Title:

Oocyte glycoproteins regulate the form and function of the follicle basal lamina and theca cells

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Short title:

Oocyte regulates the follicle basal lamina

Highlights:

- Oocyte glycoproteins regulate the extracellular matrix proteins of the follicle basal lamina
- The structure and function of the follicle basal lamina is regulated by the oocyte
- A thicker basal lamina, due to modified oocyte glycans, has relevance to disease
- The oocyte has a role in regulating theca cell function and follicle integrity
- We propose a model for multiple-oocyte follicle formation

Abstract

Maintaining follicle integrity during development, whereby each follicle is a functional unit containing a single oocyte, is essential for the generation of healthy oocytes. However, the mechanisms that regulate this critical function have not been determined. In this paper we investigate the role of the oocyte in maintaining follicle development. To investigate this role, we use a mouse model with oocyte-specific deletion of *C1galt1* which is required for the generation of core 1-derived *O*-glycans. The loss of oocyte-generated *O*-glycans results in the joining of follicles and the generation of multiple-oocyte follicles (MOFs). The aim was to determine how Mutant follicle development is modified thus enabling follicles to join. Extracellular matrix and follicle permeability were studied using histology, immunohistochemistry and electron microscopy (EM). In ovaries containing Mutant oocytes, the follicle basal lamina (FBL) is altered both functionally and structurally from the primary stage onwards with Mutant follicles possessing unexpectedly thicker FBL. In Mutant ovaries, the theca cell layer is also modified with intermingling of theca between adjacent follicles. MOF function was analysed but despite increased numbers of preantral MOFs in Mutants, these do not reach the preovulatory stage after gonadotrophin stimulation. We propose a model describing how oocyte initiated changes in FBL and theca cells result in follicles joining. These data reveal new and important roles for the oocyte in follicle development and follicle integrity.

Key words:

Oocyte, ovary, follicle, core 1 *O*-glycans, *C1galt1*, T-synthase, extracellular matrix, electron microscopy, basal lamina, multiple-oocyte follicle, collagen, laminin, ZP3 Cre recombinase, floxed, loxP.

INTRODUCTION

Ovarian follicle development is intricately regulated resulting in the generation of numerous functional follicles from which the appropriate number for each species is selected to ovulate. The role of the oocyte in follicle development is complex and has been gradually unravelled during the last 20-30 years. Initial studies were able to determine that the oocyte had a role in cumulus expansion (Buccione et al., 1990; Vanderhyden et al., 1990) and granulosa cells proliferation, differentiation and steroidogensis (Vanderhyden, 1996; Vanderhyden et al., 1990; Vanderhyden et al., 1993; Vanderhyden and Macdonald, 1998). However, in 1996, the oocyte was revealed as critical to follicle development since follicles containing oocytes lacking GDF-9 failed to develop beyond the primary stage (Dong et al., 1996). Furthermore, elegant studies by Eppig's group in 2002 using reaggregated ovaries revealed that the rate of follicle development was oocyte regulated (Eppig et al., 2002). The oocyte also has many other important and potentially subtle influences on follicle development including regulation of the growth and differentiation of granulosa cells in multiple species (McNatty et al., 2005a, b). Thus the oocyte has a critical role in follicle development and can affect its own destiny.

More recently it has been shown that glycans of glycoproteins generated by the oocyte also have important roles in follicle development. These glycans not only alter follicle development but also ovulation rate. Glycoproteins are proteins with carbohydrate molecules attached at specific motifs in a process known as glycosylation (Marino et al., 2010). Glycosylation is a highly organised process that requires specific enzymes for the addition of specific sugars in a stepwise manner to generate specific structures (reviewed in Stanley, 2011); complex *O*-glycans are one such type of structure (Fig. 1). Glycosylation is the most common form of post-translational modification and various glycoproteins are produced by many different cell types including secreted proteins, receptor proteins, and binding proteins. Glycosylation not only modifies the structure of the protein but can also affect protein function such as secretion, half-life, and receptor binding (Jiang et al., 2014; Marino et al., 2010). Many molecules critical for female reproduction are glycoproteins including FSH and LH from the pituitary and their receptors (Jiang et al., 2014), granulosa cell secreted AMH (Pankhurst and McLennan, 2013) and oocyte-secreted GDF-9 (Hayashi et al., 1999) and the zona pellucida proteins (Bleil and Wassarman, 1980).

Important roles for oocyte generated *O*- and *N*- glycans in follicle development and ovarian function have been revealed in more recent years regulating many aspects including zona morphology (Shi et al., 2004; Williams et al., 2007), transzonal process (TZP) quantity (Williams and Stanley, 2009b), granulosa cell luteinisation (Williams and Stanley, 2009b), follicle development and selection (Grasa et al., 2012; Grasa et al., 2014; Williams and Stanley, 2008), apoptosis (Grasa et al., 2014) and the maintenance of follicle integrity (Williams and Stanley, 2008). These discoveries have been achieved thought the advent of *Cre loxP* technology (Lewandoski et al., 1997) since ubiquitous ablation of these glycans causes embryonic lethality (Shi et al., 2004; Williams et al., 2007).

