

Analysis of *in vitro* follicle development during the onset of premature ovarian insufficiency in a mouse model

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Abstract

Premature ovarian insufficiency (POI) occurs in 1% of women under 40 years and is predominantly idiopathic. A transgenic mouse model of follicular POI, the Double Mutant (DM), are fertile at 6-weeks of age, become infertile by 9-weeks and exhibit POI by 3-months. DM female mice generate oocytes lacking mucin O-glycans and complex N-glycans due to deletion of *C1galt1* and *Mgat1* respectively (DM: *C1galt1*^{F/F}*Mgat1*^{F/F}:ZP3Cre; Control *C1galt1*^{F/F}*Mgat1*^{F/F}). To determine if DM follicle development could be improved in a controlled environment, follicles from DM and Controls were cultured individually and follicle growth, morphology, survival and antrum formation were evaluated. DM ovaries were more rigid than Control at 3-, 6- and 9-weeks which was exacerbated with age resulting in a failure to isolate follicles from 9-week DM females. DM follicles had decreased survival compared to Control follicles from females at 3- and 6-weeks. Furthermore, survival rate of DM follicles decreased with age between 3- and 6-weeks. DM follicles at both 3- and 6-weeks had accelerated follicle growth and altered antrum formation during the first few days of culture but after 6 days, follicles were equivalent in size to Controls. In conclusion, a population of DM follicles retain the potential to develop *in vitro* and therefore follicle culture offers a reliable method to generate antral follicles from preantral after the onset of POI in these females.

Keywords: Follicle development, premature ovarian insufficiency, *in vitro* culture, extracellular matrix, tissue rigidity

Introduction

Premature ovarian insufficiency (POI), previously known as premature ovarian failure (POF), is a clinical condition characterised by hypergonadotropic hypogonadism and amenorrhea that affects 1% to 3% of women under 40 years of age and is idiopathic in around 70% of cases (Coulam *et al.* 1986; Meskhi and Seif 2006; Shelling 2010).

The ovarian manifestation varies from a complete depletion of follicles (afollicular POI) to the presence of a variable population of follicles (follicular POI) that fail to develop (Meskhi and Seif 2006; Nelson 2009). The proportion of women with afollicular and follicular POI has been a matter of debate for several years between clinicians, and empirical studies have failed to provide reliable data. However, some studies indicate that the proportion of afollicular and follicular POI is fairly equal (Nelson *et al.* 1994; Suzuki *et al.* 2015) indicating that significant numbers of women with follicular POI could benefit from therapies focusing on reactivation of follicle development.

A mouse model of follicular POI (Williams *et al.* 2007) has been established and investigations into follicle development in this model during the onset of POI may reveal new insights for treatment development. The phenotype of this mouse model, known as the Double Mutant (DM), originates from oocyte-specific deletion of two glycosyltransferases using *Cre-LoxP* technology (Williams *et al.* 2007). The DM female mouse has an oocyte-specific deletion of the *C1galt1* and *Mgat1* alleles, which encode core 1 β 1,3-galactosyltransferase (T-synthase) and *N*-acetylglucosaminyltransferase 1 (GlcNAcT-1) enzymes, respectively. T-synthase is required for the generation of core 1- (mucin) O-glycans and GlcNAcT-1 is required for the generation of complex and hybrid *N*-glycans. The DM mouse has floxed

C1galt1 and *Mgat1* alleles, which are deleted specifically in the oocyte by a ZP3Cre recombinase transgene from the primary stage of development onwards (Philpott *et al.* 1987); the primary follicle is a follicle with a complete layer of up to 60 granulosa cells in a cross-section (Pedersen and Peters 1968). Therefore, direct effects of the mutations should only be initialised in growing follicles and the primordial follicle pool remains unaffected.

