Gene expression in mouse ovarian follicle development *in vivo* versus an *ex vivo* alginate culture system

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Abstract

Ovarian follicle maturation results from a complex interplay of endocrine, paracrine, and direct cell–cell interactions. This study compared the dynamic expression of key developmental genes during folliculogenesis *in vivo* and during *in vitro* culture in a 3D alginate hydrogel system. Candidate gene expression profiles were measured within mouse two-layered secondary follicles, multi-layered secondary follicles, and cumulus–oocyte complexes (COCs). The expression of 20 genes involved in endocrine communication, growth signaling, and oocyte development was investigated by real-time PCR. Gene product levels were compared between i) follicles of similar stage and ii) COCs derived either *in vivo* or by *in vitro* culture. For follicles cultured for 4 days, the expression pattern and the expression level of 12 genes were the same *in vivo* and *in vitro*. Some endocrine (cytochrome P450, family 19, subfamily A, polypeptide 1 (*Cyp19a1*) and inhibin β A subunit (*Inhba*)) and growth-related genes (bone morphogenetic protein 15 (*Bmp15*), kit ligand (*Kitl*), and transforming growth factor β receptor 2 (*Tgfr2*)) were downregulated relative to *in vivo* follicles. For COCs obtained from cultured follicles, endocrine-related genes (inhibin α -subunit (*Inha*) and *Inhba*) had increased expression relative to *in vivo* counterparts, whereas growth-related genes (*Bmp15*, growth differentiation factor 9, and kit oncogene (*Kit*)) and zona pellucida genes were decreased. However, most of the oocyte-specific genes (e.g. factor in the germline α (*Figla*), jagged 1 (*Jag1*), and *Nlrp5* (*Mater*)) were expressed *in vitro* at the same level and with the same pattern as *in vivo*-derived follicles. These studies establish the similarities and differences between *in vivo* and *in vitro* cultured follicles, guiding the creation of environments that maximize follicle development and oocyte quality.

Reproduction (2011) 142 309–318

Introduction

Ovarian follicle development is a highly complex process that requires coordination among the hypothalamus, the anterior pituitary, and the ovary, as well as bidirectional communication between the different cellular compartments within the follicle itself (Albertini *et al.* 2001, Erickson & Shimasaki 2001, West *et al.* 2007a). Although many factors regulating this developmental process have been identified, the explicit role of these factors during early follicle development is enigmatic (Erickson & Shimasaki 2000, Richards *et al.* 2002). Hormones that control follicle development can regulate expression of aromatase, inhibin, activin, FSH

receptor, and LH receptor (McGee & Hsueh 2000). Paracrine and autocrine signals, such as members of the transforming growth factor β (TGFB) family, insulin-like growth factor (IGF), kit ligand, and anti-Mullerian hormone, derived from the follicular somatic cells and the oocyte, communicate bidirectionally between the cellular compartments and direct follicle development (Dong *et al.* 1996, Erickson & Shimasaki 2000, Otsuka *et al.* 2000, Bristol & Woodruff 2004, Juengel & McNatty 2005, Knight & Glistler 2006). Finally, oocyte-specific gene products such as the zona pellucida proteins, factor in the germline α , and NLRP5 (MATER) have varying expression throughout development and impact follicle

and oocyte maturation (Dean 2002). Working together, genes from these diverse categories form a communication network that results in the growth and development of the follicle and the oocyte, and most importantly the formation of a healthy and fertilizable egg.

Systems for *in vitro* ovarian follicle maturation have been developed and can be used to investigate the dynamics of gene expression throughout follicle development, which may aid in investigating the complex interplay of signals needed during follicle development (Kreeger *et al.* 2005, 2006, Xu *et al.* 2006a). The 3D culture system developed by our group consists of isolation of early mouse follicles followed by culture in alginate beads for the duration of follicle development to the antral stage. The alginate hydrogel allows growth in 3D and is thus referred to as a 3D culture system, in contrast to plating directly on a flat surface (2D). 2D culture of preantral mouse ovarian follicles on collagen membranes has resulted in live birth (Eppig & Schroeder 1989, O'Brien *et al.* 2003). However, loss of the physical interactions between the oocyte and the granulosa cells that direct healthy follicle development is a concern in 2D (Cortvrindt *et al.* 1996). Supporting the natural 3D follicular structure may be more important for *in vitro* development of human ovarian follicles, which are larger than those of the mouse. The goal of our 3D model is to develop and characterize a culture system that can be successfully translated to human follicles for fertility preservation. Our system includes all of the cellular components necessary for follicular tissue development, as well as a chemical and mechanical environment that supports follicle growth and function in mouse ovarian follicles (West *et al.* 2007b). Importantly, the follicle does not require vascularization for development *in vitro*; therefore, this tissue can be cultured in isolation. The follicle maturation system supports follicle growth, steroid production, antrum formation, theca cell differentiation, and the development of mature fertilizable eggs that, following IVF and subsequent implantation into a host mother, have given rise to live, healthy, and fertile mouse pups (Xu *et al.* 2006a). Thus, this system enables a molecular-level investigation of *in vitro* follicle growth, which can be compared with freshly isolated follicles grown *in vivo*. This level of analysis provides insight into the similarities and differences in follicular development in the 3D alginate culture system versus the ovary.