Follicles exist and function within the ovary as individual units. Follicle integrity, and thus basal lamina function, is altered in mice with oocyte-specific ablation of Core 1-derived *O*-glycans because follicles containing oocytes deficient in *O*-glycans can join to form Multiple-Oocyte Follicles (MOFs; follicles containing two or more oocytes) (Williams and Stanley, 2008). Oocyte-specific ablation of Core 1-derived *O*-glycans is achieved using mice homozygous for the floxed *C1galt1* gene (previously referred to as *T-syn*) carrying a ZP3*Cre* recombinase transgene Williams (Grasa et al., 2012; Grasa et al., 2014; Shi et al., 2004; Williams and Stanley, 2008, 2009a, b, 2011; Williams et al., 2007). *C1galt1* encodes core 1 β1,3- galactosyltransferase (T-synthase) responsible for the formation of the core 1-derived *O*-glycans (Fig 1). The oocytes of *C1galt1*^{FF}:ZP3*Cre* (*C1galt1* Mutant) mice are thus unable to generate core 1-derived *O*-glycans and all oocyte-generated glycoproteins that contain the core 1 glycan structure are modified leading to the generation of MOFs. The mechanism of MOF formation in *C1galt1* Mutant mice is distinct from MOF generation formed by incomplete breakdown of the germ cell nest at birth which occurs sporadically in wild-type mice (Pepling, 2012). An alternative mechanism for MOF generation must exist in the *C1galt1* Mutant since oocyte-specific deletion of *C1galt1* is initiated by a ZP3*Cre* transgene, which is only active from the primary follicle stage onwards (Philpott et al., 1987). The numbers of MOFs are greater at preantral and antral stages in Mutant mice and they appear to form by the fusing of two adjacent follicles (Williams and Stanley, 2008). Mutant mice also have increased fertility (30-50% more pups than Controls) and an increased number of healthy follicles in the ovary (Williams and Stanley, 2008). The ovulation of multiple eggs from MOFs does not cause the increase in fertility since the number of corpora lutea (CL),

eggs ovulated, and embryos implanted were equivalent (Williams and Stanley, 2008). It remains unclear whether MOFs are able to progress to the ovulatory stage with one, if not more, of their oocytes remaining healthy.

For follicles to join, follicle integrity must be breached implying that the follicle basal lamina (FBL), a specialised barrier of extracellular matrix between the granulosa and theca cells enclosing the follicle (Irving-Rodgers et al., 2010) is altered in this mouse model. The FBL is a specialised layer of extracellular matrix that surrounds the follicles, dividing the granulosa and theca cells. The generation of the FBL begins at the primordial stage and is continually remodelled throughout follicle development to allow the follicle to expand and eventually rupture during ovulation (Irving-Rodgers et al., 2010). The FBL in the mouse ovary is comprised of numerous proteoglycans and glycoproteins, which include collagen type IV, laminin, perlecan, nidogen, fibronectin and usherin (Irving-Rodgers and Rodgers, 2006; Pearsall et al., 2002). The most abundant molecules are laminin and type IV collagen and are stabilised by forming a network with nidogen (Paulsson, 1992; Yurchenco and Schittny, 1990). However, the origin of these molecules still remains unclear, as do the signals that regulate the construction and development of the FBL. The FBL enables the development of a localized microenvironment within the follicle, essential for follicular function and development. The granulosa cells adjacent to the basal lamina are polarised and thus distinct from the inner granulosa cells. Additionally, as a consequence of its location and structure, the FBL regulates follicular growth by controlling entry of molecules into the follicle. In mice, molecules less that 100 kDa travel freely through the FBL, those between 100 kDa and 500 kDa selectively pass across, and those greater than 500 kDa do not cross the FBL (Perloff et al., 1954; Shalgi et al., 1973). The charge of molecules is also a determinant in their FBL permeability as more negatively charged molecules cross the membrane more readily (Hess et al., 1998). Since the FBL has such a critical role in the regulation of molecules entering the follicle, it is clear that changes to the composition of the FBL are likely to alter its form and also its function.

Previously, we have proposed that changes in the FBL in the *C1galt1* Mutant are involved in the generation of MOFs by either altered FBL synthesis or enhanced degradation during the course of normal remodelling (Williams and Stanley, 2008). Therefore investigating the role for oocyte-generated *O*-glycans in maintaining follicle integrity is important. The aim of this

paper was to establish if and how the FBL is modified in *C1galt1* Mutant ovaries, and the consequence of these changes on follicle development. We show here that although MOFs can become preovulatory, the vast majority of preantral MOFs and antral MOFs present in Mutant ovaries do not progress to the preovulatory stage. We also demonstrate that glycoproteins generated by Mutant oocytes have altered not only the structure and function of the FBL but also the theca cells that surround the FBL in Mutant ovaries. These results reveal a novel role for the oocyte in FBL generation and theca function and based on this we propose a new model for MOF formation.

MATERIALS AND METHODS

Mice

All animal studies using mice (*Mus musculus*) were carried out with approval of the Local Ethical Review Panel at the University of Oxford under license in accordance with the UK Animals (Scientific Procedures) Act 1986. The mice carrying the *C1galt1* floxed allele with the ZP3*Cre* transgene were maintained in a mixed genetic background of 129/SvlmJ and C57BL/6J (Williams et al., 2007). Female mice were homozygous floxed *C1galt1* with Mutants also containing one copy of the ZP3*Cre recombinase* transgene (Mutant: *C1galt1*$^{F/F}$:ZP3*Cre,* Control: *C1galt1*$^{F/F}$ as the ZP3*Cre* recombinase gene does not affect fertility (Shi et al., 2004; Williams et al., 2007). Genotyping was performed by PCR to determine carriers of the *Cre recombinase* transgene and the floxed *C1glat1* allele using genomic DNA prepared from tail or ear biopsies. The phenotype of the mice was confirmed in the colony established at the University of Oxford.