At 6-weeks of age DM females have a normal ovulation rate (Grasa *et al.* 2012) however, they produce a litter with ~50% fewer offspring than Controls (Williams *et al.* 2007; Williams and Stanley 2011). In addition, DM females are unable to produce subsequent litters, accompanied by a precipitous drop in ovulation rate at 9-weeks of age and aberrant follicle development (Grasa *et al.* 2012). Follicle development in these mice deteriorates with age and culminates in POI by 3 months of age when mice exhibit an increase in gonadotrophins and a decrease in sex steroids, all characteristic symptoms of POI in women (Williams and Stanley 2011).

Glycans, such as *N*- and *O*-glycans of the DM POI model, have several important cellular functions, including to provide structural components to the extracellular matrix, modify protein properties such as stability and solubility, regulate protein trafficking and can mediate cell signalling by affecting protein half-life and receptor binding (Axford 2001; Sinclair and Elliott 2005). The absence of complex *N*- and *O*-glycans in the oocyte of DM females leads to impaired ovulation and modified follicle development (Williams and Stanley 2011). Since the genetic modification is oocyte-specific, and females were initially fertile at 6-weeks of age, it is conceivable that follicle development is compromised by extra-follicular factors such as extracellular

matrix (ECM) or gonadotrophin balance. Therefore, if intra-follicular mechanisms are still intact in DM follicles after the onset of POI, it is likely that follicle growth could resume if the extra-follicular blocking signalling is removed.

The aim of this study was to evaluate if under controlled *in vitro* conditions, follicle development can be normalised in follicles from POI mice.

Material and methods

Mice

Female mice carrying floxed *Mgat1* and *C1galt1* alleles and a ZP3Cre transgene were used as experimental females whereas females carrying the floxed *Mgat1* and *C1galt1* alleles but not the ZP3Cre transgene were used as controls since the floxed alleles function as wild-type genes and the ZP3Cre transgene does not affect fertility (Shi *et al.* 2004; Williams *et al.* 2007). Animals were maintained in a 12h-12h light-darkness regime and provided with food and water *ad libitum*. All experiments were approved by the Home Office and the Clinical Medical Local Ethical Review Committee.

Follicle culture

Littermate 3-, 6- and 9-week Control and DM female pairs were selected for follicle culture. In both the 6- and 9-week groups, females were age-matched littermates and therefore variations due to differences in oestrous cycle are equally represented in both groups. Ovaries were dissected and placed in warm Leibovitz's L-15 culture medium (HyClone-Thermo Fisher Scientific, Loughborough, UK) supplemented with 3mg/ml of bovine serum albumin (BSA fraction V, Fisher Scientific, Loughborough,

UK). Preantral follicles were microdissected using 30G needles and acupuncture needles and cultured as previously described (Grasa *et al.* 2015). For the procedure, selected follicles were preantral, ~140 to 200 µm in diameter, with an undamaged basal lamina and some theca tissue surrounding the follicle. Follicles were placed in individual wells of a 96-well plate containing 30 µl of minimum essential medium (MEM Alpha Modification, HyClone-Thermo Scientific) supplemented with 140 mM ascorbic acid (Sigma-Aldrich, St Louis, USA), 5% fetal bovine serum (FBS, Biosera, Ringmer, UK; same lot number used in all experiments) and 2.5 IU/ml recombinant human FSH (r-hFSH, Gonal-F, Merk Serono, Feltham, UK). A preliminary dose response experiment was carried out using 2.5, 5 and 7.5 IU of rFSH, follicle development was equivalent under all conditions and therefore, a concentration of 2.5 IU of rFSH was selected for use (data not shown). The culture medium, was filtered using a 22 µm pore syringe filter, placed in the well covered with 75 µl of silicone oil (Dow Corning, BDH, VWR international, Lutterworth, UK) and equilibrated in the incubator at 37°C, 5%CO₂ in air for at least 2 h before use. Follicles were cultured for up to 6 days with daily transfer to a new well with freshly prepared culture medium. Images were taken daily with a Leica M125 microscope (Leica Microsystems, Germany) and MicroPublisher 5.0 RTV camera (Qimaging, London, UK) to evaluate growth, morphology, survival and antrum formation. Growth was also evaluated daily by measuring the diameter of the follicle with an ocular reticle at 5x magnification. Only viable follicles that grew beyond day 3 were included for the follicular growth curve analysis.

