Studies of granulosa cell maturation in dominant and subordinate bovine follicles: novel extracellular matrix focimatrix is co-ordinately regulated with cholesterol side-chain cleavage CYP11A1

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Abstract

During growth of antral ovarian follicles granulosa cells first become associated with a novel type of extracellular matrix, focimatrix, and at larger sizes follicles become either subordinate or dominant. To examine this, bovine subordinate ($9.0 \pm \text{s.e.m. } 0.4 \text{ mm}$; $n=16$), partially dominant ($12.0 \pm 0.6 \text{ mm}$; $n=18$) and fully dominant ($15.0 \pm 0.4 \text{ mm}$; $n=14$) follicles were examined by real time RT-PCR analyses of granulosa cells and by immunohistochemistry of focimatrix. Changes in the expression of FSH receptor, LH receptor, cholesterol side-chain cleavage (*CYP11A1*), 3β -hydroxysteroid dehydrogenase, aromatase (*CYP19A1*) and inhibin- α and β -B were observed as expected for follicle sizes examined. After adjusting for size differences, only *CYP11A1* was significantly different between the groups, and elevated in dominant follicles. Also after adjusting for differences in size there were no significant differences in expression of focimatrix components collagen type IV α -1 (*COL4A1*), laminin β -2, nidogen 1 (*NID1*), and perlecan (*HSPG2*) or the volume density of *NID1* and -2 and *HSPG2*. The volume density of focimatrix components in laminin 111 was significantly elevated in dominant follicles. Adjusting for analysis of more than one follicle per animal and for multiple correlations, *CYP11A1* mRNA levels were highly correlated with the focimatrix genes *COL4A1*, *NID1* and -2 and *HSPG2*. Thus, focimatrix may potentially regulate *CYP11A1* expression, and the regulation of both could be important in follicular dominance.

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Introduction

Growth and development of ovarian follicles from primordial to large antral stages is essential for production of mature oocytes and secretion of ovarian steroid hormones. However, the growth and development of follicles within ovaries involves complex dynamics. During growth, follicles sequentially undergo recruitment (initial and cyclic), selection, dominance and ovulation (or atresia). Initial recruitment is a slow growth phase in comparison with cyclic recruitment (Lussier *et al.* 1987). Once cyclic recruitment commences groups of antral follicles grow as in a 'wave'. The first wave of an oestrous cycle is initiated 1–2 days after ovulation (Valdez *et al.* 2005) and the number of follicular waves per cycle (2 or 3) is determined by the duration of the luteal phase (Fortune 1994). Large variation exists between animals in terms of number of follicles per wave (range 11–54), principally due to differences in the numbers of small follicles (those $\sim 3 \text{ mm}$ in diameter). The number of follicles per wave is relatively consistent within individual cows and does

not correlate with either two or three wave patterns per oestrous cycle, or with dominant follicle development (Burns *et al.* 2005). In each follicular wave, the future dominant follicle enters the wave $\sim 6 \text{ h}$ earlier than other follicles in the cohort, which translates into a size advantage at the end of the common growth phase (Jaiswal *et al.* 2004). Since all follicles of the common growth phase have potential for future dominance, selection of the dominant follicle is likely to involve suppression of other (subordinate) follicles (Ginther *et al.* 2003). As the wave progresses and the dominant follicle grows the subordinate follicles undergo atresia. If the wave is in the luteal phase no surge release of LH can occur and the dominant follicle becomes atretic. A new wave of follicle development follows, and if this is at the end of the luteal phase then that dominant follicle can ovulate in response to a surge release of LH.

The principal hormone controlling follicular growth is FSH, which in turn is negatively controlled by oestradiol and inhibin- α (INHA) secreted from the large-dominant follicle (Campbell *et al.* 2003). Thus, the FSH

concentration is lower (Ginther *et al.* 1997, 2003, Berisha *et al.* 2000) when deviation in the growth rate between dominant and subordinate follicles manifests as a size difference between them. During follicle selection, increasing expression of the FSH receptor (FSHR) in granulosa cells precedes increased expression of the LH receptor (LHCGR; Berisha *et al.* 2000) which is first detected only in healthy follicles >9 mm in diameter (Xu *et al.* 1995). Thus, LHCGR expression in granulosa cells occurs after selection and ensures the continued growth of the dominant follicle as the circulating concentration of FSH falls and subordinate follicles regress. However, FSH is still important as exposure to FSH in the absence of LH can still support the growth of follicles >10 mm in diameter (Crowe *et al.* 2001, Hampton *et al.* 2004). LH also stimulates thecal cell androgen secretion and combined with increasing cytochrome P450aromatase (CYP19A1) expression and activity in granulosa cells, oestradiol production from the dominant follicle increases (Fortune *et al.* 2001). As the dominant follicle matures granulosa cells also express cholesterol side-chain cleavage cytochrome P450 (CYP11A1) and 3 β -hydroxysteroid dehydrogenase (HSD3B) increasing their ability also to produce progesterone (Tian *et al.* 1995). These changes in granulosa cells whereby they become steroidogenic are generally referred to as maturation.