Genotyping

Genotyping was carried out adapted from (Williams et al., 2007) as follows. Each 25µl PCR reaction contained 2.5 µl of 10x PCR buffer (Bioline, London UK), 0.75 µl of 50 mM magnesium chloride solution (Bioline, London UK), 0.5 µl of DNTP (Roche, Mannheim, Germany), 0.5 µl of each primer (Eurogentec, Liege Science Park, Belgium), 1 µl of genomic DNA (ear or tail), and 0.5 µl of Taq polymerase (Bioline) for detection of the *Cre* transgene, or 0.15µl of Taq polymerase for the detection of floxed *C1galt1*. To detect the floxed *C1galt1* allele either primers TS1 (Williams et al., 2007) and TS8 (TCTGCATTGAAGTTCATCTGT) or FB33 and FB34 (Batista et al., 2012) were used. To

detect the *Cre* transgene, primers PS502 (GGACATGTTCAGGGATCGCCAGGCG) and PS607 (CCATGAGTGAACGAACCTGG) were used (Shi et al., 2004).

Stimulation with gonadotrophins

Mice between 6 and 8 weeks of age were selected for superovulation because this age is when Mutants ovulate the highest number of eggs (Williams and Stanley, 2008). Superovulation was carried out as described previously (Stephenson et al., 2012). Females were injected intra-peritoneally with 5 IU of pregnant mare's serum gonadatrophin (PMSG; Centaur Services, Castle Cary, UK), followed 48 h later by 5 IU of human chorionic gonadotrophin (hCG; Chorulon, Centaur Services) and sacrificed 9 h later to collect the ovaries prior to ovulation.

Tissue collection, histology, and MOF analysis

Control and Mutant ovaries were collected, weighed, fixed with 10% buffered formalin (Sigma Aldrich, Gillingham, UK) for 8 h, paraffin embedded and sectioned at 5 μm as described by Williams and Stanley (Williams and Stanley, 2008). Every 5th section was collected, stained with hematoxylin (Shadon Gill 2 hematoxylin, Fisher Scientific, Loughborough, UK) and imaged. All healthy follicles were classified according to the Peters and Pederson classification method by determining granulosa cell numbers in the central section through the oocyte nucleus (Pedersen and Peters, 1968). Follicles were excluded if they exhibited signs of atresia including detached or pyknotic granulosa cells, or blebbing of the oocyte (Williams and Stanley, 2008). Primary includes 3a and 3b follicles (≤20-60 cuboidal granulosa cells), secondary follicles are described as type 4 (61-100 granulosa cells), preantral follicles contain Type 5a and 5b follicles (101-400 granulosa cells) and antral follicles contain type 6 and 7 follicles (401-≥600 granulosa cells). Preovulatory follicles were analysed for the presence of multiple oocytes by tracking the follicle though every 5th serial of the 5μm serial sections collected. Images were obtained and the oocytes within each follicle identified. The frequency of the images analysed, every 25 μm ensured that if an oocyte (~100 μm) was present it would be seen in at least one section analysed.

Periodic Acid Schiff staining of ovary sections

The continuity of the FBL, number of adjacent follicles and the measuring of FBL and theca length was determined by visualisation of the FBL with a PAS staining kit (Sigma Aldrich,

Dorset, UK). PAS reacts with proteoglycans of the FBL colouring it pink (Bedaiwy et al., 2006). After staining with PAS, the sections were photographed and follicles where the oocyte nucleus was visible (thus the follicle stage was able to be ascertained) were examined at a higher magnification. Images were taken from a spread through the ovary so no follicle would be counted twice.

Immunohistochemistry for detection of collagen and laminin

Immunohistochemistry was carried out as previously described (Williams and Stanley, 2011) with some minor modifications. Briefly ovaries were collected, fixed in 10% buffered formalin, paraffin embedded and sectioned (5μm). Endogenous peroxidase was blocked with 3% hydrogen peroxide (Fisher Scientific) for 5 min. The slides were washed in dH_2O, then 0.1M Tris (Sigma Aldrich) Buffered Saline pH 7.5 (TBS) with 0.05% Tween-20 (Fisher Scientific) (TBST). Non-specific antibody-binding was blocked with normal goat serum (NGS; Vectastain ABC Elite kit PK-6100, Vector Laboratories, Peterborough, UK) diluted in TBS for 30 min at room temperature. Antibodies were diluted with NGS/TBS (1:500 for laminin, rabbit anti-laminin Sigma L9393; 1:100 for collagen IV, rabbit anti-collagen IV Millipore AB8201) and added to one section on each slide and NGS/TBS used as control. Slides for the detection of laminin and collagen were incubated at 4°C overnight. Sections were washed 3 times for 3 min each with TBST and secondary antibody (biotinylated anti-rabbit IgG; Vectastain ABC Elite kit) added and incubated for 30 min. Slides were washed with TBST 3 times for 3 min each. All slides were incubated with Vectastain ABC for 2h and then washed in PBS and 0.05% Tween-20 for 3 times 5 min. Antibody binding was visualised with DAB SK-4100, Vector Laboratories, Peterborough, UK); all sections in an experiment were incubated for exactly the same time. Sections were counterstained with hematoxylin, and mounted with depex (VWR, Lutterworth, UK). Only follicles where the oocyte was clearly visible were imaged and analysed. Follicles were classed as 'indistinct' if a part of the FBL was not distinct and those with 100% of the FBL distinct were classified 'distinct'.

Immunohistochemistry for detection of IgG to investigate follicle permeability

Immunohistochemistry was carried out as described above (Williams and Stanley, 2011) with minor modifications detailed below. The sections were subjected to proteinase K antigen (Roche) retrieval in PBS for 15 min before washing in dH_2O 3 times. Biotinylated anti-mouse

IgG antibody (Sigma B7264) was diluted with NGS/TBS (1:10). Slides for the detection of IgG were incubated for 4h at room temperature and at 4°C overnight. Only follicles where the oocyte was clearly visible were imaged and analysed.

