Oocyte-specific ablation of *N*- and *O*-glycans leads to altered cumulus cell signalling and extracellular matrix composition

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Abridged title: Cumulus cell signalling and matrix composition

Short Summary: Cumulus-oocyte complex expansion is essential for ovulation and fertilisation. This study revealed that cumulus area is not altered in mice with oocyte-specific deletion of *C1galt1* and *Mgat1*, despite a reduction in hyaluronan and other molecules associated with cumulus expansion. A minimum threshold may exist for supporting cumulus expansion. Oocyte-secreted factors may be affected by the deletions causing altered cumulus composition.

Abstract

Cumulus-oocyte complex (COC) expansion is essential for ovulation, fertilisation and is linked to oocyte quality. Hyaluronan (HA), the major matrix constituent, is cross-linked via inter-α-inhibitor heavy chains (HCs), pentraxin 3 (PTX3) and tumour necrosis factor-stimulated gene 6 (TSG-6). All except HCs are secreted by cumulus cells in response to oocyte-secreted factors (OSFs) which signal via SMAD pathways. The Double Mutant (DM) mouse generates oocytes lacking complex *N*- and *O*-glycans due to oocyte-specific deletion of *C1galt1* and *Mgat1*, and has modified cumulus expansion. We compared COCs prior to expansion (48h-post-PMSG) and at late-stage expansion (9h-post-hCG) (Control n=3 nice, DM n=3/group). Using histochemistry, we assessed the levels of HA, HCs, PTX3, TSG-6, and phosphorylated-SMAD1/5/8 and -SMAD2 (12-25 COCs/group). DM COCs did not differ from Controls in cumulus size or cell density at 9h-post-hCG; however, HA, HC levels and phosphorylated-SMAD1/5/8 were reduced. Furthermore, no correlations were found between the levels of matrix molecules and cumulus area in DM or Control samples. These data suggest that HA and HCs can support cumulus expansion provided that they are present above minimum threshold levels. We propose that oocyte-specific ablation of *C1galt1* and *Mgat1* may affect BMP15 synthesis or bioactivity, thereby reducing SMAD1/5/8 phosphorylation and HA production.

Key words:

Cumulus expansion, cumulus ECM, mouse, cumulus oophorus, *C1galt1*, core 1-derived *O*-glycans, *Mgat1*, complex *N*-glycans

1.1 Introduction

Ovarian follicle development culminates in the ovulation of an egg surrounded by cumulus cells (CCs) (Dunlop and Anderson, 2014). The CCs and their associated cumulus extracellular matrix (cECM), also known as the cumulus oophorus, have essential roles in oocyte development, ovulation, transportation to the oviduct and fertilisation (Chen *et al.*, 1993, 1996; Sanchez and Smitz, 2012). Most cECM molecules are secreted by CCs after the luteinising hormone (LH) surge resulting in cumulus expansion (Fulop *et al.*, 2003; Russell and Robker, 2007; Sugiura *et al.*, 2009). The degree of cumulus expansion has been linked to oocyte quality in humans (Ng *et al.*, 1999).

The major constituent of the cECM, hyaluronan (HA), is essential for maintaining the structure and viscoelastic properties of the expanded cumulus oophorous (Russell and Robker, 2007; Sugiura *et al.*, 2009). The stability of the matrix is dependent on the cross-linking of HA via the activities of the heavy chains (HCs) from inter-α-inhibitor (IαI), pentraxin 3 (PTX3) and tumour necrosis factor-stimulated gene 6 (TSG-6) (Sato *et al.*, 2001; Zhuo *et al.*, 2001; Fulop *et al.*, 2003; Ochsner *et al.*, 2003a; Salustri *et al.*, 2004; Russell and Robker, 2007; Baranova *et al.*, 2014). As the source of HCs, the liver-derived IαI (Chen *et al.*, 1992; Zhuo *et al.*, 2001) is able to cross the basal lamina into the preovulatory follicular fluid following the LH surge (McClure *et al.*, 1994; Irving-Rodgers *et al.*, 2002; Scarchilli *et al.*, 2007). The covalent transfer of HCs onto HA is then catalysed by TSG-6 (Rugg *et al.*, 2005; Briggs *et al.*, 2015), which is expressed by CCs and granulosa cells (GCs) in response to the LH surge (Carrette *et al.*, 2001; Mukhopadhyay *et al.*, 2001). Further stabilisation of the cECM is enabled by another CC-synthesised molecule, PTX3, which crosslinks HC•HA complexes by binding to HCs (Scarchilli *et al.*, 2007; Baranova *et al.*, 2014). Failure of cECM assembly has been observed in mice lacking

TSG-6, *PTX3* or the bikunin chain of IαI (Sato *et al.*, 2001; Zhuo *et al.*, 2001; Varani *et al.*, 2002; Fulop *et al.*, 2003; Salustri *et al.*, 2004).

Cumulus cell differentiation and proliferation and cumulus-oocyte complex (COC) expansion are dependent on paracrine signalling by oocyte-secreted factors (OSFs) such as growth and differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15). Both GDF9 and BMP15 are members of the TGFβ family (Diaz *et al.*, 2007) and are expressed during ovulation (Galloway *et al.*, 2000; Yan *et al.*, 2001). These OSFs act synergistically to promote cumulus expansion, either as homodimers (Su *et al.*, 2004) or heterodimers (Peng *et al.*, 2013). Once BMP15 and GDF9 bind to their corresponding receptors on CCs, intracellular signaling molecules SMAD1/5/8 and SMAD2/3 are phosphorylated (pSMAD1/5/8, pSMAD2/3) and activated. Therefore, intracellular levels of pSMADs are indicators of both the levels of OSFs and their effects on the cumulus (Kaivo-oja *et al.*, 2006).