In this study, we investigated the steady-state mRNA expression levels by real-time PCR of 20 developmental genes in ovarian follicles that were freshly isolated or cultured *in vitro*. The genes chosen for study are involved in endocrine signaling (cytochrome P450, family 19, subfamily A, polypeptide 1 (*Cyp19a1*), follicle-stimulating hormone receptor (*Fshr*), inhibin α -subunit (*Inha*), inhibin β A subunit (*Inhba*), and LH/choriogonadotropin receptor (*Lhcgr*)), follicular growth (insulin-like growth factor 1 (*Igf1*), kit oncogene (*Kit*), kit ligand (*Kitl*), and

transforming growth factor β receptor 2 (*Tgfb2*)), and oocyte-specific functions (bone morphogenetic protein 15 (*Bmp15*), growth differentiation factor 9 (*Gdf9*), fibroblast growth factor 8 (*Fgf8*), factor in the germline α (*Figla*), jagged 1 (*Jag1*), NLR family, pyrin domain containing 5 (*Nlrp5*, also known as *Mater*), nobox oogenesis homeobox (*Nobox*), DEAD box polypeptide 4 (*Ddx4*, also known as *Vasa*), zona pellucida glycoprotein 1 (*Zp1*, *Zp2*, and *Zp3*). This study provides a detailed analysis of the critical molecular events involved in the transition between follicle stages, which can be utilized to improve culture environments and thus maximize oocyte quality. In addition, this study quantifies the extent to which the culture system replicates physiology and tests the validity of the system for modeling follicular development.

Results

In vivo and *in vitro* folliculogenesis

The objective of the studies was to investigate the expression of genes associated with endocrine communication, paracrine signaling, and oocyte development during follicle growth. For *in vivo* studies, expression was analyzed for two-layered secondary (2LS) follicles, multi-layered secondary (MLS) follicles, and cumulus–oocyte complexes (COCs; Fig. 1A).

Figure 1 Two-layered secondary follicle growth in the 0.25% alginate culture system. Schematic illustrating *in vivo* follicular development versus development *in vitro* in 0.25% alginate beads, starting from 2LS follicles from 12 dpp mice (A). Freshly isolated follicles had an average diameter of $119.1 \pm 1.14 \mu\text{m}$ and after 12 days of culture grew to $329.07 \pm 13.38 \mu\text{m}$ in diameter (B).

2LS follicles, which have two layers of granulosa cells surrounding the oocyte and a diameter ranging from 100 to 130 μm , were isolated from day 12 mouse ovaries. MLS follicles were isolated from day 16 mouse ovaries and have multiple layers of granulosa cells surrounding the oocyte and a diameter ranging from 150 to 180 μm . For the *in vitro* cultures, gene expression was analyzed in follicles that had been cultured within 0.25% alginate hydrogels for 4 days, which have similar diameter and morphology to MLS follicles isolated *in vivo* from the day 16 ovary (Fig. 1A). In addition, gene expression was analyzed in COCs isolated from follicles that had been cultured for 12 days in 0.25% alginate.

During the 12-day culture period, the follicles increased in size and developed antral cavities. The alginate culture system maintained the 3D structure of the follicles (Kreeger *et al.* 2006), and follicles increased in size from an initial diameter of 119.1 ± 1.1 to 329.1 ± 13.4 μm after 12 days of culture (Fig. 1B). The size of antral follicles achieved *in vitro* was smaller than antral follicle diameter *in vivo*, which is around 424 μm (Griffin *et al.* 2006). Antrum formation was also observed in this culture system, with antra developing after 8–10 days of culture (Fig. 1A). This growth curve is consistent with the follicle growth and development that has previously been described (Xu *et al.* 2006b).

Gene expression analysis

To compare follicle development *in vitro* and *in vivo*, gene expression *in vivo* in MLS follicles isolated from the day 16 ovary was compared to *in vitro* follicles isolated from the day 12 ovary that had been cultured for 4 days (Fig. 1A). In addition, gene expression from *in vivo* COCs was compared to expression within COCs obtained from follicles after 12 days of culture.