EM analysis of FBL

For transmission electron microscopy (EM) studies, ovaries were prepared using standard methods (Christian et al., 2007). Briefly, ovaries were fixed overnight in glutaraldehyde (TAAB, Aldermaston, UK) (2.5%, v/v, in PBS), washed in 0.1 M Phosphate buffer (pH7.2), postfixed in 1% Osmium Tetroxide (TAAB) in buffer for 1-2 hours and washed in nH_2O. Block staining was carried out using 2% uranyl Acetate (TAAB) in nH_2O for 45 min-1 hour, followed by a wash in nH_2O. Next, the ovaries were dehydrated through a series of increasingly concentrated EtOH (VWR) (70–90%) for 15 min each, then three 15 min washes in 100% EtOH. Finally ovaries were embedded in Spurr resin (TAAB) overnight. Ultrathin sections (50nm thick) were cut and stained with 2% uranyl acetate in nH_2O and lead citrate (TAAB). Ultrathin sections were viewed using a JEOL transmission electron microscope (JEOL-1010). Images were taken of the FBL at 4 equidistant points around each preantral follicle at x25,000 magnification. The thickness of the FBL was measured at three equidistant points in each image using Image J.

Analysis

Analyses were carried out blind to genotype. Theca and FBL length were determined using Image J with PAS stained sections. Two-tailed t-tests were carried out using Microsoft Office Excel to analyse preovulatory follicle numbers, the percent of follicle theca cells intermingled (arcsin transformed before t-test), mean number of follicles adjacent and FBL thickness. The Fishers test was used to analyse all categorical data (IgG, PAS, laminin, collagen, EM conventional versus non-conventional FBL) using graphpad prism 5 (GraphPad Software Inc., CA, USA).

RESULTS

The majority of MOFs in *C1galt1* Mutant ovaries do not progress to the preovulatory stage

The number of preovulatory MOFs in 6-8 wk mice stimulated with exogenous hormones was obtained to determine if follicles containing multiple oocytes are capable of ovulation.

Unexpectedly the number and proportion of Mutant preovulatory follicles that were MOFs did not differ to Control ovaries (Table 1). Therefore although Mutant ovaries contain significant numbers of MOFs at the preantral and antral stages of development (Williams and Stanley, 2008), the majority do not progress through the preovulatory stage.

Preovulatory MOFs do not contain multiple oocytes at the same stage of maturation

Of the MOFs that developed to preovulatory follicles, each contained only one oocyte with an expanded cumulus mass. The other oocytes were visible at the periphery of the follicle embedded within the granulosa cells (Fig. 2). This was the case for MOFs in both Control and Mutant ovaries.

The function of the FBL is modified in Mutant ovaries

The permeability of the Mutant FBL was investigated by identifying IgG, an endogenous molecule that can cross the FBL and be detected within the follicle (Zhou et al., 2007). This molecule has a size of 155KDa (Hess et al., 1998). IgG was detected in Control and Mutant follicles (Fig. 3A) and quantified using an arbitrary scale into two categorise, high and low (Fig 3B).

In both Mutant and Control follicles, an increase in intrafollicular IgG corresponds with follicle maturation as demonstrated by an increase in the proportion of follicles in the high category with stage of development (Fig. 3C). However, the proportion of follicles with high levels of intrafollicular IgG was decreased for Mutant follicles compared to Control for primary ($P<0.05$) and preantral follicles ($P<0.0001$) indicating that Mutant follicles are less permeable to IgG than Control (Fig. 3C).

The FBL is modified in Mutant ovaries

To visualize and evaluate the FBL, ovary sections were stained with periodic acid-Schiff (PAS) or subjected to immunohistochemistry to detect laminin and collagen IV. For each analysis, follicles with a clearly visible oocyte were photographed and images assessed for the presence of the FBL. All follicles were classified into one of two categories according to FBL continuity: (i) FBL distinct - when the FBL can be clearly followed around the follicle (Fig. 4A, A'), and (ii) FBL indistinct - where it cannot (Fig. 4B, B'). FBL were classified as indistinct when one or more locations of the FBL appeared to either fuse with the stroma, or

was too thin to be clearly visible. The proportion of Mutant follicles in sections stained with PAS with an indistinct FBL increased as follicles develop, whereas the proportion of Control follicles in this category remained constant from the secondary stage onwards (Fig. 4C). The difference resulted in a smaller proportion of Mutant secondary follicles in the indistinct category compared to Control follicles. In contrast there is a greater proportion of Mutant antral follicles that were indistinct compared to the Control antral follicles (Fig. 4C). Indistinct follicles exhibited either a 'fused' phenotype or a 'thin' phenotype, and the proportion of either did not differ between the Control and Mutant groups (fused: Control 83% Mutant 81%, too thin: Control 17% Mutant 19%).

Laminin in the FBL is modified in Mutant follicles

Immunohistochemistry was used to evaluate the presence of laminin in the FBL. If laminin detected around the entire FBL was distinct from granulosa and theca extracellular matrix, it was classified as 'laminin distinct' (Figs. 4D, D'). All follicles with a portion of FBL that was not visible were termed 'laminin indistinct' (Figs. 4E, E').

The percentage of Control follicles with a laminin indistinct FBL remained relatively constant at all follicle stages (Fig. 4F). The proportion of Mutant follicles classified as laminin indistinct was comparable to Control follicles at the primary and secondary stages of development. However, the proportion of FBL that were laminin indistinct increased dramatically in Mutant preantral (79% versus 43%; P=0.069) and antral follicles (86% versus 56%; P<0.05) (Fig. 4F).