Modified cumuli oophori, with cells remaining attached to ovulated eggs after hyaluronidase treatment, occurs in a mouse model of follicular premature ovarian failure (POF) that results from oocyte-specific deletion of *C1galt1* and *Mgat1* (Williams *et al.*, 2007; Williams and Stanley, 2011); referred to here as the Double Mutant (DM). *C1galt1* encodes T-synthase, which is required for the generation of complex *O*-glycans (Ju *et al.*, 2002), while *Mgat1* encodes GlcNAc-TI, which is required for producing complex and hybrid *N*-glycans (Chen and Stanley, 2003). Both forms of glycosylation play a range of roles in cells (reviewed in Ohtsubo and Marth (2006)), for example, *O*-glycosylation has been implicated in receptor signalling (Wagner *et al.*, 2007) and cell-matrix interactions (Tian *et al.*, 2012), while *N*-glycosylation is involved with protein folding (Trombetta, 2003) and in embryo compaction and pre-implantation development (Shi *et al.*, 2004). Deletion of *C1galt1* and/or *Mgat1* will affect the glycosylation of all oocyte-expressed proteins with *N*-

and/or *O*-linked glycans. Proteins which could be affected include BMP15 and GDF9; there are 5 potential *N*-linked glycosylation sites (Dube *et al.*, 1998) and two *O*-glycosylation sites (Saito *et al.*, 2008) on BMP15, while GDF9 has one *N*-glycosylated site in the mature protein (Hayashi *et al.*, 1999) and one potentially *O*-glycosylated (Lokman *et al.*, 2010).

Interestingly, oocyte-specific deletions of *Mgat1* (Shi *et al.*, 2004; Williams and Stanley, 2009) or *C1galt1* (Williams and Stanley, 2008) both result in reduced cumulus expansion. COCs from *C1galt1* Mutant mice have altered cECM composition in addition to reduced expansion (Ploutarchou *et al.*, 2015); however, these animals have increased fertility compared to Controls (Williams *et al.*, 2007; Williams and Stanley, 2008). Conversely, *Mgat1* Mutant mice ovulated fewer (and more compact) COCs than Controls, and had less HA present in their cECM despite increased COC resistance to hyaluronidase treatment (Williams and Stanley, 2009). No other studies on cECM composition of *Mgat1* Mutant COCs have been performed thus far. In DM mice, fertilisation is not impaired, but COCs are resistant to hyaluronidase treatment, with CCs remaining attached to ovulated eggs even after prolonged incubations compared to Controls, indicative of abnormal cumulus expansion (Williams *et al.*, 2007). These observations indicate that oocyte-specific deletion of *C1galt1* and/or *Mgat1* might result in altered cECM organisation with a greater level of HA crosslinking.

Since the genotype of the DM mouse is oocyte-specific, it can be assumed that any changes associated with cumulus matrix formation in these animals are due to changes in oocyte-derived factors. This study aims to determine whether the lack of complex *N*- and *O*-glycans in the oocyte affects the levels and/or distribution of the cECM components HA, HCs, PTX3 and TSG-6, and/or SMAD signalling pathways during late stage COC expansion.

1.2 Materials and Methods

1.2.1 Mice

Six-week-old Control (*C1galt1*^{FF}*Mgat1*^{FF}) and DM (*C1galt1*^{FF}*Mgat1*^{FF}:ZP3*Cre*) females, on a mixed background (C57BL/6 x 129), were used here; Control females did not carry the ZP3*Cre* transgene; in addition, the transgene has been previously shown to not affect fertility (Shi *et al.*, 2004; Williams *et al.*, 2007). All experiments were approved by the Home Office and the Local Ethical Review Committee.

1.2.2 Superovulation

Ovaries were collected from mice pre- and at late stage cumulus expansion. Ovaries containing COCs pre-expansion were collected 48 h post injection with 5 IU pregnant mare serum gonadotropin (PMSG) (Biosupply, Bradford, UK); hereafter referred to as "48 h post-PMSG". Ovaries containing COCs at late stage expansion were collected 9 h after mice were injected with 5 IU human chorionic gonadotropin (hCG; Chorulon) (Biosupply, Bradford, UK), i.e. 48 h post-PMSG; hereafter referred to as "9 h post-hCG". Ovaries were fixed in 10% (v/v) buffered formalin (Sigma-Aldrich, Dorset, UK) for 8 h and then stored at 4ºC in 70% (v/v) ethanol until paraffin-embedding prior to sectioning (5 µm).

1.2.3 Location of preovulatory follicles

Every 10th serial section was stained with haematoxylin (Shandon Gill 2 Haematoxylin; Fisher Scientific, Loughborough, UK) and mounted with Depex (VWR, Leicestershire, UK). Images were taken using a Leica DM 2500 microscope, (Microscope Services Ltd., Woodstock, UK) and a MicroPublisher 5.0 RTV camera (Qimaging; Microscope Services Ltd.) to determine the location and number of follicles present in each ovary.

Based on classification by Pedersen and Peters (1968), late antral (Type 7) follicles were selected for analysis at 48 h post-PMSG stimulation, and preovulatory follicles (Type 8) were selected for analysis at 9 h post-hCG. Type 7 follicles contain a single antral follicle with ≥600 granulosa cells, while Type 8 follicles also have a well-formed cumulus stalk.