Morphology assessment

Morphological features such as an intact basal lamina, a visible oocyte and antrum formation were evaluated daily by observation under the microscope and confirmed by analysing the daily images taken throughout the culture period. To avoid any bias, during analysis the investigator was blind to the genotype of the mice. Follicle diameter was determined by taking the mean of multiple measurements from the edge of the basal lamina using a precalibrated ocular micrometer and images were taken to confirm measurements using ImageJ software (version 1.46r, National Institute of Health, USA).

Statistical analysis

Statistical analysis was carried out using Prism software version 4.0b (GraphPad Software, Inc., version 4.0b, 2004. La Jolla, CA, USA). D'Agostino & Pearson normality test was applied to test Gaussian (normal) distribution of the samples. The Mann–Whitney U test was performed to detect differences between groups with non-parametrical distribution and equal variances. Unpaired T test was used to analyse samples with normal distribution.

Results

Insufficient numbers of preantral follicles were obtained for culture from 9 week old DM ovaries

One distinctive characteristic noticed when dissecting the DM ovaries, especially at 9-weeks of age, was that the ovarian tissue was more rigid and fibrous compared with Control ovaries (Figure 1). Ovaries from 3- and 6-week DM ovaries were less rigid than 9-week DM ovaries. When dissecting follicles, we were able to tear the Control ovaries between follicles, which allowed isolation of numerous intact follicles

from each pair of ovaries at all ages (n=12 follicles per mouse). However, we were unable to tear the 9-week old DM ovaries between follicles, as we could in Controls, as it resulted in the shredding of follicles in the process. Attempting to isolate follicles from the 9-week old DM ovaries using microdissection scissors was equally limited. Therefore, although significant numbers of intact follicles were isolated from 9-week Control ovaries (n=36 follicles, n=3 mice), 30% of DM females yielded zero follicles, 55% of DM females one follicle, and one female yielded two follicles (total DM females; n=9).

Follicles from 3- and 6-week old DM females have a decreased survival rate *in vitro*

Twelve ovarian follicles were dissected from each 3- and 6-week DM (3-weeks n=8 mice and 6-weeks n=4 mice) and Control female (3-weeks n=7 mice and 6-weeks n=4 mice) and cultured for 6 days (Figure 2). Follicles that that stopped growing on day 0 or stopped growing by day 3 of culture were classified as unviable and were excluded from the growth analysis.

The survival rate of follicles was significantly decreased in DM compared with Controls at both, 3-weeks (DM $87.18 \pm 4.2\%$ versus Control $98.81 \pm 1.2\%$) and 6-weeks (DM $50.57 \pm 8.5\%$ versus Controls $97.92 \pm 2.1\%$) however the survival rate of DM follicles decreased from 3- to 6-weeks (Figure 3A). Next, we analysed the day on which the follicles stopped growing. Since only one Control follicle stopped growing at both the 3- and 6-week cultures, we were unable to compare Control and Mutant data. Analysis of DM follicle growth termination between 3- and 6-weeks revealed no difference in the day the follicles stopped growing (Figure 3B).

The diameter at collection (d0) of the unviable follicles was compared with the diameter of the follicles that grew normally in order to assess whether the survival rate was related with differences in the type of follicle collected in both groups or was intrinsic to the mice genotype. However, no differences were detected in the diameter between viable and unviable Control and DM follicles collected at 3- and 6-weeks (3 weeks: Control viable $183.7 \pm 1.5 \mu\text{m}$ n=69, DM viable $182.5 \pm 1.9 \mu\text{m}$ n=56, Control unviable $182.4 \pm 4.6 \mu\text{m}$ n=8, DM unviable $175.8 \pm 2.1 \mu\text{m}$ n=25; 6 weeks: Control viable $185.3 \pm 2.0 \mu\text{m}$ n=42, DM viable $188.0 \pm 4.8 \mu\text{m}$ n=23, Control unviable $195.4 \pm 8.3 \mu\text{m}$ n=4, DM unviable $177.7 \pm 4.0 \mu\text{m}$ n=24).