Endocrine-related genes

The expression of five genes involved in endocrine communication was quantified during *in vivo* and *in vitro* development of ovarian follicles. Steady-state mRNA encoding aromatase (*Cyp19a1*), the FSH receptor (*Fshr*), *Inha*, *Inhba*, and *Lhcgr* were assessed. *In vivo*, all endocrine-related genes, with the exception of *Lhcgr*, increased in expression from 2LS to MLS with a fold change of at least 2 (Fig. 2A). *Lhcgr* did not exhibit a significant change in expression. *In vitro*, three of the five endocrine genes had similar patterns of expression as the *in vivo* isolated follicles. *Fshr* was expressed in both *in vivo* and *in vitro* MLS follicles with at least two times the expression level of 2LS follicles and *Inha* expression was two times greater in all MLS follicles than in 2LS follicles (Fig. 2A and B). *Lhcgr* expression levels did not change during *in vitro* culture or in the *in vivo* condition. However, expression levels of *Inhba* did not change

Figure 2 Fold changes in gene expression as measured by real-time PCR for genes involved in endocrine signaling. Changes in gene expression are shown as follicles develop from 2LS follicles isolated from 12 dpp ovaries to MLS follicles isolated from 16 dpp ovaries (*in vivo* developed) (A) or from 2LS follicles isolated from 12 dpp ovaries and cultured to the MLS stage over 4 days (*in vitro* developed) (B). Note *Lhcgr* and *Inhba* overlap in (B). Fold changes in gene expression relative to 2LS follicles are directly compared between *in vivo* MLS follicles (black bars) and follicles cultured for 4 days *in vitro* (gray bars) (C) and between *in vivo* COCs from antral follicles (black bars) and COCs from follicles cultured for 12 days *in vitro* (gray bars) (D). Error bars represent the S.E.M. * $P < 0.05$ and *** $P < 0.001$. Note log scale for (C) and (D). $N = 3$ for all experiments.

between 2LS and MLS *in vitro*, in contrast with the *in vivo* results (Fig. 2C). Also in contrast with *in vivo* results, *Cyp19a1* levels declined between 2LS and MLS follicles *in vitro* (Fig. 2B). *Cyp19a1* and *Inhba* had significantly (6.5- and 5-fold) reduced expression levels within the MLS follicles *in vitro* relative to *in vivo*. Thus, *Fshr*, *Inha*, and *Lhcgr* had the same pattern of expression *in vivo* as *in vitro*, but *Cyp19a1* and *Inhba* differed between the *in vivo* and *in vitro* conditions.

For COCs, the expression levels varied between the *in vitro* and the *in vivo* conditions for three of the five factors. *Cyp19a1* and *Lhcgr* had similar expression levels *in vitro* as *in vivo* (Fig. 2D). *Inha* and *Inhba* were upregulated in *in vitro* COCs compared to *in vivo*, which contrasts with the MLS studies that indicated no change or downregulation for *in vitro* relative to *in vivo* follicles. *Fshr* had twofold reduced expression *in vitro* compared with *in vivo* ($P < 0.05$).

Growth-related genes

We subsequently investigated the expression patterns of four genes involved in growth signaling: *Igf1*, *Tgfb2*, *Kit*, and *Kitl* (Fig. 3). *In vivo*, *Igf1* levels substantially increased (4.5-fold) between the 2LS and the MLS stages whereas *in vitro* *Igf1* levels increased 1.8-fold. *Tgfb2* and *Kit* levels *in vivo* increased to a lesser extent (1.4- and 1.8-fold respectively), and expression levels of *Kitl* did not significantly change. *Kit* had a similar expression pattern *in vitro* as *in vivo*, with levels slightly increased during development to the MLS stage. *Kitl* and *Tgfb2* had decreased expression after 4 days of culture (2-fold and 4.5-fold respectively, relative to 2LS follicles), which contrasts with the *in vivo* results (Fig. 3B). Relative to *in vivo* follicles, *Kitl* and *Tgfb2* were downregulated in cultured MLS follicles 6-fold and 2.4-fold respectively (Fig. 3C). *Igf1* was the only non-oocyte-specific growth-related gene to be expressed at least twofold higher in MLS follicles compared with 2LS follicles *in vivo*. For COCs, only one of the four genes had a significantly different expression level between cultured and freshly isolated follicles. *Kit* expression differed between *in vitro* and *in vivo* conditions, whereas *Igf1*, *Kitl*, and *Tgfb2* were expressed similarly between *in vivo* and *in vitro* COCs (Fig. 3D).

