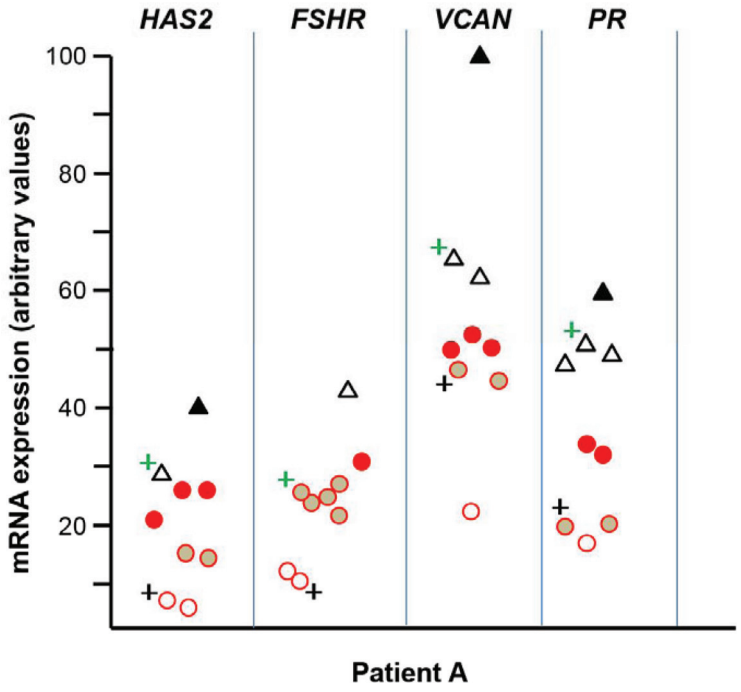

Figure 3. Rankings of individual MII oocytes based on relative cumulus cell (CC) gene expression levels for *HAS2, FSHR, VCAN* and *PR* according to their numerical order, assigned at the time of oocyte collection. Green crosses are associated with a fertilized oocyte that was transferred and resulted in a live birth. The black crosses were associated with a fertilized oocyte that developed into a good blastocyst but was frozen. Each of the symbols (circles, triangles) denotes a different ranking with respect to gene expression: 1 = red circle with white fill; 2 = red circle with grey fill; 3 = red circle with red fill; 4 = triangle with white fill; 5 = triangle with grey fill; 6 = triangle with black fill. This data has been modified with permission from a previous study.[69]

that at any moment in time few, if any, antral follicles, are functionally similar and that follicles develop in a hierarchical manner.[6,14] Given that exogenous gonadotropins do not effectively over-ride hierarchical development, the challenge to find alternative methods to consistently improve the yields of quality oocytes without disrupting the developmental synchrony between the follicular somatic cells and the oocyte has been a very significant one. The recent demonstrations that the concentrations of oocyte-derived GDF9 and/or BMP15 influence ovulation rate and oocyte yield, but not gonadotropin or ovarian steroid secretions, suggest that antagonists to BMP15, GDF9 or both may prove to be more suitable alternatives to exogenous gonadotropins. Variations in BMP15 and GDF9 concentrations appear not to disrupt hierarchical follicular development nor compromise the developmental synchrony between the oocyte and GC.

In recent years, the identity of candidate genes in CC associated with oocytes having a beneficial outcome after IVF has continued to be elucidated. The wide variation in gene expression levels both within and between individuals is perhaps not unexpected given that most follicles are functionally different and that exogenous gonadotropin treatment adds another layer of complexity. The most recent evidence gained from ranking expression levels of candidate CC genes associated with single oocytes within each individual suggests that this may prove to be a more informative approach towards developing a molecular diagnostic test of oocyte quality.

Author Contributions

Concepts in the manuscript were inspired by the work of the late Dr Hannah Peters and were conceived by KPM. Experimental designs and analyses for all experiments were undertaken equally by JLJ, JLP and KPM. All authors contributed to, and approved the final draft of the manuscript.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests. Provenance: the authors were invited to submit this paper.

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