Follicles from 3- and 6-week old DM females show accelerated growth *in vitro*

Surprisingly, the final diameter reached by DM follicles, was similar to the Control group in both 3- and 6-week follicles demonstrating that DM follicles are able to grow and develop *in vitro* (Figure 4A and B). However, the growth curve of DM follicles differed to Controls. Follicles collected from 3-week DM (n=56) females grew significantly larger for the duration of the culture but had reduced growth during the final day of culture and thus were the same size as Controls (n=69) at the end of the culture period (Figure 4A). A similar situation was observed in follicles isolated from 6-week DM (n=23) females, however follicles were significantly larger than Controls (n=42) for the first 3 days of culture only (Figure 4B).

***In vitro* antrum development of DM follicles differs to Controls at 3- and 6-weeks**

The day and the size of the follicle when the antrum was first detected was assessed; there was no difference in the day that a follicular antrum developed between Control and DM in both 3- and 6-weeks follicles (Figure 4C and D), however the diameter of DM follicles when the antrum was first visible, was increased compared with Controls at both ages (DM 263.0 ± 2.2 μm and Controls 246.1 ± 2.4 μm for 3-week follicles and DM 288.6 ± 6.4 μm and Controls 266.7 ± 5.9 μm , respectively for 6-week follicles) (Figure 4E and F).

Discussion

Understanding follicle development during the onset of POI and thus developing therapies focusing on reactivation of follicle development for women with follicular POI is essential (Nelson *et al.* 1994; Suzuki *et al.* 2015). Oocyte-specific deletion of *C1galt1* and *Mgat1* alleles in DM females produce a rapid deterioration of ovarian function from fertile females at 6-weeks to complete ovarian dysfunction and POI by 3-months (Williams and Stanley 2011; Grasa *et al.* 2012). The development of POI is characterized by a sharp decrease in ovulation rate from 6- to 9-weeks (Grasa *et al.* 2012) with an altered endocrine profile and impaired follicle development by 3-months of age (Williams and Stanley 2011). Infertility in DM females occurs from 9-weeks onwards and even though the total number of follicles is comparable to 3-month Control ovaries, the majority of the follicles in DM ovaries are arrested at the 3a stage of primary follicle development with ovaries lacking follicles from the secondary stage onwards (Williams and Stanley 2011). Thus, the DM mouse presents a POI phenotype comparable with follicular POI displayed by women and therefore provides a useful model to study POI follicle development *in vitro*.

Ovarian follicle development is regulated by a balance between extra and intra-ovarian factors. Several intra-ovarian factors such as epidermal growth factor (EGF), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs) and also members of the transforming growth factor beta (TGF- β) family, for example GDF-9 and anti-Müllerian hormone (AMH), have been implicated in normal and pathological follicle development, such as women with polycystic ovary syndrome (PCOS) (Qiao and Feng 2011). In addition, early studies assessed the possibility that follicles could secrete factors that affect directly the development of the adjacent follicles within the ovary. Co-culture of early antral and late preantral follicles *in vitro* has demonstrated that larger follicles retard the growth of smaller follicles (Baker *et al.* 2001) and therefore mouse follicles secrete factors that can affect the development of other follicles when cultured from the late preantral to Graafian stages of follicle development (Spears *et al.* 2002). In addition, glycans have important functions, including providing structural components to the ECM, modification of protein properties, mediating protein trafficking and cell signalling (Axford 2001; Sinclair and Elliott 2005). Oocyte-specific deletion of *Mgat1* and *C1galt1* and therefore the absence of hybrid and complex N- and core 1-derived O-glycans, is likely to be disturbing intra-ovarian signalling in DM ovaries affecting follicle progression.