1.2.4 Histochemistry and immunohistochemistry

Histochemistry to detect HA, and immunohistochemistry (IHC) to detect pSMAD1/5/8, pSMAD2, PTX3, HCs and TSG-6, were performed on sections from Control and DM ovaries 9 h post-hCG as previously described (Ploutarchou *et al.*, 2015). Sections were dewaxed and rehydrated, endogenous peroxidase was blocked using 3% (v/v) H_2O_2 (Fisher Scientific) in PBS for 5 min and slides were washed with water, followed by Tris-buffered saline with 0.05% (v/v) Tween 20 (TBST) (histochemistry) or PBS (IHC). Antigen retrieval was performed for pSMAD1/5/8 detection using 0.01 M citrate buffer. Non-specific binding was blocked for HA using 2% (v/v) FCS (Sigma-Aldrich) in PBS for 1 h; for HCs using 5% (w/v) dry milk (Alcafe, Reading, UK) in PBS for 2 h; for PTX3 using 10% (w/v) dry milk in PBS for 1 h; for pSMAD1/5/8 using 5% (w/v) BSA (Fisher Scientific) in PBS for 1 h; for pSMAD2 and TSG-6 using 1.5% (v/v) normal goat serum (Vectastain ABC Kit; Vector Laboratories, Peterborough, UK) in PBS for 1 h. Sections were incubated at 4ºC overnight, with either biotinylated hyaluronan binding protein (bHABP (Clark *et al.*, 2011), Cosmo Bio Co., Tokyo, Japan) at 1:50, rabbit anti-human IαI/PαI polyclonal antibody (Ab) (indicative of HC levels) (Carrette *et al.*, 2001; Mukhopadhyay *et al.*, 2001); Dako, Glostrup, Denmark) at 1:100, 1 mg/ml rabbit anti-human PTX3 polyclonal Ab at 1:100 ((Scarchilli *et al.*, 2007), generously provided by Antonio Inforzato) at 1:200, rabbit anti-mouse TSG-6 polyclonal anti-sera (Carrette *et al.*, 2001) at 1:150, anti-pSMAD1/5/8 polyclonal Ab (Cell Signaling Technology, Beverly, Massachusetts, USA) at 1:250, or rabbit anti-pSMAD2 polyclonal Ab (0.25 mg/ml; Life Technologies,

Invitrogen, Paisley, UK) at 1:100, at 4˚C overnight. All dilutions were in the relevant blocking solutions and Control sections lacking Ab or bHABP were incubated with their respective blocking solutions. The following day, sections were washed and incubated with biotinylated anti-rabbit IgG secondary antibody (Vectastain ABC Elite Kit) for 30 min, followed by ABC solution (Vectastain ABC Elite Kit) for 30 min at room temperature. Antigen-specific detection was revealed using diaminobenzidine (DAB, peroxidase substrate kit; Vector Laboratories). The sections were dehydrated with decreasing ethanol concentrations, mounted with Depex (VWR) and imaged using a light microscope.

Following IHC or histochemistry, slides were soaked in xylene to aid cover slip removal. Sections were then rehydrated and counterstained with haematoxylin for 2 min, washed in water for 5 min, destained with acid alcohol for 5 s, washed again in water, dehydrated with ethanol, remounted using Depex and reimaged.

1.2.5 Quantification and analysis of HA, HC, PTX3, TSG-6, pSMAD1/5/8 and pSMAD2

To enable quantification of both cECM and intracellular molecules, total pixel intensities (TPI), numbers of pixels and mean pixel intensities (MPI) were determined using ImageJ (National Institutes of Health, Bethesda, Maryland, USA). We elected to use IHC methods to determine pSMAD expression within COCs, as this had previously been performed to assess protein within murine GCs and CCs (Tian *et al.*, 2010; Ploutarchou *et al.*, 2015). MPI was expressed as a percentage, where 0% reflects white and 100% reflects black. TPI, calculated as MPI multiplied by number of pixels in the cumulus complex, was normalized to CC number.

1.2.6 Morphometric analysis

Oocyte area, cumulus complex area, CC number and distance between the corona radiata (CR) cells and the oocyte were then measured in sections stained with haematoxylin using ImageJ. Within the CR, cell numbers and distances between cells were measured using Image J. For each follicle, cumulus complex size was determined using the section with maximum oocyte area (i.e. from the centre of the oocyte); if this was not clearly visible due to folding of the section or other artifact, this follicle was excluded.

For follicles at 48 h post-PMSG, the cumulus mass was assessed using regions that had clearly discernable separation between GCs and CCs. Therefore, the circumference of the entire COC and the circumference of the oocyte in each of these clearly distinguishable regions were measured. To estimate the total CC number and cumulus complex area in each COC, measurements from each region were added together and corrected using a calculated factor (total circumference of oocyte/sum of measured oocyte circumference in areas).

1.2.6 Statistical analysis

Prism GraphPad software version 6.0 (GraphPad Software, La Jolla, California, USA) was used for all statistical analyses. Data were tested for normality using the D'Agostino-Pearson omnibus normality test (GraphPad Software; normally distributed data were analyzed using unpaired *t*-tests with Welch's correction, whereas non-normally distributed data were analyzed using the Mann-Whitney test (GraphPad Software). A *P* value of <0.05 was considered significant. Correlations between datasets were determined using linear regression analyses, where the coefficient of determination (r^2) was calculated to measure the fit of the linear regression model to the plotted data. An r^2 value of >0.8 was considered to indicate a strong correlation between the linear model and the plotted data.

1.3 Results

1.3.1 Oocyte size and cumulus expansion in DM COCs

COCs from mice with oocyte-specific deletions of *C1galt1* and *Mgat1* have abnormal cumulus matrix that is resistant to hyaluronidase treatment. To investigate this phenotype further, oocyte size, cumulus size and CC number were compared for DM and Control mice. Ovaries collected at 48 h post-PMSG and 9 h post-hCG from both DM (n=3) and Control (n=3) animals were sectioned, and sections taken through the centers of individual oocytes were analyzed to ensure equivalent assessment of follicles (Figures 1A-D).

No significant differences in oocyte area were seen between Control and DM follicles at either 48 h post-PMSG or 9 h post-hCG (Figures 1E-F). At both time points, the cumulus area of DM COCs was not significantly different to Controls although there was a trend (p=0.0693) towards a decrease in expansion (Figures 1G, H), however, this trend was not reflected in CC numbers (Figure 1I, 1J). Normalization of the cumulus area to the CC number (i.e. CC cell density) showed that the cumulus mass in DM COCs was denser than the Control at 48 h post-PMSG (Figure 1K), i.e. prior to expansion; however, no difference between DM and Control follicles was observed at 9 h post-hCG (Figure 1L).