While externally follicle growth and development and granulosa cell maturation are under the control of the pituitary gonadotrophins, internally growth factors and cell–cell and cell–matrix contacts are also important. Very little research has been conducted on the roles of matrix. However, a novel type of basal lamina matrix, called focimatrix (focal intra-epithelial matrix; Irving-Rodgers *et al.* 2004), developmentally appears about 10 days before ovulation in the cow. Focimatrix (illustrated in Fig. 1) is deposited as plaques or aggregates of basal lamina material between the epithelial granulosa cells in follicles >5 mm in diameter in bovine and its abundance increases as follicles enlarge. Although discovered in the bovine, focimatrix also exists in sheep (Huet *et al.* 1997) and humans (Yamada *et al.* 1999, Alexopoulos *et al.* 2000). The novel aspect of this matrix is its conformation. Basal laminae are normally a sheet of matrix ‘wrapped’ around a cell or a group of cells (epithelia or endothelia). Therefore, basal laminae make compartments within tissues. Focimatrix, however, does not form a continuous layer and thus it cannot perform known basal lamina functions. Focimatrix in the cow is composed of aggregates of basal lamina material (collagen type IV α -1 (COL4A1) and α -2, laminin chains α -1, β -2 (LAMB2) and γ -1, perlecan (HSPG2), and nidogens 1 and 2 (NID1 and -2). It is degraded at ovulation (Irving-Rodgers *et al.* 2006a) as granulosa cells commence terminal differentiation into luteal cells and hence does not exist in human or bovine corpora lutea (Irving-Rodgers *et al.* 2004, 2006b). Thus, focimatrix is

Figure 1 Laminin 111 components are localised (red) to focimatrix (open arrows) within the membrana granulosa, the follicular basal lamina (arrows) and subendothelial basal laminae in the theca interna (TI). Nuclei; DAPI counterstain (blue). Scale bar=20 μ m.

developmentally regulated and is present before, and then during, the follicular phase when granulosa cells mature to produce progesterone and oestradiol and at the same time when follicles become dominant or subordinate.

To investigate whether focimatrix has a role either in the selection and dominance of ovarian follicles and/or in the maturation of granulosa cells, we examined healthy subordinate, partially dominant (the largest healthy follicle, but next largest is still healthy) and fully dominant (the largest healthy follicle, but next largest is atretic) bovine follicles. We undertook immunohistochemical analysis of focimatrix components in the membrana granulosa of these follicles, and examined expression of markers of granulosa cell maturation (gonadotrophin receptors, inhibins, steroidogenic enzymes) and focimatrix components.

Results

Fully dominant follicles were significantly ($P=0.0002$) larger (16.0 ± 0.5 mm; mean $\pm$ s.e.m.) than partially dominant follicles (12.0 ± 0.5 mm), which in turn were significantly ($P<0.0001$) larger than subordinate follicles (9.5 ± 0.5 mm; Fig. 2A). The diameter of dissected follicles can be up to 3 mm greater than that estimated by ultrasound, since the latter measures only the antral cavity (Quirk *et al.* 1986, Evans & Fortune 1997). Real time RT-PCR analyses showed that mRNA levels for the *FSHR* did not differ between these follicle groups (Fig. 2B) while mRNA levels for the *LHCGR* were significantly greater in fully dominant follicles in comparison with partially dominant ($P=0.0014$) and subordinate follicles ($P=0.0002$; Fig. 2C). However,

Figure 2 Mean $\pm$ S.E.M. diameter of follicles (A), and the levels of mRNA relative to 18S rRNA in granulosa cells of (B) *FSHR*, (C) *LHCGR* (log scale), (D) *CYP11A1*, (E) *HSD3B*, (F) *CYP19A1*, (G) *INHA* and (H) *INHBB* in subordinate, partially dominant and fully dominant follicles (numbers of follicles are shown in parentheses). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ significantly different prior to adjusting for differences in diameters between groups. For statistical analyses the data in (C), (D), (E) and (F) were log transformed.

after adjusting for differences in follicle diameter, there were no longer any significant differences between the three follicle groups. mRNA levels for *CYP11A1* were significantly lower in subordinate follicles in comparison with dominant follicles ($P = 0.004$; Fig. 2D) and this effect was maintained following adjustment for follicle size ($P = 0.01$). mRNA levels for *HSD3B* and *CYP19A1* were significantly lower in subordinate follicles in comparison with either partially or fully dominant follicles (Fig. 2E and F) but after adjusting for follicle diameter there were no longer any significant differences. mRNA levels for *INHA* ($P = 0.071$) and *INHBB* ($P = 0.72$) did not differ significantly between follicles (Fig. 2G and H).