Oocyte-specific genes

The expression levels of 11 genes that, in the ovarian follicle, are specifically expressed in the oocyte were measured. The designation 'oocyte-specific' is used to indicate that these factors are expressed within the oocyte and does not capture the multi-functional roles of these genes in follicle development and oocyte maturation. *In vivo*, levels of *Bmp15*, *Nobox*, *Ddx4*, and *Zp2* increased by factors of 1.7-, 1.7-, 2.6-, and 1.9-fold from

Figure 3 Fold changes in gene expression as measured by real-time PCR for genes involved in growth signaling. Changes in gene expression are shown as follicles develop from 2LS follicles isolated from 12 dpp ovaries to MLS follicles isolated from 16 dpp ovaries (*in vivo* developed) (A) or from 2LS follicles isolated from 12 dpp follicles and cultured to the MLS stage over 4 days (*in vitro* developed) (B). Fold changes in gene expression relative to 2LS follicles are directly compared between *in vivo* MLS follicles (black bars) and follicles cultured for 4 days *in vitro* (gray bars) (C) and between *in vivo* COCs from antral follicles (black bars) and COCs from follicles cultured for 12 days *in vitro* (gray bars) (D). Error bars represent the S.E.M. ** $P < 0.01$ and *** $P < 0.001$. Note log scale for (C) and (D). $N = 3$ for all experiments.

Figure 4 Fold changes in gene expression as measured by real-time PCR for genes involved in oocyte development that are expressed *in vitro* at the same level as *in vivo*. Changes in gene expression are shown as follicles develop from 2LS follicles isolated from 12 dpp ovaries to MLS follicles isolated from 16 dpp ovaries (*in vivo*-developed) (A) or from 2LS follicles isolated from 12 dpp follicles and cultured to the MLS stage over 4 days (*in vitro* developed) (B). Note *Nobox* and *Ddx4* overlaid each other in (B). Fold changes in gene expression relative to 2LS follicles are directly compared between *in vivo* MLS follicles (black bars) and follicles cultured for 4 days *in vitro* (gray bars) (C) and between *in vivo* COCs from antral follicles (black bars) and COCs from follicles cultured for 12 days *in vitro* (gray bars) (D). Although *Figla* was significantly downregulated at the MLS stage *in vitro*, there was no significant difference *in vitro* versus *in vivo* by the COC stage. Error bars represent the S.E.M. Note log scale for (C) and (D). * $P < 0.05$. $N = 3$ for all experiments.

the 2LS to MLS stage (Fig. 4A and C; Fig. 5A and C). Levels of *Figla*, *Gdf9*, *Jag1*, *Nlrp5*, *Zp1*, and *Zp3* varied by 33% or less between 2LS and MLS. *Fgf8* levels were significantly downregulated in MLS follicles compared to 2LS follicles. For *in vitro* culture to the MLS stage, the expression of *Nobox*, *Ddx4*, and *Zp2* had significantly increased expression between 2LS and MLS, with *Nobox* expression having a greater increase *in vitro* than *in vivo* (Fig. 4A and B; Fig. 5B). The expression of *Nlrp5*, *Jag1*, *Bmp15*, *Zp1*, and *Zp3* changed little over the 4-day culture period (Fig. 4B; Fig. 5B). Expression of *Nlrp5* and *Jag1* also changed minimally *in vivo* over the same time period. However, *in vitro* levels of *Bmp15*, *Zp1*, and *Zp3* were significantly different between *in vivo* and *in vitro* MLS follicles (Fig. 5C). *Figla*, *Fgf8*, and *Gdf9* had decreasing levels of expression between the 2LS and the MLS stages *in vitro*, which was only observed with *Fgf8* *in vivo*. Of the 11 genes, four had significantly different transcript levels between MLS follicles *in vitro* and *in vivo*: *Bmp15*, *Figla*, *Zp1*, and *Zp3*. *Zp3* had an increased level of expression *in vitro* relative to *in vivo*, whereas the remainder had decreased expression levels *in vitro*.

The expression of *Bmp15*, *Fgf8*, *Gdf9*, *Zp1*, *Zp2*, and *Zp3* was significantly different for *in vivo* and *in vitro*-derived COCs. *Fgf8* had increased expression levels *in vitro* relative to *in vivo*, whereas the other five factors had expression that was reduced *in vitro* relative to *in vivo* (Fig. 5D). The expression levels of *Figla*, *Nlrp5*, *Nobox*, *Ddx4*, and *Jag1* were not significantly different between *in vivo* COCs and COCs obtained from antral follicles at the end of culture (Fig. 4D).

Discussion

This study investigated expression patterns of genes known to be important in folliculogenesis during *in vivo* follicle development and during *in vitro* culture. The expression levels of 20 genes involved in follicle development were quantified in 2LS, MLS, and COCs derived from antral follicles. Table 1 shows the gene regulation patterns, categorized as increased, decreased, or unchanged relative to 2LS follicles, for MLS follicles *in vivo*. For the endocrine-related genes in *in vivo* isolated follicles, *Cyp19a1* expression increased from the 2LS to the MLS state, which is consistent with induction within the granulosa cells of preovulatory follicles (Hickey *et al.* 1988). *Fshr* is solely expressed in granulosa cells of developing follicles, with twofold increased expression in preovulatory follicles (LaPolt *et al.* 1992, Sites *et al.* 1994) as is consistent with this report. In addition, the expression of both inhibins and activins has been reported to be maximal in ovaries in which secondary follicles dominate (Woodruff *et al.* 1988, Findlay *et al.* 2001, Pangas *et al.* 2002), which is also consistent with our mRNA data. Finally, LH receptor expression is induced in mural cells of preovulatory