Collagen in the FBL is modified in Mutant follicles

A second molecule, collagen IV, present in the FBL at all stages of follicle development was detected using immunohistochemistry. Follicles were categorized as 'collagen distinct' or 'collagen indistinct' (Figs. 4G, G', H, H' respectively) as described above for laminin. At the preantral follicle stage there were less collagen indistinct Mutant FBL compared to Controls, but no differences were observed at any other stage (Figs. 4I). This shows that collagen IV laid down in the FBL of preantral follicles has been altered.

To summarize, these data reveal modifications of the FBL in Mutant follicles as they develop. Furthermore, the specific differences observed by analysis of different molecules at

352 different stages of follicle development reveal stage-specific modifications of FBL
353 generation, with many of these changes occurring at the preantral follicle stage.
354
355 **Mutant theca structure is modified**
356 For follicles to join, the FBL as well as the adjacent theca cells must be modified. To
357 determine if the theca cells adjacent to the FBL were altered, preantral follicles in PAS
358 stained sections from 3-week ovaries were analysed. Theca cells within 2 cell layers of the
359 FBL were assessed. For MOFs to be generated follicles must be adjacent and therefore the
360 number of adjacent follicles was determined. Mutant preantral follicles have more follicles
361 adjacent to them than Control follicles (Fig. 5A) (Control: 2.2±0.2 versus Mutant: 2.7±0.2;
362 mean±sem, $P<0.05$). The length of theca in direct contact with each adjacent follicle's theca
363 was determined and was classified as either 'separate' if clear definitions existed, or
364 'intermingled' if the follicle that 'owned' the theca could not be determined (Fig. 5B).
365 Intermingled theca length was measured and the length of intermingled theca expressed as a
366 percentage of the total length of theca between the two adjacent follicles. This analysis
367 revealed that the proportion of adjacent theca that was intermingled was greater in the Mutant
368 (Fig. 5C). Therefore, these data demonstrate that oocyte glycoproteins have a role in theca
369 layer formation and retaining the integrity of the follicle as a single unit. Furthermore, theca
370 cells of follicles containing Mutant oocytes mix more readily with theca cells of neighbouring
371 follicles thus facilitating a route for follicle fusion and MOF generation.
372
373 **Ultrastructure of Mutant preantral FBL is more variable**
374 To examine FBL morphology in more detail, FBL ultrastructure at the preantral stage of
375 Control follicles, Mutant single-oocyte follicles (SOFs) and Mutant MOFs were examined
376 using transmission electron microscopy (TEM) (Fig. 6). Four TEM images were obtained at
377 equidistant points of the FBL for each preantral follicle, and these revealed a variety of
378 morphologies which were termed 'conventional', 'thick', 'multilayer', 'indistinct' and
379 'loopy' (Fig. 6A) based on previous BL studies (Da Silva-Buttkus et al., 2008; Rodgers and
380 Irving-Rodgers, 2010). A follicle was categorized as having 'conventional' morphology if ≥3
381 of the 4 FBL points per follicle were 'conventional' or 'nonconventional' if ≥2 of the 4 FBL
382 points per follicle were not conventional (Fig. 6B). The percent of follicles in each category
383 was different when comparing Control to Mutant SOFs ($P<0.05$) and when compared to
384 Mutant MOFs ($P<0.0001$) (Fig. 6B). The increase in nonconventional FBL morphology in

Mutant SOFs and Mutant MOFs can chiefly be attributed to an increase in 'thick' and 'multiple layer' morphologies (Fig. 6C). These morphologies indicate that Mutants have a greater proportion of non-conventional FBL, with more in Mutant MOFs than SOFs.

The BL of preantral follicles containing Mutant oocytes is thicker

To quantify FBL thickness, the FBL was measured at 12 points of each follicle (n=25 follicles for Control and Mutant preantral SOFs, n=9 follicles for Mutant preantral MOFs). The Mutant SOF FBL and Mutant MOF FBL were thicker than Control FBL ($P<0.005$ and $P<0.000005$ respectively; Fig. 7A). Furthermore, for the FBL of Mutant MOFs, this increased thickness phenotype was exacerbated beyond the FBL of Mutant SOFs ($P<0.05$).

The intra-follicle variation in FBL diameter was much greater in Mutant SOFs and MOFs compared to Controls as shown by the range in standard deviation per follicle (Fig. 7B). Further investigation by plotting the frequency of each FBL diameter across the full range of measurements for both Control and Mutant FBL revealed that the increased variability in Mutant FBL diameter was not due to an increase in the range of FBL thicknesses, but was due to an increase of thicker FBL (Fig. 7C). Furthermore, the FBL of Mutant SOFs had 4 times as many (4%) FBL>170nm than Controls (1%) all in different follicles. Of the 9 Mutant MOFs measured, 19.4% (7 of the 36) measurements were >170nm from 3 different follicles. Therefore, the MOFs FBL was thicker than Mutant SOFs or Controls and in Mutant follicles, there are specific regions thicker than the rest of the BL.

DISCUSSION

We have previously shown that oocyte glycoproteins generated by *C1galt1*$^{F/F}$:ZP3*Cre* female mice affect follicle integrity enabling adjacent follicles to join resulting in the generation of follicles containing multiple oocytes (MOFs) and a mechanism was proposed for this novel phenotype (Williams and Stanley, 2008). In this paper we test this model by assessing BL structure and function and investigate the ovulatory potential of MOFs.

Basal laminae are common to all tissues in the body. They are involved in a wide range of diseases including diabetes, asthma, alzheimers, cancer and stroke (Abrahamson, 1986; Hamann et al., 2003; Kalaria, 2002; Martinez-Hernandez and Amenta, 1983). Here we have described novel oocyte-glycoprotein mediated structural and functional modifications of the

FBL. We have demonstrated that glycan-deficient oocyte glycoproteins have, either directly or indirectly, altered both the FBL structure and theca cells to enable follicles to join. We have also revealed that although a very small proportion of MOFs can become preovulatory, the MOFs that do, contain only one expanded oocyte-cumulus complex, not multiple. Therefore, these data reveal important new roles for the oocyte in the generation and regulation of ECM and thecal cell function.