The mRNA levels of *COL4A1*, *LAMB2*, *HSPG2* and *NID1* did not differ significantly between follicles (Fig. 3A–D), however, mRNA levels for *NID2* were significantly ($P < 0.01$) lower in partially dominant follicles in comparison with fully dominant ($P = 0.0072$) or subordinate ($P = 0.0018$) follicles, and this effect was still significant after adjusting for follicle size ($P = 0.0182$ and $P = 0.0323$ respectively; Fig. 3E).

Estimation of the volume density of extracellular matrix (laminin 111, NID1 and -2, and HSPG2) by image analysis of immunostained sections showed that the amount of laminin (as detected with anti-laminin 111) was significantly lower in subordinate follicles in comparison with partially ($P < 0.001$) or fully ($P = 0.0007$) dominant follicles (Fig. 4A). Following adjustment for follicle size this effect remained ($P = 0.07$ and $P = 0.001$ respectively). The amounts of NID1 and -2 and HSPG2 did not differ significantly ($P > 0.05$) between groups (Fig. 4B–D).

Correlation analyses were conducted using follicle size and gene expression data, accounting for dependence between observations from the same animal (since 2 or 3 follicles were collected per animal), and

Figure 3 Mean $\pm$ S.E.M. levels of mRNA relative to 18S rRNA in granulosa cells of (A) *COL4A1*, (B) *LAMB2*, (C) *HSPG2*, (D) *NID1* and (E) *NID2* in subordinate, partially dominant and fully dominant follicles (numbers of follicles are shown in parentheses). ** $P < 0.01$ significantly different prior to adjusting for differences in diameters between groups. For statistical analyses the data in (C), (D) and (E) were log transformed.

Figure 4 Volume density of (A) laminin (detected using anti EHS laminin 111), (B) nidogen 1 (C) nidogen 2 and (D) perlecan in the membrana granulosa of subordinate, partially dominant and fully dominant follicles (numbers of follicles are shown in brackets). *** $P < 0.001$; **** $P < 0.0001$ significantly different prior to adjust for differences in diameters between groups. For statistical analyses the data in (D) were initially log transformed.

corrected for multiple comparisons. The correlation coefficients and their significance values are shown in Table 1 and examples shown in Fig. 5. The expression of levels of *COL4A1*, *NID1* and -2 and *HSPG2* were very significantly correlated with each other (Table 1 and Fig. 5), and *LAMB2* was less significantly correlated with *COL4A1* and *HSPG2*. *LHCGR* correlated with follicle size and extracellular matrix components (*COL4A1*, *NID1* and -2 and *HSPG2*, but not *LAMB2*; Table 1 and Fig. 5). Interestingly, *CYP11A1* correlated with *CYP19A1* and also with *LAMB2*, *HSPG2* and *COL4A1* (Table 1 and Fig. 5). Neither *FSHR* nor *LHCGR* correlated with any steroidogenic enzyme (Table 1).

By accounting for dependence between observations from the same animal these statistical analyses effectively average observations across all the follicles for each animal. Thus, individual correlations were additionally conducted separately for each follicle group thereby ensuring that only one follicle per animal was included in each analysis (Table 2 shows data for partially dominant follicles). Qualitatively similar correlation relationships were observed in the partially dominant subset of follicles, but additionally *CYP19A1* expression correlated with *LHCGR*, *NID1* and -2, *HSPG2* and *COL4A1* but not *LAMB2*. In subordinate follicles, there was no association between *CYP11A1* and *CYP19A1* with the focimatrix components (shown in Supplementary Data, which can be viewed online at www.reproduction-online.org/supplemental/) and no significant correlations were observed in the fully dominant follicles (shown in Supplementary Data).

Table 1 Correlation coefficients for correlations between genes and follicle size using all data, and accounting for dependence between observations from the same animal and correcting for multiple comparisons using the Sidak stepdown method.