Figure 5 Fold changes in gene expression as measured by real-time PCR for genes involved in oocyte development that are expressed differentially between *in vivo* and *in vitro* conditions. Changes in gene expression are shown as follicles develop from 2LS follicles isolated from 12 dpp ovaries to MLS follicles isolated from 16 dpp ovaries (*in vivo* developed) (A) or from 2LS follicles isolated from 12 dpp follicles and cultured to the MLS stage over 4 days (*in vitro* developed) (B). Fold changes in gene expression relative to 2LS follicles are directly compared between *in vivo* MLS follicles (black bars) and follicles cultured for 4 days *in vitro* (gray bars) (C) and between *in vivo* COCs from antral follicles (black bars) and COCs from follicles cultured for 12 days *in vitro* (gray bars) (D). Error bars represent the S.E.M. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Note log scale for (C) and (D). $N = 3$ for all experiments.

follicles. For the transition from 2LS to MLS, *Lhcgr* levels were unchanged, which was expected given the early stage of follicle development (Segaloff *et al.* 1990, Lei *et al.* 2001).

Substantial growth of the follicle and development of the oocyte occur during development from the 2LS state to ultimately produce a COC. Thus, we investigated the expression of multiple growth factor, as well as oocyte-specific transcripts. A significant increase in expression of *Igf1* at the MLS follicle stage was observed (Table 1), consistent with its role in oocyte maturation (McGee & Hsueh 2000). For the *in vivo* isolated follicles, the expression of many growth-related genes reported here (*Bmp15*, *Gdf9*, *Kit*, *Kitl*, and *Tgfb2*) changed less than twofold from the 2LS to the MLS follicle stage (Table 1), consistent with the roles of these genes in the regulation of cell fate decisions during the preantral phase of folliculogenesis (Parrott & Skinner 1997, Driancourt *et al.* 2000, Knight & Glister 2006, Thomas & Vanderhyden 2006). Patterns of oocyte-specific gene expression observed within the *in vivo* isolated follicles (Table 1) are also supported by previous reports of gene function (Suzumori *et al.* 2002, Shimasaki *et al.* 2004, Knight & Glister 2006). *Nlrp5* and *Jag1* are the only two genes that have steady or increased expression at the COC stage compared to the 2LS follicle stage (Fig. 4C), consistent with their roles in oocyte growth. *Nlrp5* expression reportedly persists into the blastocyst stage of embryonic development (Dean 2002), and *Jag1* is an important factor during all stages of folliculogenesis (Vorontchikhina *et al.* 2005). The expression patterns of *Figla* and the genes encoding the zona pellucida proteins *in vivo* (Table 1, Fig. 4A and C, Fig. 5A and C) are also consistent with previous reports that *Figla* regulates and is required for zona pellucida gene expression and that this expression increases and then decreases as the oocyte increases in diameter (Epifano *et al.* 1995).

In this study, we investigated gene expression that occurs during *in vivo* and *in vitro* follicle growth as a means to identify the supportive and limiting components of the alginate culture environment. In comparing the patterns of expression, we determined that 60% of *in vitro* candidate gene patterns investigated are similar to their corresponding *in vivo* patterns of expression at the MLS stage (Table 2), whereas 50% of the factors had similar expression patterns in COCs *in vitro* and *in vivo* (Table 3). As the culture system supports follicle growth, antrum formation, steroid production, and ultimately the production of fertilizable oocytes, it is expected that many of the gene expression patterns in our culture system are the same as those *in vivo*. At the MLS stage, *Fgf8*, *Fshr*, *Gdf9*, *Igf1*, *Inha*, *Jag1*, *Kit*, *Lhcgr*, *Nlrp5*, *Nobox*, *Ddx4*, and *Zp2* were expressed at similar levels *in vivo* and *in vitro*. Of these, expression levels of *Fgf8*, *Fshr*, *Gdf9*, *Kit*, and *Zp2* had diverged by the COC stage (Table 3). However, *Cyp19a1*, *Figla*, *Kitl*, and *Tgfb2* levels were not

Table 1 Patterns of gene expression as follicles develop *in vivo* from the two-layered secondary to multi-layered secondary stages. Expression changes of twofold or greater at the multi-layered secondary follicle stage are considered increased or decreased from the two-layered secondary follicle stage, whereas changes of less than twofold are reported here as unchanged.