In order to eliminate potential suppressing intra-ovarian factors, we isolated preantral follicles from 3- and 6-week old DM ovaries for *in vitro* culture. The survival rate of DM follicles exacerbates with age with less follicles surviving at 6-weeks, showing an age-dependent decrease in survival. Furthermore, survival rate of DM follicles was less than Controls indicating that intrinsic follicular factors are present in preantral follicles before and after puberty in DM females preventing follicle development.

271 Interestingly, DM follicles that did survive initially grew faster than Control follicles but
272 reached an equivalent final size to Controls. Since the growth of DM follicles slows
273 towards the end of culture, it is possible that if cultured for longer periods of time,
274 they may stop growing altogether. However this is also the case for Controls as
275 follicle size is finite and shortly after day 6 of culture using this system, they either
276 ovulate an egg and structurally collapse, or die. Therefore, it is unknown if this
277 decrease in DM follicle growth rate was due to follicle deterioration or due to
278 physiological constraints and the fact that a larger size was attained prematurely.
279 However, although Control or DM follicle growth or egg competence was not
280 assessed beyond 6-days of culture, culture of wildtype preantral follicles for 6-days is
281 sufficient for maturation of oocytes *in vitro* (Davidson *et al.* 2013) and therefore it is
282 not unreasonable to assume that eggs could be retrieved from DM follicles at day 6.
283
284 Successful follicle development requires interactions between the cells that comprise
285 the follicle and the surrounding ECM resulting in remodelling of the ECM to adapt to
286 the growing follicle. Differences in the composition of the ovarian cortex and medulla
287 ECM are thought to play a critical role in follicle development. The cortex is collagen-
288 rich and provides rigid support for quiescent primordial follicles (Hornick *et al.* 2012).
289 Primordial follicles are thought to activate as they migrate to the less rigid medulla
290 which is a more supportive environment for the development and expansion of
291 follicles (Woodruff and Shea 2011). Changes in the ECM composition of the ovary
292 lead to a disruption of follicle development and anovulation. This is evident in women
293 with PCOS who have a denser ovarian cortex due to higher levels of collagen than
294 the cortex of normal ovaries (Hughesdon 1982).

295

296 The finding that DM ovaries have an increased rigidity and density, compared with
297 Controls at 3-, 6- and 9-weeks of age, that is exacerbated with age and coincides
298 with the decreased survival rate of DM follicles, indicating that components of the
299 ECM are affected and therefore it is likely that intra-ovarian factors and
300 communication is abnormal in the DM mouse. The rigidity observed in DM ovaries is
301 unlike ovaries of aged mice, which are fibrous due to the loss of growing follicles
302 which changes the relative proportions of cortex:medulla, and is therefore
303 considered abnormal. This also indicates that these modifications accumulate with
304 age and are coincident with the change in the DM phenotype from fertile to infertile
305 and may provide a cause for the previously reported block in primary follicle
306 development (Williams and Stanley 2011) and also for the ovary dysfunction as
307 observed in PCOS. Changes in ECM components such as laminin and collagen IV
308 have been reported in mice with an oocyte-specific deletion of *C1galt1* (Christensen
309 *et al.* 2015) and therefore it is likely that these and other components of the
310 extracellular matrix are compromised in the DM ovary.