	Diameter	FSHR	Log LHCGR	INHBB	INHA	Log HSD3B	Log CYP11A1	Log CYP19A1	LAMB2	Log NID1	Log NID2	Log HSPG2
Diameter	1.000											
FSHR	-0.478	1.000										
Log LHCGR	0.715 [†]	-0.420	1.000									
INHBB	-0.419	0.402	-0.316	1.000								
INHA	-0.039	0.198	0.150	0.549	1.000							
Log HSD3B	0.409	-0.170	0.276	0.258	0.495	1.000						
Log CYP11A1	0.016	0.144	0.477	0.424	0.673 [†]	0.580	1.000					
Log CYP19A1	0.474	0.096	0.588	0.136	0.541	0.627	0.711 [†]	1.000				
LAMB2	0.034	0.055	0.458	0.220	0.510	0.270	0.609 [*]	0.394	1.000			
Log NID1	0.322	-0.146	0.778 [§]	-0.117	0.148	-0.068	0.490	0.467	0.467	1.000		
Log NID2	0.428	-0.397	0.821 [§]	-0.165	0.062	-0.007	0.300	0.307	0.415	0.864 [§]	1.000	
Log HSPG2	-0.093	-0.010	0.668 [†]	0.088	0.344	-0.038	0.642 [†]	0.319	0.613 [*]	0.922 [§]	0.701 [†]	1.000
Log COL4A1	0.231	-0.127	0.791 [§]	-0.070	0.289	0.043	0.578 [*]	0.467	0.630 [*]	0.866 [§]	0.780 [§]	0.836 [§]

* $P < 0.05$, [†] $P < 0.01$, [§] $P < 0.0001$.

Figure 5 Scatter plots of mRNA levels of (A) Ln *NID2* versus Ln *LHCGR*, (B) Ln *HSPG2* versus Ln *NID2*, and (C) Ln *HSPG2* versus Ln *CYP11A1*. circles, subordinate follicles; triangles, partially dominant follicles; diamonds, fully dominant follicles.

Discussion

During the later stages of follicle development, we measured in granulosa cells the level of expression of markers of maturation (*FSHR*, *LHCGR*, *CYP11A1*, *HSD3B*, *CYP19A1*, *INHA* and *INHBB*) and components of focimatrix (*COL4A1*, *LAMB2*, *HSPG2*, *NID1* and -2). We also measured the volume density of components of focimatrix (components of laminin 111, *HSPG2*, *NID1* and -2) in the membrana granulosa. With a view to identifying a role of focimatrix either in follicle dominance and/or granulosa cell maturation, we conducted our statistical analyses after removing the effect of follicle size. These analyses revealed that *CYP11A1* expression was upregulated in dominant follicles independently of increased size, and that *CYP11A1* expression was highly and positively correlated with a number of focimatrix components, but not *FSHR* or *LHCGR*. This suggests either that focimatrix genes and *CYP11A1* are all co-ordinately regulated, or that focimatrix induces the expression of *CYP11A1* during follicle maturation. The latter is more likely as focimatrix is first observed at smaller sizes of follicles (5 mm follicle diameter) than is *CYP11A1* (Irving-Rodgers *et al.* 2004).

Studies of follicular dominance have generally been conducted by examining known follicular genes

Table 2 Correlation coefficients of correlations between genes and follicle size in the partially dominant group, corrected for multiple comparisons using the Sidak stepdown method.

	Diameter	<i>FSHR</i>	Log <i>LHCGR</i>	<i>INHBB</i>	<i>INHA</i>	Log <i>HSD3B</i>	Log <i>CYP11A1</i>	Log <i>CYP19A1</i>	<i>LAMB2</i>	Log <i>NID1</i>	Log <i>NID2</i>	Log <i>HSPG2</i>
Diameter	1.000											
<i>FSHR</i>	-0.493	1.000										
Log <i>LHCGR</i>	0.784*	-0.295	1.000									
<i>INHBB</i>	-0.596	0.483	-0.388	1.000								
<i>INHA</i>	-0.151	0.346	0.350	0.281	1.000							
Log <i>HSD3B</i>	0.181	-0.156	0.119	-0.175	-0.213	1.000						
Log <i>CYP11A1</i>	0.155	0.225	0.615	0.233	0.381	-0.070	1.000					
Log <i>CYP19A1</i>	0.556	-0.186	0.922 [§]	-0.186	0.282	0.266	0.754*	1.000				
<i>LAMB2</i>	0.497	0.094	0.764*	-0.047	0.343	-0.021	0.711	0.711	1.000			
Log <i>NID1</i>	0.461	-0.030	0.855 [†]	-0.051	0.589	-0.195	0.802*	0.803*	0.762	1.000		
Log <i>NID2</i>	0.538	-0.023	0.860 [†]	0.032	0.228	-0.039	0.739	0.828 [†]	0.703	0.928 [§]	1.000	
Log <i>HSPG2</i>	0.374	0.100	0.809*	0.058	0.359	-0.098	0.891 [†]	0.833 [†]	0.786*	0.954 [§]	0.891 [†]	1.000
Log <i>COL4A1</i>	0.568	-0.043	0.914 [§]	-0.041	0.280	0.110	0.797*	0.929 [§]	0.773*	0.895 [†]	0.924 [§]	0.904 [§]

* $P < 0.05$, [†] $P < 0.01$, [§] $P < 0.001$, [§] $P < 0.0001$.