	Increase	Decrease	No change
<i>In vivo</i>			
Endocrine	<i>Cyp19a1</i> <i>Fshr</i> <i>Inha</i> <i>Inhba</i> <i>Igf1</i>		<i>Lhcgr</i>
Growth			<i>Bmp15</i> <i>Gdf9</i> <i>Kit</i> <i>Kitl</i> <i>Tgfb2</i> <i>Figla</i> <i>Jag1</i> <i>Nlrp5</i> <i>Nobox</i> <i>Zp1</i> <i>Zp2</i> <i>Zp3</i>
Oocyte	<i>Ddx4</i>	<i>Fgf8</i>	

significantly different between *in vivo* and *in vitro* COCs. The oocyte has been described as orchestrating folliculogenesis as well as its own maturation, and the expression of these genes in similar patterns and at comparable levels between *in vitro* and *in vivo* is consistent with the generation of healthy, viable, and meiotically competent oocytes obtained from our culture system (Dean 2002, Eppig *et al.* 2002, Xu *et al.* 2006a, 2006b).

While *in vitro* culture does promote follicle growth and oocyte development, the follicles cultured *in vitro* do not reach the same final diameter as *in vivo* grown follicles and the oocytes do not have the same potential for blastocyst development and production of live births. These observations motivated this study to identify differences between *in vitro* cultured follicles and *in vivo* follicles, which were observed primarily as quantitative differences in expression level and not a complete absence of expression. Differences were expected due to isolating follicles from their native environment, which may alter the signals that normally regulate follicle development. For example, signaling through the hypothalamic–pituitary–gonadal axis is disrupted, surrounding follicles and somatic cells are removed and oxygen concentrations differ. While only 15% of the tested genes were more highly expressed in *in vitro* COCs compared with *in vivo* COCs, 35% of the tested genes had lower expression levels *in vitro* compared with *in vivo* (Table 3). Of the endocrine-related genes, only *Lhcgr* was expressed in the same manner and at the same level of expression between *in vivo* and *in vitro* cultured follicles and COCs. *Inha* and *Inhba* were upregulated in the cultured COCs compared to *in vivo* COCs, which suggest some disruption of

normal endocrine regulation of development. In addition, increased inhibin levels may be required for *in vitro* follicle development, in order to accommodate the decrease in the external stimuli that normally regulate development.

Substantial differences in transcript levels of oocyte-specific growth factor and zona pellucida genes were observed (Table 3), as well as a difference in overall expression patterns of *Fgf8*, *Kitl*, *Tgfb2*, and *Zp3* (Table 2). Decreased expression of the growth-related genes, such as *Bmp15*, *Gdf9*, and *Kit*, may be a consequence of removing the follicle from growth inhibitory signals imparted by surrounding follicles and stromal cells. In the absence of this inhibition, the reduced expression of these genes may be sufficient to promote follicle development. However, these variations in expression level between *in vitro* and *in vivo* may also underlie the differences in the follicle and oocyte properties that are obtained at the conclusion of culture.

This culture system provides an enabling tool to investigate the differences in order to understand the contributions of the environment to follicle development. Importantly, we note that the *in vitro* and *in vivo* environment has significant differences from each other, as well as the normal cycle. Pregnant mare serum gonadotropin (PMSG) raises the level of FSH above normal in order to induce the development of pre-ovulatory follicles. Although this technique is effective, it is not reflective of the normal cycle. For our *in vitro* culture, FSH levels are constant, which is also not reflective of the normal cycling mouse. Although these approaches to obtain mature oocytes differ and may underlie some of the differences in gene expression, a number of similarities are present in the measurements of gene expression.

Table 2 Patterns of gene expression as follicles develop *in vitro* from the two-layered secondary to multi-layered secondary stages. Bolded genes indicate significant differences relative to the *in vivo* condition.

Expression changes of twofold or greater at the multi-layered secondary follicle stage are considered increased or decreased from the two-layered secondary follicle stage, whereas changes of less than twofold are reported here as unchanged.

	Increase	Decrease	No change
<i>In vitro</i>			
Endocrine	<i>Fshr</i> <i>Inha</i>	<i>Cyp19a1</i>	<i>Inhba</i> <i>Lhcgr</i>
Growth		<i>Kitl</i> <i>Tgfb2</i>	<i>Bmp15</i> <i>Fgf8</i> <i>Gdf9</i> <i>Kit</i> <i>Igf1</i> <i>Figla</i> <i>Jag1</i> <i>Nlrp5</i> <i>Zp1</i> <i>Zp2</i> <i>Zp3</i>
Oocyte	<i>Nobox</i> <i>Ddx4</i>		

Table 3 Summary of mRNAs that are higher or lower under *in vitro* versus *in vivo* conditions at the multi-layered secondary follicle (MLS) stage or the cumulus-oocyte complex (COC) stage.