1.3.2 Corona radiata cells in DM COCs

The highest concentrations of OSFs are found in the CR, where the concentrations of OSFs to which CCs are exposed decrease with distance from the oocyte. To assess whether any differences were apparent between Control and DM COCs at the level of the corona radiata, i.e. indicative of changes in expansion due to OSFs, the distance between the CR cells and the number of CR cells were determined for each COC (Figures 2A-D). Significantly fewer CR cells were found in DM COCs at 48 h post-PMSG (Figure 2E), but this was not observed at 9 h post-

hCG (Figure 2F). There were no differences in the distances between CR cells at either 48 h post-PMSG (Figure 2G) or 9 h post-hCG (Figure 2H).

1.3.3 Quantification of HA, HCs, PTX3 and TSG-6 in the cumulus matrix

Given the cumulus associated with ovulated eggs from DM mice were resistant to hyaluronidase treatment compared to Controls, it is likely that the DM cECM is modified. We investigated the levels and distributions of the cumulus matrix molecules HA, HCs, PTX3 and TSG-6 in COCs during late stage expansion to quantify the differences in cECM between mice deficient in oocyte *N*- and *O*-glycans and Controls.

HA was detected throughout the cECM and associated with CCs and mural GCs in both Control (Figure 3A) and DM (Figure 3B) COCs. Quantification of HA revealed significantly lower levels in DM COCs, even when normalised to CC number (Figures 3C-D). IαI immunostaining (likely corresponding to HCs) showed similar distributions to HA (Figures 3E, F) and was also present at reduced levels in DM COCs, even when TPI was normalised to CC number (Figures 3G, H). Although PTX3 appeared less widely distributed in DM COCs compared to controls (Figures 3I, J), there was no difference in intensity between Control and DM COCs (Figures 3K, L). This was also the case for TSG-6 (Figures 3M, N, O, P).

1.3.4 Quantification of pSMAD1/5/8 and pSMAD2 in cumulus cells

The OSFs BMP15 and GDF9 play key roles in cumulus expansion, where they bind to cumulus cells and signal via SMAD1/5/8 and SMAD2/3, respectively. We elected to use IHC methods to determine pSMAD expression within COCs, as this had previously been performed to assess expression within murine GCs and CCs (Tian *et al.*, 2010; Ploutarchou *et al.*, 2015). In addition, obtaining samples from live, healthy follicles undergoing cumulus expansion to localise and quantify CC pSMAD

expression would be challenging. Here, cell-associated localisation of pSMAD1/5/8 was seen in both Control (Figure 4A) and DM (Figure 4B) COCs at 9 h post-hCG. However, levels of pSMAD1/5/8 were significantly reduced in DM COCs compared to Controls when normalised to CC number (Figure 4C). In contrast, whilst pSMAD2 was detected in cells from both DM and control COCs (Figures 4D, E) with no significant differences in pSMAD2 levels (Figure 4F).

1.3.5 Correlations between cumulus area and cumulus molecules

The use of linear regression analyses revealed no significant correlations between the levels of any of the cECM molecules or pSMADs investigated and the extent of cumulus expansion for either Control or DM COCs (data not shown). Similarly, no significant correlations were seen between levels of individual matrix molecules and either pSMAD1/5/8 or pSMAD2 (data not shown).

1.4 Discussion

The formation of a functional cumulus complex, containing its necessary structural components, is linked to oocyte development (McKenzie *et al.*, 2004; Cillo *et al.*, 2007; Gebhardt *et al.*, 2011; Ekart *et al.*, 2013), ovulation (Varani *et al.*, 2002; Fulop *et al.*, 2003; Ochsner *et al.*, 2003b; Brown *et al.*, 2006), fertilisation (Tanghe *et al.*, 2002; Salustri *et al.*, 2004) as well as subsequent embryo development (McKenzie *et al.*, 2004). Given the poor success rate of assisted reproductive techniques (ART) such as in *vitro* maturation of oocytes (<35%, (Ellenbogen *et al.*, 2014)), further insight into the role of the cumulus and the cECM may provide markers for assessing and potentially improving oocyte competency in ART. The results presented here reveal that despite increased hyaluronidase resistance (Williams *et al.*, 2007) and altered cECM composition in COCs with oocyte-specific deletions of *C1galt1* and *Mgat1*, only subtle (~10%) decreases were observed in cumulus area, CC number and CC density.

Aside from being the major constituent of the cECM (Russell and Robker, 2007; Sugiura *et al.*, 2009), HA is also hypothesised to be associated with oocyte maturation (Yokoo and Sato, 2011). HA synthesis by cumulus cells is dependent on the expression of *HAS2* (Sugiura *et al.*, 2009), which is upregulated in response to hCG (Park *et al.*, 2004), BMP15 (Caixeta *et al.*, 2013; Fenwick *et al.*, 2013) and GDF9 (Dragovic *et al.*, 2005; Fenwick *et al.*, 2013). hCG, an analogue of LH, stimulates the production of epidermal growth factor-like (EGF-L) peptides from mural GCs, which bind to their receptors on CCs to induce *HAS2* expression (Park *et al.*, 2004). Since both Controls and DM were given the same dose of hCG, a decrease in HA secretion could result from altered secretion of EGF-L peptides by the granulosa cells, or reduced levels of EGF-L receptors or a modified response to EGF-L binding by the CCs. In addition, *HAS2* expression is also influenced by BMP15 (Caixeta *et al.*, 2013; Fenwick *et al.*, 2013) via phosphorylated SMAD1/5/8 (pSMAD1/5/8), which was reduced in DM COCs (Li *et al.*, 2009; Peng *et al.*, 2013). The reduced levels of pSMAD1/5/8 detected here in DM CCs could reflect a reduction in BMP15 synthesis or bioactivity (Saito *et al.*, 2008), but do not completely abolishing the signal since the DM model does not phenocopy the *BMP15*$^{-/-}$ mice in this regard. In contrast, GDF9 signalling may be unimpaired by the oocyte-specific deletion of *N*- and *O*-glycans, since pSMAD2/3 levels appear to be unaffected. Aside from *HAS2*, BMP15 and GDF9 also regulate the expression of *PTX3* and *TSG-6* in cumulus cells (Varani *et al.*, 2002; Yoshino *et al.*, 2006; Li *et al.*, 2009); however, levels of both molecules were unchanged in DM COCs.