MOFs from both Mutants and Controls were observed at the preovulatory stage, demonstrating that such development of MOFs is possible. Despite the high number of preantral and antral MOFs in Mutants (Williams and Stanley, 2008), there was no difference in the number of Mutant preovulatory MOFs compared to Control indicating that most Mutant MOFs cannot progress to the preovulatory stage. This finding confirms our previous hypothesis that MOFs do not ovulate multiple functional eggs (Williams and Stanley, 2008). Occasional MOFs exist in wildtype ovaries due to incomplete primordial follicle nest breakdown soon after birth (Pepling, 2012) and these develop as a single follicle. These are distinct from MOFs that have been created from the joining of two follicles, most likely at different stages of development. We therefore hypothesise that the MOFs that exist due to aberrant follicle nest breakdown are the ones that become preovulatory. Therefore, Mutant and Control ovaries contain the same number of preovulatory MOFs because MOFs formed through the joining of follicles do not reach the preovulatory stage. There are many possible reasons for such poor survival of MOFs. We have previously postulated that since follicles joining are likely to be at different stages of development, it is likely the differing function of the cells from the two follicles cannot be reconciled to function correctly as a single unit (Williams and Stanley, 2008). A second possibility relates to the affects a change in size will have on follicle developmental dynamics. Follicles that have joined will have an altered surface area to volume ratio, this has implications for follicle nutrition since larger follicles have a relatively smaller surface area for nutrients to enter the avascular granulosa cells beneath the basal lamina. There will also be a proportionally smaller number of basal granulosa cells, which may compromise development. The few MOFs that were preovulatory contained one oocyte with an expanded cumulus mass, whilst the other oocytes were at the periphery of the follicle embedded within the granulosa cells (Fig. 2). This strongly suggests that one oocyte alone is dominant within a MOF indicative of oocyte-to-oocyte interactions.

The FBL regulates the molecules that enter the follicle based on size and charge (Hess et al., 1998). A greater proportion of preantral Mutant follicles contained lower amounts of IgG (a large serum-derived molecule) indicating a decrease in the permeability of Mutant follicles. The decrease in FBL permeability in Mutant primary follicles was unexpected since this is when *C1galt1* is deleted in the oocyte. However primary follicle development extends for several days and if deletion occurs early, changes in permeability may well be evident in late primary follicles. Alternatively, primary follicle permeability may be decreased due to signals from more developed neighbouring follicles, which have affected the FBL of primary follicles. Indeed follicle-to-follicle signalling has been observed *in vitro* and almost certainly occurs *in vivo* (Da Silva-Buttkus et al., 2009; Spears et al., 1996). Alternatively, IgG may pass into the follicle but cannot leave, and therefore it accumulates as follicles mature. The lack of difference at the antral stage may reflect a limit in the follicle's permeability, reached by both Mutant and Control follicles. However, changes in FBL permeability most likely result from alterations in FBL structure and composition; therefore FBL structure was investigated.

FBL analysis using PAS and detecting laminin and collagen IV, the two most prevalent FBL scaffold molecules (Berkholtz et al., 2006a; Rodgers et al., 1999), revealed the FBL structure to be modified in a stage dependent pattern indicating complex regulation of FBL generation by the oocyte. However, the majority of the modifications were at the preantral stage which is the same stage that MOFs were first observed in significant numbers in Mutant ovaries (Williams and Stanley, 2008). MOF generation in Mutants is also likely to be facilitated by the increase in indistinct FBL with theca intermingled with adjacent follicles. Our results reveal that the ablation of *C1galt1* in the oocyte alters the presence of both laminin and collagen IV in the FBL. During follicle development, the FBL continuously changes in composition and physical properties. Laminin and collagen IV are present in different proportions as the follicle develops (Rodgers et al., 2003). Collagen is very rigid (Black et al., 2008) and thus changes in collagen IV likely alter the mechanical properties of the FBL. Collagen IV is decreased in Mutant preantral follicles which could lead to a decrease in FBL integrity and accentuate MOF formation specifically at the preantral stage. The decrease in laminin may also compromise the FBL structure. Although both laminin and collagen IV have *O*-glycans, since the deletion of *C1glat1* is oocyte-specific we do not anticipate the phenotype to be due to defective ECM proteins since there is no evidence the oocyte