	MLS follicles		COCs	
	Up <i>in vitro</i>	Down <i>in vitro</i>	Up <i>in vitro</i>	Down <i>in vitro</i>
Endocrine		<i>Cyp19a1</i> <i>Inhba</i>	<i>Inha</i> <i>Inhba</i>	<i>Fshr</i>
Growth		<i>Bmp15</i> <i>Kitl</i> <i>Tgfb2</i>		<i>Bmp15</i> <i>Gdf9</i> <i>Kit</i>
Oocyte	<i>Zp3</i>	<i>Figla</i> <i>Zp1</i>	<i>Fgf8</i>	<i>Zp1</i> <i>Zp2</i> <i>Zp3</i>

In this study, the 3D alginate culture system was shown to support follicular gene expression similar, in part, to that of follicles grown *in vivo*. These similarities between *in vitro* and *in vivo* validate the use of the 3D culture system as a convergent tool for investigating ovarian follicular development and modeling follicle biology. The results of this study suggest that the 3D alginate culture system could be a powerful tool for modeling other physiologic processes, which depend on a 3D architecture and which are affected by environmental rigidity. For example, culture in 2D versus 3D alters the differentiation potential of embryonic stem cells (Dawson *et al.* 2008) and matrix rigidity plays a role in the differentiation potential of stem cells and cancer progression. A 3D alginate culture system of varying rigidity, combined with appropriate growth factors, can be used as a synthetic environment to molecularly dissect tissue formation.

There were also many differences in gene expression between follicles grown in the alginate culture system and those grown *in vivo*. Because follicles in culture are removed from the context of endocrine axes and the paracrine effects of neighboring follicles, gene expression patterns supporting optimized follicle growth may have differed from those *in vivo*. Thus, it cannot be assumed that all differences seen between *in vitro* and *in vivo* follicles are maladaptive. Nevertheless, genes crucial for healthy follicle development, which are expressed differently in the alginate culture system, are potential targets of, and markers for, improved culture conditions that support a higher frequency of fertilizable oocyte development.

In conclusion, the abundance and changing transcription patterns of gene expression during both *in vivo* and *in vitro* folliculogenesis were directly compared within MLS follicles and COCs. For the genes tested, 60% had the same *in vitro* patterns of gene expression during the first 4 days of culture as follicles isolated *in vivo* with a similar size and morphology. In addition, half of the factors had the same expression level within COCs obtained *in vivo* and following *in vitro* culture. Interestingly, oocyte-specific genes such as *Figla*, *Jag1*, *Nlrp5*, *Nobox*, and *Ddx4* were all expressed *in vitro* at

the same level and with the same pattern as they were *in vivo*. Despite the many similarities, several differences in expression profile were noted. Endocrine-related genes, such as *Cyp19a1* and *Inhba*, as well as growth-related genes, such as *Bmp15*, *Kitl*, and *Tgfb2*, had lower levels of expression *in vitro* than *in vivo* after 4 days of culture. At the culmination of culture, COCs obtained *in vitro* expressed endocrine-related genes (*Fshr*, *Inha*, and *Inhba*), growth-related genes (*Kit* and the oocyte-specific genes *Bmp15* and *Gdf9*), and other oocyte-specific genes (*Fgf8*, *Zp1*, *Zp2*, and *Zp3*) at aberrant levels compared to *in vivo* COCs. These studies provide a set of gene expression profiles indicating the similarities and differences between *in vivo* and *in vitro* cultured follicles, providing a basis for investigating follicular development and the creation of environments that promote follicle development and maximize oocyte quality.

Materials and Methods

Animals and materials

Male CBA and female C57BL/6 mouse breeding pairs were housed in a temperature- and light-controlled environment (12 h light:12 h darkness) and provided with food and water *ad libitum*. Animals were fed Teklad Global irradiated 2919 chow, free of phyto-estrogens. Animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and protocols approved by the Institutional Animal Care and Use Committee at Northwestern University.

Alginate preparation

Sodium alginate (55–65% glucuronic acid) was provided by FMC BioPolymers (Philadelphia, PA, USA). Charcoal-stripped and sterilized alginate was reconstituted with 1×PBS to a concentration of 0.25% (w/v).

Follicle isolation, encapsulation, and culture

2LS (follicular diameter of 100–130 µm) and MLS (follicular diameter of 150–180 µm) were mechanically isolated from ovaries of C57BL/6xCBA F1 mice 12 and 16 days *postpartum* (dpp) respectively (Xu *et al.* 2006a). 2LS and MLS follicles were immediately frozen in clean tubes with minimal media for real-time PCR analysis of *in vivo* gene expression. For analysis of gene expression in the alginate culture system, 2LS follicles were encapsulated into 0.25% (w/v) alginate beads for culture, as described previously (Xu *et al.* 2006b). Growth medium was prepared using α-MEM with 10 mIU/ml recombinant FSH (Organon, Roseland, NJ, USA), 3 mg/ml BSA, 1 mg/ml bovine fetuin (Sigma–Aldrich), 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium. Beads containing follicles were cultured at 37 °C and 5% CO₂ in individual wells of a 96-well plate containing 100 µl growth medium for 4–12 days, with half of the medium replaced with fresh medium every second day.