Although cumulus area was not affected by the reduction in HA, the reduced levels of HC observed may be due to limited availability of HA with which to form complexes. Both HA and HC showed similarly reduced levels when comparing DM and Control COCs (28% and 24% decrease, respectively), suggesting that levels of covalently

attached HCs may be influenced by the levels of HA. It should be noted that in the *C1galt1* single Mutant we observed an increase in HC levels in COCs that could be due to a more permeable basal lamina, allowing more IαI to enter the follicular fluid (Ploutarchou *et al.*, 2015). It is possible that the deletion of both *C1galt1* and *Mgat1* could give rise to changes in the basal lamina that reduce its permeability to IαI. Despite the reduction of HA and HC in DM COCs, the levels of both molecules remained above the threshold required for normal COC expansion; a threshold we have previously proposed (Ploutarchou *et al.*, 2015). Although the HA levels are decreased in DM COCs, the resistance to hyaluronidase treatment may be due to the unchanged levels of PTX3 in DM COCs; more PTX3 may be bound to HA in the cECM, increasing its stability (Baranova *et al.*, 2014).

Interestingly, our findings here for DM COCs contrast with those from the single *C1galt1* deletion. In the *C1galt1* Mutant, the levels of HA, HC and PTX3 in the cECM were higher than Controls, however, pSMAD2 and pSMAD1/5/8 levels were similar in Mutant and Control COCs (Ploutarchou *et al.*, 2015). Thus, it seems that it is the lack of *N*-glycans in the DM mutant that results in lower levels of HA and HCs in the cECM. The only cECM molecule that does not appear to be affected by the oocyte-specific deletion of either *C1galt1* or both *C1galt1* and *Mgat1* is TSG-6, which plays a catalytic role in HC•HA formation but does not have a major structural role in matrix stability (Briggs *et al.*, 2015). TSG-6 levels may not be affected in DM COCs, as its expression can also be stimulated by GDF9 via the production of prostaglandin E_2 (PGE_2) and PGE_2 receptor (Elvin *et al.*, 2000); thus if there is sufficient GDF9 stimulation, this pathway may rescue TSG-6 expression.

To summarize, this study supports our previous work (Ploutarchou *et al.*, 2015) demonstrating that levels of HA, HC, PTX3, TSG-6 and SMAD phosphorylation do not correlate with the extent of cumulus expansion in Controls. The observation

that reduced levels of HA and HC in DM COCs have no detectable effect on cumulus expansion suggests that these molecules are present at concentrations that exceed the minimum threshold required, or that there are other compensatory mechanisms in DM mice. Increased crosslinking between HC•HA and PTX3 in the DM, which could affect elasticity and stiffness, may explain the increased resistance of the cumulus oophorus to hyaluronidase treatment (Williams *et al.*, 2007). Additionally, the flexibility in cECM formation is sufficient at these levels to allow comparable fertilisation rates between DM and Control mice (Grasa *et al.*, 2012). Further analysis of the effects of oocyte-specific *C1galt1* and *Mgat1* deletion on BMP15 and GDF9 synthesis and bioactivity may help to explain our results and shed further light on the mechanisms behind cumulus expansion.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Baranova N. S., Inforzato A., Briggs D. C., Tilakaratna V., Enghild J. J., Thakar D., Milner C. M., Day A. J. and Richter R. P. (2014) Incorporation of pentraxin 3 into hyaluronan matrices is tightly regulated and promotes matrix cross-linking. *J Biol Chem* **289** 30481–30498.

Briggs D. C., Birchenough H. L., Ali T., Rugg M. R., Waltho J. P., Ievoli E., Jowitt T.

A., Enghild J. J., Richter R. P., Salustri A. *et al.* (2015) Metal ion-dependent heavy chain transfer activity of TSG-6 mediates assembly of the cumulus oocyte matrix. *J Biol Chem* **290** 28708–28723.

Brown H. M., Dunning K. R., Robker R. L., Pritchard M. and Russell D. L. (2006) Requirement for ADAMTS-1 in extracellular matrix remodeling during ovarian folliculogenesis and lymphangiogenesis. *Developmental Biology* **300** 699–709.

Caixeta E. S., Sutton-McDowall M. L., Gilchrist R. B., Thompson J. G., Price C. A., Machado M. F., Lima P. F. and Buratini J. (2013) Bone morphogenetic protein 15 and fibroblast growth factor 10 enhance cumulus expansion, glucose uptake, and expression of genes in the ovulatory cascade during in vitro maturation of bovine cumulus-oocyte complexes. *Reproduction*.

Carrette O., Nemade R. V, Day A. J., Brickner A. and Larsen W. J. (2001) TSG-6 is concentrated in the extracellular matrix of mouse cumulus oocyte complexes through hyaluronan and inter-alpha-inhibitor binding. *Biol Reprod* **65** 301–308.

Chen W. and Stanley P. (2003) Five Lec1 CHO cell mutants have distinct Mgat1 gene mutations that encode truncated N-acetylglucosaminyltransferase I. *Glycobiology* **13** 43–50.

Chen L., Mao S. J. and Larsen W. J. (1992) Identification of a factor in fetal bovine serum that stabilizes the cumulus extracellular matrix. A role for a member of the inter-alpha-trypsin inhibitor family. *J Biol Chem* **267** 12380–12386.