(Bao *et al.* 1997, Yuan *et al.* 1998) or using gene expression profiling (Malhi *et al.* 2005, Zielak *et al.* 2007) with a view to understand the regulation of dominance. However, follicular dominance is also associated with a greater increase in size of the dominant follicle over the subordinate follicle leading eventually to its atresia. Before the onset of atresia in the subordinate follicle both dominant and subordinate follicles are increasing in size. Therefore, in the current study, subordinate, partially dominant and fully dominant follicles were first identified on size and histological assessment of the health of the three largest follicles in each pair of ovaries. In agreement with previously published data (reviewed in (Bao & Garverick 1998, Webb *et al.* 1999, Fortune *et al.* 2001, Knight & Glister 2003, Beg & Ginther 2006)) follicle size increased across these groups and the expression of *FSHR* declined while *LHCGR*, *CYP11A1*, *HSD3B* and *CYP19A1* all increased and no changes were observed in *INH1* and *INH2*. We then statistically removed the effect of size differential between the groups. This enabled us to identify molecules that were differentially altered specifically due to the degree of dominance, independent of changes in follicle size. Only *CYP11A1* remained significantly different and was elevated in dominant follicles compared with subordinate follicles. No significant differences were observed between the groups in the expression of *COL4A1*, *LAMB2*, *HSPG2* and *NID1*, but *NID2* was significantly lower in the partially dominant group, even after adjusting for size. The volume density of focimatrix obtained by immunostaining with antisera against laminin 111 (which identifies any of the laminin chains α -1, β -1 or γ -1) was significantly elevated in dominant follicles even after adjusting for a size differential. No significant differences were observed in volume density for *HSPG2* and *NID1* or -2. Collectively, these results suggest that many of the markers of granulosa cell maturation identified here and previously in many other studies differed between dominant and subordinate follicles merely because the follicles were different in size. However, since both *CYP11A1* expression and the focimatrix components found in laminin 111 were significantly elevated in dominant follicles, independent of size, it is suggested that these molecules could be important in either gaining or maintaining dominance.

Correlation analyses were also undertaken to gain insight into which genes could be co-ordinately regulated during the process of dominance. These analyses took into account the fact that more than one follicle was examined per animal, and that multiple correlations were conducted. Interestingly, *CYP11A1* expression was correlated not only with *CYP19A1* but also with *LAMB2*, *HSPG2* and *COL4A1* expression across all three groups of follicles. Additionally, in partially dominant follicles, *NID1* and -2, *HSPG2* and *COL4A1* were significantly correlated with *CYP19A1*, suggesting that at least in the transition to gain

dominance there is also an association between focimatrix and *CYP19A1* expression. Recent studies have suggested that β -catenin can upregulate both *CYP11A1* and *CYP19A1* in rat granulosa cells (Parakh *et al.* 2006) and that β -catenin is an important co-activator of FSH-regulated oestrogen synthesis (Hernandez Gifford *et al.* 2009). β -catenin also plays a pivotal role in epithelial-mesenchymal transition (EMT; Shook & Keller 2003, Oloumi *et al.* 2004). In epithelia, it associates with the junctions at the cell surface as part of focal adhesions, and in mesenchymal cells it is phosphorylated and translocated into the nucleus where it acts as a cofactor in activating gene transcription. While most of the literature focuses on cell-cell junctions and cadherins there is evidence for a role of integrin-linked kinase in EMT (Oloumi *et al.* 2004). Granulosa cell interaction with focimatrix is likely to be via integrins, and thus β -catenin could be the functional link between focimatrix and expression of steroidogenic enzymes.

Expression levels of *COL4A1*, *NID1* and -2, and *HSPG2* were highly correlated with each other, while *LAMB2* was also significantly correlated with *COL4A1* and *HSPG2*. These genes encode different components of focimatrix which has the ultrastructural appearance of a basal lamina but without forming a sheet as conventional basal laminae do (Irving-Rodgers *et al.* 2004). Thus, focimatrix would be expected to contain basal lamina components in sufficiently correct proportions for basal lamina assembly. That *LAMB2* was not as tightly correlated with all other focimatrix components is not surprising as there is more than one type of laminin in follicular basal laminae (van Wezel *et al.* 1998). Overall, these results suggest co-regulation of the molecules that constitute focimatrix.