484 generates these ECM proteins. Furthermore, it is unlikely that oocyte-generated proteins
485 would migrate from the oocyte to the FBL. Thus, the modifications in ECM proteins in the
486 Mutant are due to either direct or indirect signals from the oocyte.
487
488 Detailed EM analyses unexpectedly revealed that the Mutant FBL has increased variation in
489 both thickness and morphology. This was against expectations based on the IHC and
490 histology data. Most Control FBL would be classified as "thin basal lamina" as it is between
491 50-100nm wide and comprised of a single layer of molecules (Yurchenco, 2011). However in
492 Mutant MOFs and SOFs, the basal lamina becomes thicker with the FBL comprised of
493 multiple layers of networked molecules. Some of the ultrastructural morphologies ('thick',
494 'multiple layers' and 'loopy') observed in *C1galt1* mice have been previously described in
495 bovine and human preantral and antral follicles (Irving-Rodgers et al., 2009; Irving-Rodgers
496 and Rodgers, 2000). A decline in oocyte competency has been linked to the presence of a
497 'loopy' FBL in bovine follicles (Irving-Rodgers et al., 2009), which presents a novel insight
498 into the role of the FBL in oocyte development.
499
500 Thicker basal laminas occur in a variety of diseases including the reticular basement
501 membrane of asthmatics (Saglani et al., 2006), and capillary basement membrane width has
502 been linked to diabetes (Ganda et al., 1983). In malignant cancers, the integrity of the BL
503 around the tumour is often used to grade the tumour (Guarino, 2007). Modification of the
504 basal lamina also has been shown to have a role in the prevention and treatment of illness.
505 Exercise decreases the basement membrane width of muscle capillaries in diabetics and non-
506 diabetic patients to the width of younger patients (Williamson et al., 1996). In addition in the
507 treatment of cancer, modification of the extracellular matrix using nanoparticles is now being
508 considered to prevent cancer invasion and reduce the rate of growth of the tumour
509 (Kanapathipillai et al., 2012). The 'thick' and 'multiple layer' morphologies are also seen in
510 other organs in relation to either a diseased state ('thick' morphology in diabetics) or
511 developmental changes ('multiple layers' in newborn rat kidney eventually fuse to become a
512 single layer in older animals (Abrahamson, 1986)). This suggests that *C1galt1* FBL
513 morphologies may be equally relevant to the function and development of the follicle, and
514 aberrant thickness and morphology most likely contribute to the MOF generation and altered
515 follicle development seen in *C1galt1* Mutants. Furthermore, in the ovarian pathology
516 polycystic ovarian syndrome, the rigidity of the ovary is increased due to modified

517 expression of extracellular matrix molecules (Oksjoki et al., 2004) and it has been proposed
518 that mechanical modifications may explain in part the altered follicle development and lack
519 of ovulation (Woodruff and Shea, 2011). Determining the structural and functional changes
520 of the FBL will improve our understanding of the FBL's role in follicle development and
521 potentially lead to improvements in follicle culture for fertility treatment.
522
523 The modified structure of the Mutant FBL most likely explains the decrease in follicle
524 permeability and we would assume leads to a difference in the permeability of other
525 molecules. Furthermore, growth factors involved in follicle development such as fibroblast
526 growth factors (FGF), transforming growth factor-beta (TGF-B), and bone morphogenetic
527 proteins (BMPs) can bind and accumulate on other constituents of the basal lamina including
528 heparin sulphate proteoglycans agrin, perlecan and collagen type XVIII (Yurchenco, 2011).
529 Therefore, the changes in follicle permeability and potential growth factor binding and thus
530 bioavailability implicates a vast array of potential molecules which may contribute to the
531 MOF or increased fertility phenotypes (Grasa et al., 2014; Williams and Stanley, 2008).
532
533 The changes observed in the Mutant FBL most likely also have a mechanical influence on
534 follicle development. Extracellular matrix molecules have an impact on the proliferation rate,
535 survival and hormone production of granulosa cells (Berkholtz et al., 2006b). Therefore the
536 changes in ECM we observe in this Mutant mouse are most likely modifying follicle growth
537 and function. *In vitro*, the environment surrounding the follicle affects follicle growth. Mouse
538 follicles cultured on increasing concentrations of alginate gels, thus mimicking increasing
539 rigidity of the ECM, demonstrated reduced follicle growth with increasing rigidity (Xu et al.,
540 2006);(West et al., 2007). Therefore, the FBL provides an important mechanical barrier that
541 confines the dividing granulosa cells and forces them to divide in an inward direction as to
542 increase follicular cell layers, whilst expanding in a regulated manner to allow follicular
543 growth (Da Silva-Buttkus et al., 2008).
544
545 From the findings of this paper we suggest a novel model for MOF formation in *C1galt1*
546 mice (Fig. 8). If two adjacent follicles with a thick point in their FBL are in the correct
547 orientation to touch this may lead to fusion of the follicles (Fig. 8A). As the follicles develop,
548 the theca of the follicles become closer and due to modified development resulting from
549 oocyte signalling, fuse together (Fig. 8B). Changes to the theca allow the FBL to come into

contact with each other. Thicker FBL areas are more likely to 'touch' at these points (Fig. 8C). Intrinsic FBL changes in Mutant follicles allows the FBL to fuse. A thicker, but potentially less stable FBL, may result in an FBL less able to withstand the mechanical forces exerted by increasing granulosa cell numbers. This results in the FBL being breached and mingling of the granulosa cells from the two follicles resulting in the generation of a MOF (Fig. 8D). Furthermore, the necessity for the 'correct orientation' of follicles may be why not all adjacent Mutant follicles fuse to form MOFs.

MOFs have also been observed in mouse models with altered molecules from the TGFβ superfamily of growth factors, for example, *BMP15*$^{-/-}$ *GDF9*$^{+/-}$ mice have an increased number of MOFs (Yan et al., 2001). Furthermore these two important oocyte-specific growth factors have been implicated in the *C1galt1* Mutant phenotype (Grasa et al., 2014).

In conclusion, we have demonstrated that the structure of the FBL in mice with glycan-deficient oocytes has been altered. The influence that oocytes have on follicle development is substantial and integral to follicle maturation and ovulation. As an element of follicle development, the FBL is essential to the selective movement of signalling molecules in and out of the follicle and undergoes dynamic changes as the follicle matures. These results underline the integral role of the oocyte in follicle development.

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Figure Legends

Fig 1. Generation of Core 1 derived O-glycans. The glycosyltransferase T-synthase encoded by *C1galt1* is required for the generation of the core 1 *O*-glycan by transferring galactose (Gal) to N-acetylgalactosamine (GalNAc) on serine/threonine residues (S/T). This core 1 structure can be further extended by other specified glycosyltransferases.