311

312 Recently, the role of the environmental rigidity in follicle development has been
313 investigated through the use of alginate in follicle culture systems. Follicles cultured
314 in a less rigid matrix exhibit an increased follicular growth and a higher rate of antrum
315 and theca formation than follicles cultured in a more rigid environment (Xu *et al.*
316 2006; West *et al.* 2007). The increased density observed in DM ovaries could
317 potentially act as a growth-restriction agent to limit the expansion and development
318 of the DM follicles. Our results demonstrate that the removal of DM follicles from their
319 rigid ovarian environment to a less-rigid environment in culture appears to have
320 allowed DM follicles to have better development as they initially grew faster. We

speculate that the more rigid ECM surrounding the isolated DM follicles provides support for the follicle in culture mimicking the normal ovarian environment, whereas removing Control follicles from the ovary to a culture environment results in the disruption of their follicular architecture and removal of structural support, limiting their ability to grow as well in culture initially. This hypothesis is supported by the fact that DM follicles grew faster during first days of culture and were larger than Control follicles when they developed an antrum. The antrum cavity is filled with follicular fluid composed of serum transudate as well as secretory products of granulosa cells such as mucopolysaccharides, steroids and hormones (Andersen *et al.* 1976; Grimek *et al.* 1984; Cristol *et al.* 1985). The composition of the follicular fluid is related to the physiological status of the follicle and has been suggested to reflect the fertility potential of the oocyte inside in both, humans (Mendoza *et al.* 2002; Das *et al.* 2008; Piomboni *et al.* 2014) and cattle (Bender *et al.* 2010).

In summary, DM follicles are able to grow *in vitro* reaching the same size as Control follicles at 3- and 6-weeks of age. However, the survival rate of DM follicles is compromised with age and also when compared to Control follicle survival at both ages. Furthermore, the increased rigidity of the ECM in the DM ovary could be affecting follicle development *in vivo* since the growth curve of DM follicles differs to Controls demonstrated by an initial acceleration in follicle growth and a different pattern in antrum development. Despite these uncertainties, our results demonstrate that a population of DM follicles retain the potential to develop *in vitro* and therefore follicle culture offers a reliable method to generate antral follicles from preantral after the onset of POI in these females.

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Figure legends

Figure 1: Images of Control and DM ovaries. Representative images of Control and DM ovaries from 3-, 6- and 9-week old females. Scale bar 200 μ m.

Figure 2: Ovarian follicle development *in vitro*. Representative images of follicle grown *in vitro* from 3-week old (Control n=69 follicles n=7 mice, DM n=56 follicles n=8 mice) and 6-week old (Control n=42 follicles n=4 mice, DM n=23 n=4 mice) Control and DM females. Ovarian follicles were dissected (d0) and cultured with 2.5 IU of r-hFSH for up to 6 days. Scale bar 200 μ m.

Figure 3: Survival rate and growth arrest of Control and DM follicles during *in vitro* culture. (A) Survival rate of Control and DM ovarian follicles dissected from 3- (Control n=69 follicles n=7 mice, DM n=56 follicles n=8 mice) and 6-week (Control n=42 follicles n=4 mice, DM n=23 n=4 mice) old females. Growth arrest of follicles dissected from (B) 3- and 6-week old females and assessed as the day when follicle growth stopped during culture. Results are expressed as mean \pm SEM of follicles. * $P\leq 0.05$, ** $P\leq 0.01$. The Mann–Whitney U test was analyse data in Figure 3A.

Figure 4: Assessment of Control and DM follicle development and antrum formation *in vitro*. (A-B) Growth curve of Control and DM follicles dissected from 3- (Control n=69 follicles n=7 mice, DM n=56 follicles n=8 mice) and 6-week old (Control n=42 follicles n=4 mice, DM n=23 n=4 mice) females; results are expressed as mean \pm SEM. (C-D) Day of culture when Control and DM follicles developed antrum. (E-F) Size of Control and DM follicles when an antrum was first detected. (C-F) Results are expressed as mean \pm SD. * $P\leq 0.05$, ** $P\leq 0.01$, *** $P\leq 0.001$, **** $P\leq 0.0001$.

500 The Mann–Whitney U test was used to analyse data in Figures 4A, 4B, 4C and 4E.

501 An unpaired t-test was used to analyse data in Figures 4D and F.