Images of the follicles were taken every 4 days using a Leica DM IL light microscope (Leica, Wetzlar, Germany) equipped with a heated stage, and a Spot Insight 2 Megapixel Color Mosaic camera with Spot software (Spot Diagnostic Instruments, Sterling Heights, MI, USA). Follicle diameters were measured using ImageJ Software (National Institutes of Health, Bethesda, MD, USA). After the culture period, the medium was replaced with 100 μ l L15 medium containing 10 IU/ml alginate lyase (Sigma–Aldrich) for 30 min at 37 °C and 5% CO₂ to degrade the alginate bead and release the follicle into the medium. For each RNA sample, an average of 30 follicles were removed from medium and immediately frozen in clean tubes in minimal medium for RNA isolation. For each time point, three independent samples were collected. Each sample consisted of follicles isolated from multiple mice from the same litter. Separate samples, either for *in vivo* analysis or for *in vitro* culture, were isolated on different days from different litters.

COC isolation

For *in vivo* COC isolation, C57BL/6xCBA F1 mice 18 dpp were i.p. injected with 5 IU PMSG. Ovaries were collected 40 h post-injection, and COCs were mechanically isolated from antral follicles of the stimulated ovaries. COCs were also isolated from antral follicles derived from culturing 2LS follicles from 12 dpp mice for 12 days, as described above; thus, these antral follicles were 24 dpp at the time of isolation. Approximately 15–20 antral follicles for RNA isolation were punctured to release the COC for each sample. COCs were pipetted into clean tubes and immediately frozen in minimal media for RNA isolation. In total, three independent samples were collected as for 2LS and MLS follicles above.

Real-time PCR

Total RNA from frozen uncultured or cultured follicles was isolated using the Absolutely RNA Microprep Kit (Stratagene, Cedar Creek, TX, USA), following the manufacturer's instructions. RNA was reverse transcribed into first-strand cDNA using the SuperScript First Strand Synthesis System (Invitrogen) with random hexamer primers. Real-time PCR using Taqman Gene Expression Assays and the Applied Biosystems 7300 System (Applied Biosystems, Foster City, CA, USA) was used to compare steady-state mRNA levels. Taqman Gene Expression Assays contain two unlabeled primers specific to the gene of interest used at a final concentration of 900 nM each, as well as a FAM dye-labeled, minor groove binder (MGB)-containing probe used at a final concentration of 250 nM. All assays have an amplification efficiency of 100% within measurement error ($\pm 10\%$). All assays used have been optimized for specificity, sensitivity, and efficiency by the manufacturer. Sequences of primers and alignment maps are referenced on the Applied Biosystems website. mRNA transcript levels of the following genes were assayed; gene symbols, followed by the Taqman Assay ID number of each primer/probe set, are in parentheses: *Bmp15* (assay ID Mm00437797_m1), *Cyp19a1* (Mm00484049_m1), DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (*Ddx4*; Mm00802445_m1), *Figla* (Mm00488691_m1), *Fgf8*

(Mm00438922_m1), FSH receptor (*Fshr*; Mm00442819_m1), *Gdf9* (Mm00433565_m1), *Igf1* (Mm00439561_m1), *Inha* (Mm00439683_m1), *Inhba* (Mm00434338_m1), *Jag1* (Mm00496902_m1), *Kit* (Mm00445212_m1), *Kitl* (Mm00442972_m1), *Lhcgr* (Mm00442931_m1), NLR family, pyrin domain containing 5 (*Nlrp5*; Mm00488691_m1), *Nobox* (Mm00453743_m1), *Tgfb2* (Mm00436977_m1), *Zp1* (Mm00494367_m1), *Zp2* (Mm00442173_m1), and *Zp3* (Mm00442176_m1). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; 4352339E) was used as an endogenous control. In total, three independent samples were each run in triplicate, with *in vivo* samples isolated from different mice and *in vitro* samples isolated from different mice and cultured separately as described above. Reactions run without reverse transcriptase served as negative controls.

Statistical analysis

Follicle size, survival rate, antral growth rate, and gene expression data were collected for three independent cultures for each time point. Gene expression relative to 2LS follicles was determined using the comparative C_t method. Statistical significance for gene expression was analyzed using ANOVA followed by Tukey *post hoc* test.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work has been funded by the NIH U54 HD041857 grant.

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Received 23 November 2010

First decision 14 January 2011

Revised manuscript received 10 May 2011

Accepted 24 May 2011