Chen L., Russell P. T. and Larsen W. J. (1993) Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. *Mol Reprod Dev* **34** 87–93.

Chen L., Zhang H., Powers R. W., Russell P. T. and Larsen W. J. (1996) Covalent linkage between proteins of the inter-alpha-inhibitor family and hyaluronic acid is mediated by a factor produced by granulosa cells. *J Biol Chem* **271** 19409–

19414.

Cillo F., Brevini T. A. L., Antonini S., Paffoni A., Ragni G. and Gandolfi F. (2007) Association between human oocyte developmental competence and expression levels of some cumulus genes. *Reproduction (Cambridge, England)* **134** 645–650.

Clark S. J., Keenan T. D., Fielder H. L., Collinson L. J., Holley R. J., Merry C. L., van Kuppevelt T. H., Day A. J. and Bishop P. N. (2011) Mapping the differential distribution of glycosaminoglycans in the adult human retina, choroid, and sclera. *Invest Ophthalmol Vis Sci* **52** 6511–6521.

Diaz F. J., Wigglesworth K. and Eppig J. J. (2007) Oocytes determine cumulus cell lineage in mouse ovarian follicles. *J Cell Sci* **120** 1330–1340.

Dragovic R. A., Ritter L. J., Schulz S. J., Amato F., Armstrong D. T. and Gilchrist R. B. (2005) Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion. *Endocrinology* **146** 2798–2806.

Dube J. L., Wang P., Elvin J., Lyons K. M., Celeste A. J. and Matzuk M. M. (1998) The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. *Mol Endocrinol* **12** 1809–1817.

Dunlop C. E. and Anderson R. A. (2014) The regulation and assessment of follicular growth. *Scand J Clin Lab Invest Suppl* **244** 13–7; discussion 17.

Ekart J., McNatty K., Hutton J. and Pitman J. (2013) Ranking and selection of MII oocytes in human ICSI cycles using gene expression levels from associated cumulus cells. *Human Reproduction* **28** 2930–2942.

Ellenbogen A., Shavit T. and Shalom-Paz E. (2014) IVM results are comparable and may have advantages over standard IVF. *Facts, Views & Vision in ObGyn* **6** 77–80.

Elvin J. A., Yan C. and Matzuk M. M. (2000) Growth differentiation factor-9 stimulates progesterone synthesis in granulosa cells via a prostaglandin E2/EP2 receptor pathway. *Proc Natl Acad Sci U S A* **97** 10288–10293.

Fenwick M. A., Mora J. M., Mansour Y. T., Baithun C., Franks S. and Hardy K. (2013) Investigations of TGF-β Signaling in Preantral Follicles of Female Mice Reveal Differential Roles for Bone Morphogenetic Protein 15. *Endocrinology* **154** 3423–3436.

Fulop C., Szanto S., Mukhopadhyay D., Bardos T., Kamath R. V, Rugg M. S., Day A. J., Salustri A., Hascall V. C., Glant T. T. *et al.* (2003) Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. *Development* **130** 2253–2261.

Galloway S. M., McNatty K. P., Cambridge L. M., Laitinen M. P., Juengel J. L., Jokiranta T. S., McLaren R. J., Luiro K., Dodds K. G., Montgomery G. W. *et al.* (2000) Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat Genet* **25** 279–283.

Gebhardt K. M., Feil D. K., Dunning K. R., Lane M. and Russell D. L. (2011) Human cumulus cell gene expression as a biomarker of pregnancy outcome after single embryo transfer. *Fertility and Sterility* **96** 47–52.e2.

Grasa P., Kaune H. and Williams S. A. (2012) Embryos generated from oocytes lacking complex N- and O-glycans have compromised development and implantation. *Reproduction* **144** 455–465.

Hayashi M., McGee E. A., Min G., Klein C., Rose U. M., van Duin M. and Hsueh A. J. (1999) Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles. *Endocrinology* **140** 1236–1244.

Irving-Rodgers H. F., Mussard M. L., Kinder J. E. and Rodgers R. J. (2002) Composition and morphology of the follicular basal lamina during atresia of bovine antral follicles. *Reproduction* **123** 97–106.

Ju T., Cummings R. D. and Canfield W. M. (2002) Purification, characterization, and subunit structure of rat core 1 Beta1,3-galactosyltransferase. *J Biol Chem* **277** 169–177.

Kaivo-oja N., Jeffery L. A., Ritvos O. and Mottershead D. G. (2006) Smad signalling in the ovary. *Reprod Biol Endocrinol* **4** 21.

Li Q., Rajanahally S., Edson M. A. and Matzuk M. M. (2009) Stable expression and characterization of N-terminal tagged recombinant human bone morphogenetic protein 15. *Mol Hum Reprod* **15** 779–788.

Lokman P. M., Kazeto Y., Ozaki Y., Ijiri S., Tosaka R., Kohara M., Divers S. L., Matsubara H., Moore L. G. and Adachi S. (2010) Effects of reproductive stage, GH, and 11-ketotestosterone on expression of growth differentiation factor-9 in the ovary of the eel, Anguilla australis. *Reproduction* **139** 71–83.

McClure N., Macpherson A. M., Healy D. L., Wreford N. and Rogers P. A. (1994) An immunohistochemical study of the vascularization of the human Graafian follicle. *Hum Reprod* **9** 1401–1405.

McKenzie L., Pangas S., Cisneros P., Amato P., Matzuk M. and Carson S. (2004) Human granulosa cell gene expression: A predictor of fertilization and embryo selection in women undergoing in vitro fertilization. *Fertility and Sterility* **82** S73.

Mukhopadhyay D., Hascall V. C., Day A. J., Salustri A. and Fulop C. (2001) Two distinct populations of tumor necrosis factor-stimulated gene-6 protein in the extracellular matrix of expanded mouse cumulus cell-oocyte complexes. *Arch Biochem Biophys* **394** 173–181.