Highly significant correlations with *LHCGR* expression and *COL4A1*, *NID1* and -2, and *HSPG2* were also observed and may indicate that LH could upregulate focimatrix production, or that focimatrix could upregulate *LHCGR*. The latter is more likely as focimatrix appears during follicular development at 5 mm diameter (Irving-Rodgers *et al.* 2004), before the upregulation of *LHCGR*. Furthermore, in other preliminary unpublished results, we have been unable to demonstrate LH induction of focimatrix *in vitro*. Alternatively, induction of focimatrix and upregulation of *LHCGR* may occur coincidentally via independent mechanisms.

Nidogens function to cross-link molecules within basal lamina. *NID1* and -2 can bind laminin γ -1, *COL4* and *HSPG2*, thus cross-linking these components makes the basal lamina more rigid. They have overlapping functions, but are not identical as *NID2* has lower affinity for laminin and can bind endostatin, whereas *NID1* does not (Ho *et al.* 2008). During follicular development, these molecules are differentially regulated and are absent from the follicular basal lamina in the primordial

follicles but appear at the early antral stage (McArthur *et al.* 2000, Irving-Rodgers & Rodgers 2006). This is despite the follicular basal lamina of primordial follicles having all six collagen type IV chains and at least the laminin chains α -1, β -2 and γ -1 (Rodgers *et al.* 1998, van Wezel *et al.* 1998). Nidogens are universally produced by mesenchymal cells even when present as a component of epithelial basal lamina (Ho *et al.* 2008). However, in the present study, we have shown that the granulosa cells express *NID1* and -2, with *NID2* mRNA levels being an order of magnitude greater than *NID1*. Other components of focimatrix, *COL4A1*, *LAMB2* and *HSPG2*, were also expressed by granulosa cells, suggesting that the focimatrix is derived from the granulosa cells themselves. In the present study, there was not precise agreement between the results obtained for the mRNA levels and the volume density of the focimatrix. For example, *NID2* mRNA levels were significantly different between the three groups whereas the volume density of *NID2* was not. This may not be unexpected since extracellular matrices can have relatively long half lives.

Thus, in summary, we observed that dominant follicles independent of increasing follicle size have increased expression of *CYP11A1* and focimatrix components contained in laminin 111. Additionally, *CYP11A1* is highly correlated with the expression of a number of focimatrix genes. Since focimatrix precedes *CYP11A1* expression during follicle growth (Irving-Rodgers *et al.* 2004) and first appears at follicular sizes smaller than follicular deviation, focimatrix may induce the expression of *CYP11A1* and this process may be important for development or maintenance of follicular dominance. However, direct proof of this is still required.

Materials and Methods

Tissue processing

Pairs of ovaries were collected at the abattoir from 41 *Bos taurus* cows, visually assessed as non-pregnant. The largest two or three follicles were identified and dissected from the ovaries of 18 and 23 cows respectively. Each pair of ovaries had one corpus luteum at stage II and III (Ireland *et al.* 1980), indicating that the follicles were not in the ovulatory wave of the cycle, and thus eliminating any confounding effect of an LH surge. The external follicle diameter was measured with callipers and then cut open and a portion through the follicle wall ($\sim 2 \times 2 \times 2$ mm) was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer. An additional small section through the follicle wall ($\sim 2 \times 2 \times 2$ mm) was placed in Tissue Tek OCT compound (Miles Inc., Elkhart, IN, USA) and snap-frozen for immunohistochemistry. The remainder of the follicle was removed to Hanks balanced salt solution without calcium or magnesium (H 2387; Sigma Bio Science) and granulosa cells isolated, then placed in 500 μ l Trizol (Invitrogen Aust. Pty Ltd) for extraction of RNA.

Follicle classification

For follicles collected from the same cow, the largest was classified as dominant and smaller follicle(s) classified as subordinate. Follicles were assessed as healthy or atretic based upon the morphology of the membrana granulosa and the presence or absence of apoptotic cells, as previously described (Irving-Rodgers *et al.* 2001, 2003). These morphological methods have previously been shown to classify follicles correctly as healthy or atretic on the basis of follicular fluid oestradiol levels (Irving-Rodgers *et al.* 2001, 2003). Follicles were assigned to one of three groups based upon the following classification regime: Group 1, largest follicle healthy and subordinate follicles atretic ($n=14$); Group 2, all follicles healthy ($n=7$); Group 3, largest follicle atretic and subordinate follicle(s) healthy ($n=11$). Only healthy follicles were included in the study. The largest healthy follicles from Group 1 were designated as fully dominant ($n=14$) since subordinate follicles in this group were atretic. The largest healthy follicles from Groups 2 and 3 were designated as partially dominant ($n=18$), since subordinate follicles in these groups were healthy. The subordinate follicle class was derived from healthy follicles of Groups 2 and 3 ($n=16$).