Fig 2. Preovulatory MOFs in Controls and Mutant follicles do not contain multiple oocytes at the same stage of maturation. (A) Control preovulatory MOF. Two oocytes in the Control MOF (A1-A2) are adjacent to the granulosa cell wall and exhibit no cumulus expansion (closed arrows), whereas the oocyte in the centre (A3) exhibits a small degree of cumulus expansion (open arrow). (B-C) Two sections through the same Mutant preovulatory MOF. (B1) One Mutant oocyte is adjacent to the granulosa cell wall and exhibits no cumulus expansion (closed arrow) whereas the Mutant oocyte in the centre (C1) has a fully expanded cumulus matrix (open arrow). *expanded cumulus cell mass, #non-expanded cumulus cell mass.

Fig 3. Analysis of follicular IgG using immunohistochemistry with Control and Mutant ovary sections. (A) Immunohistochemistry revealed IgG in Control and Mutant ovaries with a complete absence of stain observed in the section where the primary antibody was omitted. (B) The amount of IgG detected in each follicle, as indicated by brown colouration, was classified as ‘low’ or ‘high’. (C) As Control and Mutant follicles develop, the proportion of follicles with high follicular IgG increased. The percent of Mutant follicles with high IgG also increased with follicle development however the increase was of less magnitude than Control follicles at the primary, secondary and preantral stages. Control and Mutant; n=3 ovaries from 3 mice. The number in the bar is the number of follicles analysed. Statistics were calculated on raw values; *P<0.05, **P<0.0001**.** MOFs were not included in these data as they were not present in large enough numbers (Control n=2, Mutant n=6).

Fig 4. Analysis of FBL morphology using Periodic Acid Schiff (PAS) stain, and IHC detecting laminin and collagen. (A, B) Ovary sections stained with PAS reveal follicles have either a distinct FBL or an indistinct FBL. (A’, B’) Higher magnification of distinct and indistinct FBL between arrows revealed by PAS staining. (C) PAS staining revealed an

altered FBL in Mutant secondary and antral follicles compared to Controls. (D, E) Laminin detection in ovary sections using IHC revealed follicles with a 'laminin distinct' or 'laminin indistinct' FBL. (D' and E') Higher magnification of laminin distinct and laminin indistinct FBL between arrows. (F) The proportion of follicles with a laminin indistinct FBL was higher in Mutant antral follicles compared to Controls. (G, H) Collagen IV detection in ovary sections using IHC revealed a 'collagen distinct' or 'collagen indistinct' FBL. (G' and H') Higher magnification of collagen distinct and indistinct FBL between arrows. (I) The FBL containing collagen was indistinct in less preantral follicles in Mutants compared to Controls. The number in each bar is the number of follicles analysed (PAS Control ovaries, n=3 Mutant ovaries, n=3; Laminin Control ovaries: n=3, Mutant ovaries, n=3, Collagen Control ovaries: n=4, Mutant ovaries, n=6). Statistics were calculated on numbers of follicles; *P<0.05, #P=0.069.

Fig 5. Mutant follicles have more adjacent follicles with increased amounts of intermingled theca. (A) Mutant preantral follicles have more follicles adjacent to them compared with Control. (Control follicles n=61, Mutant follicles n=67) (B) A follicle with the FBL outlined in white and the perimeter of the theca outlined in black. The box with the broken line highlights an area where the theca is separate of the adjacent follicle and the box with the unbroken line highlights an area of theca which is intermingled with the adjacent follicle. (C) A greater percentage of the theca between adjacent follicles is intermingled in Mutant ovaries compared to Control (mean±SEM; *P<0.005) (Control follicles n=57, Mutant follicles n=61). Control ovaries, n=3 Mutant ovaries, n=3. Number within the columns represent the number of follicles analysed.

Fig 6. Characterization of preantral FBL morphology by use of EM. (A) Preantral FBL were revealed to have five different morphologies: conventional, thick, multilayer, indistinct and loopy. (G; granulosa cell). The FBL lies between the white arrows. (B) Percentage of Control, Mutant SOFs and Mutant MOFs with conventional morphology (≥3 of the 4 images per follicle were 'conventional') or nonconventional morphology (≤2 of the 4 images per follicle were conventional). Control and Mutant SOF: 25 follicles with 4 images per follicle, Mutant MOF: 9 follicles with 4 images per follicle. (C) Percentage of Control, Mutant SOF and Mutant MOF nonconventional FBL images by morphology. Control and Mutant SOF: 100 images each, Mutant MOF: 36 images total.

Fig 7. The FBL of Mutant follicles is thicker and more variable in diameter. (A) The average thickness of the FBL for each follicle was greater for Mutant follicles than Control. MOFs FBL is thicker than both Mutant SOFs and Control FBL. The numbers in the bars are the number of follicles analysed (the value used for each follicle was the average of the 12 measurements) *P<0.005, **P<0.0005, ***P<0.000005. (B) The thickness of the FBL was more variable in Mutant follicles. Each point is the standard deviation of the 12 measurements for each follicle. The bar is the mean of the STDEV's. (C) Distribution of the frequency of measurements of FBL thickness in Control and Mutant MOFs and SOFs.

Fig 8. Model for MOF generation. (A) Two Mutant follicles with thicker more variable FBL follicle are adjacent with discrete, non-intermingled theca. (B) As the follicles grow they get closer and the theca of both follicles mingle. (C) Follicles continue to grow and the thick FBL make contact. (D) Follicles connected by the FBL form a passage allowing granulosa cells to connect and a MOF is generated. This model explains why adjacent follicle do not always form MOFs because adjacent follicles can be close without fusion if the FBL of the two does not touch.