Ng S. T., Chang T. H. and Wu T. C. (1999) Prediction of the rates of fertilization,

cleavage, and pregnancy success by cumulus-coronal morphology in an in vitro fertilization program. *Fertil Steril* **72** 412–417.

Ochsner S. A., Day A. J., Rugg M. S., Breyer R. M., Gomer R. H. and Richards J. S. (2003a) Disrupted function of tumor necrosis factor-alpha-stimulated gene 6 blocks cumulus cell-oocyte complex expansion. *Endocrinology* **144** 4376–4384.

Ochsner S. A., Russell D. L., Day A. J., Breyer R. M. and Richards J. S. (2003b) Decreased expression of tumor necrosis factor-alpha-stimulated gene 6 in cumulus cells of the cyclooxygenase-2 and EP2 null mice. *Endocrinology* **144** 1008–1019.

Ohtsubo K. and Marth J. D. (2006) Glycosylation in cellular mechanisms of health and disease. *Cell* **126** 855–867.

Park J. Y., Su Y. Q., Ariga M., Law E., Jin S. L. and Conti M. (2004) EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* **303** 682–684.

Pedersen T. and Peters H. (1968) Proposal for a classification of oocytes and follicles in the mouse ovary. *J Reprod Fertil* **17** 555–557.

Peng J., Li Q., Wigglesworth K., Rangarajan A., Kattamuri C., Peterson R. T., Eppig J. J., Thompson T. B. and Matzuk M. M. (2013) Growth differentiation factor 9:bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions. *Proc Natl Acad Sci U S A* **110** E776-85.

Ploutarchou P., Melo P., Day A. J., Milner C. M. and Williams S. A. (2015) Molecular analysis of the cumulus matrix: insights from mice with O-glycan-deficient oocytes. *Reproduction* **149** 533–543.

Rugg M. S., Willis A. C., Mukhopadhyay D., Hascall V. C., Fries E., Fulop C., Milner C. M. and Day A. J. (2005) Characterization of complexes formed between TSG-6 and inter-alpha-inhibitor that act as intermediates in the covalent transfer of heavy chains onto hyaluronan. *J Biol Chem* **280** 25674–25686.

543 Russell D. L. and Robker R. L. (2007) Molecular mechanisms of ovulation: co-
544 ordination through the cumulus complex. *Hum Reprod Update* **13** 289–312.

545 Saito S., Yano K., Sharma S., McMahon H. E. and Shimasaki S. (2008)
546 Characterization of the post-translational modification of recombinant human
547 BMP-15 mature protein. *Protein Sci* **17** 362–370.

548 Salustri A., Garlanda C., Hirsch E., De Acetis M., Maccagno A., Bottazzi B., Doni A.,
549 Bastone A., Mantovani G., Beck Peccoz P. *et al.* (2004) PTX3 plays a key role
550 in the organization of the cumulus oophorus extracellular matrix and in in vivo
551 fertilization. *Development* **131** 1577–1586.

552 Sanchez F. and Smitz J. (2012) Molecular control of oogenesis. *Biochim Biophys*
553 *Acta* **1822** 1896–1912.

554 Sato H., Kajikawa S., Kuroda S., Horisawa Y., Nakamura N., Kaga N., Kakinuma C.,
555 Kato K., Morishita H., Niwa H. *et al.* (2001) Impaired fertility in female mice
556 lacking urinary trypsin inhibitor. *Biochem Biophys Res Commun* **281** 1154–
557 1160.

558 Scarchilli L., Camaioni A., Bottazzi B., Negri V., Doni A., Deban L., Bastone A.,
559 Salvatori G., Mantovani A., Siracusa G. *et al.* (2007) PTX3 interacts with inter-
560 alpha-trypsin inhibitor: implications for hyaluronan organization and cumulus
561 oophorus expansion. *J Biol Chem* **282** 30161–30170.

562 Shi S., Williams S. A., Seppo A., Kurniawan H., Chen W., Ye Z., Marth J. D. and
563 Stanley P. (2004) Inactivation of the Mgat1 gene in oocytes impairs oogenesis,
564 but embryos lacking complex and hybrid N-glycans develop and implant. *Mol*
565 *Cell Biol* **24** 9920–9929.

566 Su Y. Q., Wu X., O'Brien M. J., Pendola F. L., Denegre J. N., Matzuk M. M. and
567 Eppig J. J. (2004) Synergistic roles of BMP15 and GDF9 in the development
568 and function of the oocyte-cumulus cell complex in mice: genetic evidence for

569 an oocyte-granulosa cell regulatory loop. *Dev Biol* **276** 64–73.

570 Sugiura K., Su Y. Q. and Eppig J. J. (2009) Targeted suppression of Has2 mRNA in
571 mouse cumulus cell-oocyte complexes by adenovirus-mediated short-hairpin
572 RNA expression. *Mol Reprod Dev* **76** 537–547.

573 Tanghe S., Van Soom A., Nauwynck H., Coryn M. and de Kruif A. (2002) Minireview:
574 Functions of the cumulus oophorus during oocyte maturation, ovulation, and
575 fertilization. *Mol Reprod Dev* **61** 414–424.

576 Tian X., Halfhill A. N. and Diaz F. J. (2010) Localization of phosphorylated SMAD
577 proteins in granulosa cells, oocytes and oviduct of female mice. *Gene Expr*
578 *Patterns* **10** 105–112.

579 Tian E., Hoffman M. P. and Ten Hagen K. G. (2012) O-glycosylation modulates
580 integrin and FGF signalling by influencing the secretion of basement membrane
581 components. *Nat Commun* **3** 869.

582 Trombetta E. S. (2003) The contribution of N-glycans and their processing in the
583 endoplasmic reticulum to glycoprotein biosynthesis. *Glycobiology* **13** 77R–91R.