Histology and immunohistochemistry

For light microscopy, glutaraldehyde-fixed tissue was post-fixed in 1% osmium tetroxide and embedded in epoxy resin as previously described (Irving-Rodgers *et al.* 2002b) and 1 μ m thick sections were cut with glass knives using a Richert-Jung Ultracut E ultramicrotome (Leica Microsystems Pty Ltd, North Ryde, NSW, Australia), collected on plain glass slides and stained with aqueous 1% methylene blue in 1% sodium tetraborate. OCT-embedded follicle walls were sectioned at 10 μ m using a CM 1800 Leica cryostat (Leica Microsystems Pty Ltd), and immunohistochemistry conducted as reported previously (Irving-Rodgers *et al.* 2002a). Unfixed sections were dried under vacuum for 5 min before fixation in either formalin or 100% ethanol, or left unfixed, depending upon the primary antibody to be applied. All slides were incubated in 10% normal donkey serum (D-9663; Sigma Bio Science) in antibody diluent containing 0.55 M sodium chloride and 10 mM sodium phosphate (pH 7.2), for 20 min at room temperature. Sections were then incubated overnight with primary antibodies. The primary antibodies were rabbit anti-mouse laminin 111 from the EHS tumour (#L 9393, Sigma Bio Science, 1:100 dilution, section fixed in 100% ethanol), rat anti-human HSPG2 (#A7L6 from Upstate Biotechnologies (Lake Placid, NY, USA), 1:100 dilution, section formalin fixed), rabbit anti-mouse *NID1* (#913, Dr M Dziadek (Dziadek *et al.* 1985), 1:200 dilution, unfixed section) and rabbit anti-mouse *NID2* (#1130 from Dr T Sasaki (Salmivirta *et al.* 2002), 1:800 dilution, unfixed section). The secondary antibodies used were biotin-SP-conjugated AffiniPure donkey-rabbit IgG (Cat # 711-066-152, 1:200), donkey-mouse IgG (715-065-020, 1:100), or donkey-rat IgG (712-066-153, 1:100) depending upon primary antibody, followed by Cy3-conjugated-streptavidin (016-160-084, 1:100), from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA), in antibody diluent. All incubations were carried out at

room temperature in a humidified chamber and following fixation and incubation with primary or secondary antibodies or streptavidin-conjugated reagents, sections were washed (3×5 min) in hypertonic PBS containing 0.274 M sodium chloride, 5.4 M potassium chloride, 10 mM sodium phosphate, pH 7.2. Sections were counterstained with the nuclear stain 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (3 µm; Molecular Probes, Eugene, OR, USA) for 10 min at room temperature and mounted in Dako fluorescent mounting media (Cat. # S3023; Dako Corporation, Carpinteria, CA, USA). Sections were viewed with an Olympus BX50 microscope with epifluorescence attachment (Olympus Australia Pty Ltd, Mount Waverly, VIC, Australia) at a magnification of ×60 and images (three per section) captured with a Spot RT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). ImageJ software (<http://rsb.info.nih.gov/ij/>) was used to estimate the volume fraction of immunoreactive material in the membrana granulosa by selecting a region of interest excluding the follicular basal lamina and measuring the total area of immunopositive material per area of membrana granulosa.

RNA extraction

Total RNA was extracted from granulosa cells using Trizol reagent as per the manufacturer's recommendations. The quantity of total RNA was determined by spectrophotometry (Absorbance at 260 nm) using the Beckman DU50 u.v. spectrophotometer (Beckman Coulter Australia Pty Ltd, Gladesville, NSW, Australia). Two microgram RNA was DNase treated (DNA-free, Ambion, Applied Biosystems, Melbourne, VIC, Australia) and then used to prepare cDNA. cDNA (20 µl) was synthesised according to the manufacturer's instructions using 500 ng random hexamers (GeneWorks, Adelaide, SA, Australia), 10 nmol dNTP mix, 20 U RNaseOUT and 200 U Superscript III RT (Invitrogen).