584 Varani S., Elvin J. A., Yan C., DeMayo J., DeMayo F. J., Horton H. F., Byrne M. C.
585 and Matzuk M. M. (2002) Knockout of pentraxin 3, a downstream target of
586 growth differentiation factor-9, causes female subfertility. *Mol Endocrinol* **16**
587 1154–1167.

588 Wagner K. W., Punnoose E. A., Januario T., Lawrence D. A., Pitti R. M., Lancaster
589 K., Lee D., von Goetz M., Yee S. F., Totpal K. *et al.* (2007) Death-receptor O-
590 glycosylation controls tumor-cell sensitivity to the proapoptotic ligand
591 Apo2L/TRAIL. *Nat Med* **13** 1070–1077.

592 Williams S. A. and Stanley P. (2008) Mouse fertility is enhanced by oocyte-specific
593 loss of core 1-derived O-glycans. *FASEB J* **22** 2273–2284.

Williams S. A. and Stanley P. (2009) Oocyte-specific deletion of complex and hybrid N-glycans leads to defects in preovulatory follicle and cumulus mass development. *Reproduction* **137** 321–331.

Williams S. A. and Stanley P. (2011) Premature ovarian failure in mice with oocytes lacking core 1-derived O-glycans and complex N-glycans. *Endocrinology* **152** 1057–1066.

Williams S. A., Xia L., Cummings R. D., McEver R. P. and Stanley P. (2007) Fertilization in mouse does not require terminal galactose or N-acetylglucosamine on the zona pellucida glycans. *J Cell Sci* **120** 1341–1349.

Yan C., Wang P., DeMayo J., DeMayo F. J., Elvin J. A., Carino C., Prasad S. V, Skinner S. S., Dunbar B. S., Dube J. L. *et al.* (2001) Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Mol Endocrinol* **15** 854–866.

Yokoo M. and Sato E. (2011) Physiological function of hyaluronan in mammalian oocyte maturation. *Reproductive Medicine and Biology* **10** 221–229.

Yoshino O., McMahon H. E., Sharma S. and Shimasaki S. (2006) A unique preovulatory expression pattern plays a key role in the physiological functions of BMP-15 in the mouse. *Proc Natl Acad Sci U S A* **103** 10678–10683.

Zhuo L., Yoneda M., Zhao M., Yingsung W., Yoshida N., Kitagawa Y., Kawamura K., Suzuki T. and Kimata K. (2001) Defect in SHAP-hyaluronan complex causes severe female infertility. A study by inactivation of the bikunin gene in mice. *J Biol Chem* **276** 7693–7696.

Figure legends

Figure 1 – Cumulus expansion in Control and DM COCs. Representative images of haematoxylin-stained sections taken through the centres of oocytes are shown. The red dotted lines indicate the area of the cumulus mass. Regions of COCs where CCs are discernible from mural GCs were used to measure cumulus area for Control 48 h post-PMSG (A), DM 48 h post-PMSG (B), Control 9 h post-hCG (C) and DM 9 h post-hCG (D). Scale bar: 50 μm. Oocyte areas of Control and DM COCs at 48 h post-PMSG (E) and 9 h post-hCG (F), cumulus areas of Control and DM COCs at 48 h post-PMSG (G) and 9 h post-hCG (H), total numbers of cumulus cells in the cumulus area of Control and DM COCs at 48 h post-PMSG (I) and 9 h post-hCG (J) and cumulus cell densities of Control and DM COCs at 48 h post-PMSG (K) and 9 h post-hCG (L) were determined. Data are plotted as mean values ± SD; control 48 h post-PMSG follicles (n=23); DM 48 h post-PMSG follicles (n=12); Control 9 h post-hCG follicles (n=14); DM 9 h post-hCG follicles (n=18). Mann-Whitney test. *P<0.05.

Figure 2 – Distance between corona radiata cells as a function of expansion. Representative images of haematoxylin-stained sections cut through the centres of oocytes were used to measure the distances (shown by dotted lines between black circles) between corona radiata (CR) cells in Control COCs at 48 h post-PMSG (A) (expanded from Figure 1A), DM COCs at 48 h post-PMSG (B) (expanded from Figure 1B), Control COCs at 9 h post-hCG (C) (expanded from Figure 1C) and DM COCs at 9 h post-hCG (D) (expanded from Figure 1D). Scale bar: 25 μm. Corona cell numbers at 48 h post-PMSG (E) and 9 h post-hCG (F) and distance between CR cells at 48 h post-PMSG (G) and 9 h post-hCG (H) for Control and DM COCs are plotted as mean values ± SD. Control 48 h post-PMSG follicles (n=23); DM 48 h post-PMSG follicles (n=12); Control 9 h post-hCG follicles (n=14); DM 9 h post-hCG follicles (n=18). Mann-Whitney test. **P<0.01.

Figure 3 – Localisation and quantification of cumulus matrix molecules in COCs. Localisation, quantification (mean pixel intensity, MPI) and normalisation of total pixel intensity (TPI) to CC number were carried out following histochemical detection of hyaluronan (HA) (A-D), heavy chains (HCs) (E-H), pentraxin 3 (PTX3) (I-L) and tumour necrosis factor-stimulated gene 6 (TSG-6) (M-P) in Control and DM cumulus extracellular matrix. Control sections without antibody or bHABP are shown counterstained with haematoxylin. Scale bar: 50μm. Data are presented as mean values ± SD. The number of follicles analysed in each case (n) is shown on the bar charts. Mann-Whitney test. ***P*<0.01; ****P*<0.001.

Figure 4 – Localisation and quantification of pSMAD1/5/8 and pSMAD2 in preovulatory follicles. Localisation and quantification (total pixel intensity (TPI) per CC number were carried out for pSMAD1/5/8 (A-C) and pSMAD2 (D-F) in Control and DM preovulatory follicles. Control sections without antibody are shown counterstained with haematoxylin. Scale bar: 50μm. Data are presented as mean values ± SD. The number of follicles analysed in each case (n) is shown on the bar charts. Mann-Whitney test. ****P*<0.001.