Real time RT-PCR

Quantitative RT-PCR analysis of mRNA was performed using a Corbett RotorGene 6000 (Adelab Scientific, Adelaide, SA, Australia) and Real MasterMix, (Eppendorf, Quantum,

Brisbane, QLD, Australia) with SYBR Green as the fluorescence detector, according to the manufacturer's recommendations. The housekeeping gene was 18S rRNA. Primers were designed across an exon boundary to amplify cDNAs (Table 3). *Taq* polymerase amplified PCR products were cloned into pCR II-TOPO plasmid vector; plasmids were transformed into Top 10 F' competent *Escherichia coli*. Plasmid DNA was purified using a NucleoSpin kit (Macherey Nagel product supplied by Integrated Sciences, Sydney, NSW, Australia) and then sequenced to confirm the identity of the amplicon. PCR parameters were 95 °C for 15 s and 60 °C for 45 s for 40 cycles and data were analysed using Rotorgene software version 1.7. For quantitation of each gene the corresponding plasmid DNA was quantitated using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and concentrations were calculated from absorbance at 260 nm. Plasmid DNA for each gene was then serially diluted over 3 logs to establish a standard curve that encompassed the observed C_t values for the RNA samples (between 1 ng/µl and 1 fg/µl). Concentration of RNA for each gene was normalised to the concentration of 18S rRNA expressed as fmole mRNA/nmole 18S rRNA.

Statistical analyses

All statistical calculations were performed using SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA). Differences in expression between follicle groups (fully dominant, partially dominant and subordinate) were investigated using linear mixed effects models. Animal number was included as a random effect in the models to account for the dependence in follicles obtained from the same animal. To investigate differences in expression between the follicle groups, unrelated to follicular diameter, additional linear mixed effects models adjusted for follicular diameter were undertaken. In both adjusted and unadjusted analyses, data were examined for normality and were log transformed when necessary. All log transformed data were normally distributed. Where a significant difference was found across the three follicle groups, *post-hoc* tests were conducted to compare the follicle groups two at a time.

To calculate the correlations between the expressions of different genes, while accounting for the dependence between

Table 3 PCR primers and amplicon sizes.

Gene name	Gene symbol	Amplicon size (bp)	Forward primer	Reverse primer	GenBank accession number
18S rRNA	18S	90	5'-agaacggctaccacatccaa-3'	5'-cctgtattgttatttttcgt-3'	DQ222453
FSH receptor	FSHR	74	5'-tggcaagtgccttaacacctgtgt-3'	5'-tggvssgtgcttaatacctgtgt-3'	NM_174061
LH receptor	LHCGR	157	5'-gccactgctgtgcttttagaaa-3'	5'-ccagccactcagttcactctca-3'	U87230
Inhibin-α	INHHA	93	5'-tgagcccgaggaccaagat-3'	5'-ctctctcagcctctccagacat-3'	NM_174094
Inhibin β-B	INHBB	85	5'-cgtctccgagatcatcgctt-3'	5'-tgccctcgttgagatgaaga-3'	NM_176852
Cholesterol side-chain cleavage	CYP11A1	85	5'-cactttccgacatcgagaa-3'	5'-tgaatgatataaactgactccaattgc-3'	K02130
3β-Hydroxysteroid dehydrogenase	HSD3B	70	5'-agagaccatcatgaacgtcaatgt-3'	5'-gtacgctggcctggacaca-3'	NM_776768
Aromatase	CYP19A1	141	5'-ggctatgtggacgtgtgtgacc-3'	5'-tgagaaggagagcttgctgct-3'	NM_174305
Collagen type IV α-1	COL4A1	104	5'-tttcaggcgactcaggg-3'	5'-cgtgccataacagttccgg-3'	XM_580317
Laminin β-2	LAMB2	63	5'-cccagccctactgcatcgt-3'	5'-cgggagtcacacagaaagca-3'	XM_598402
Perlecan	HSPG2	64	5'-tcaggatcgagtcgtctctt-3'	5'-gaccacgcagttcagatcca-3'	XM_584570
Nidogen 1	NID1	106	5'-cgagttagcggcaaggtaag-3'	5'-gcccatgggtcattaccagta-3'	NM_001101155
Nidogen 2	NID2	113	5'-gctttcctgctgcttattga-3'	5'-gggaaatcgtcgtccacatact-3'	XM_600125

observations from the same animal, between-subject Pearson's correlation coefficients were calculated. This was equivalent to taking a mean of the repeated measurements from each animal and using these data to calculate the coefficient. As a large number of correlations were investigated using this approach, the *P* values were corrected for multiple comparisons using the stepdown Sidak method. Where appropriate, log transformed data were used to ensure normally distributed data were analysed. In addition to an assessment of the correlations over all follicle groups, correlations within each group of follicles (in which each follicle was from a separate animal) were undertaken.

Declaration of interest

The authors declare that they have no conflicts of interest that would affect the impartiality of the conduct and reporting of these experiments.

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