

Embryos generated from oocytes lacking complex *N*- and *O*-glycans have compromised development and implantation

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Abstract

Female mice generating oocytes lacking complex *N*- and *O*-glycans (double mutants (DM)) produce only one small litter before undergoing premature ovarian failure (POF) by 3 months. Here we investigate the basis of the small litter by evaluating ovulation rate and embryo development in DM (*Mgat1^{F/F}C1galt1^{F/F}:ZP3Cre*) and Control (*Mgat1^{F/F}C1galt1^{F/F}*) females. Surprisingly, DM ovulation rate was normal at 6 weeks, but declined dramatically by 9 weeks. *In vitro* development of zygotes to blastocysts was equivalent to Controls although all embryos from DM females lacked a normal zona pellucida (ZP) and ~30% lacked a ZP entirely. In contrast, *in vivo* preimplantation development resulted in less embryos recovered from DM females compared with Controls at 3.5 days post coitum (dpc) (3.2 ± 1.3 vs 7.0 ± 0.6). Furthermore, only 45% of mated DM females contained embryos at 3.5 dpc. Of the preimplantation embryos collected from DM females, approximately half were morulae unlike Controls where the majority were blastocysts, indicating delayed embryo development in DM females. Post-implantation development in DM females was analysed to determine whether delayed preimplantation development affected subsequent development. In DM females at 5.5 dpc, only ~40% of embryos found at 3.5 dpc had implanted. However, at 6.5 dpc, implantation sites in DM females corresponded to embryo numbers at 3.5 dpc indicating delayed implantation. At 9.5 dpc, the number of decidua corresponded to embryo numbers 6 days earlier indicating that all implanted embryos progress to midgestation. Therefore, a lack of complex *N*- and *O*-glycans in oocytes during development impairs early embryo development and viability *in vivo* leading to delayed implantation and a small litter.

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Introduction

The fertility of an individual female is a reflection of the number of eggs ovulated and their competence. The development of each oocyte occurs within a follicle which can be considered as the functional unit of the ovary. The development and growth of follicles requires complex endocrine, paracrine and autocrine regulations, as well as bidirectional signals between the oocyte and follicular cells. Each follicle consists of a central oocyte that is surrounded by layers of granulosa cells that are separated from the outer surrounding theca cells by a basal lamina. Upon antrum formation, granulosa cells differentiate into mural granulosa cells that line the follicle wall and cumulus cells that surround the oocyte. Since 1996 when the critical role of the oocyte in follicle development was revealed, elucidating the various functions of the oocyte has been the focus of much research (Dong *et al.* 1996, Matzuk *et al.* 2002, Hutt & Albertini 2007, Gilchrist *et al.* 2008, Kidder &

Vanderhyden 2010). Indeed, differentiation of the cumulus cells and their subsequent expansion before ovulation requires oocyte-secreted factors (Diaz *et al.* 2007, Sugiura *et al.* 2010).

Essential cell-to-cell signalling between the oocyte and the surrounding granulosa cells occurs via transzonal processes (TZPs). TZPs penetrate from the granulosa cells through the extracellular matrix coat that surrounds the oocyte. This extracellular matrix coat known as the zona pellucida (ZP) is composed of three glycoproteins, ZP1, ZP2 and ZP3 (Bleil & Wassarman 1980b, Wassarman 2005) in the mouse, all of which are expressed exclusively by the oocyte from the primary stage of follicle development onwards (Philpott *et al.* 1987). Glycans on ZP proteins had been postulated as sperm receptors for many years (Bleil & Wassarman 1980a, van Duin *et al.* 1994, Litscher *et al.* 2009, Han *et al.* 2010). However, mutant mice generating oocytes lacking complex *N*- and/or *O*-glycans are fertile demonstrating that these glycans are not required for zona–sperm binding in mice although

the ZP is thinner and more fragile if it lacks *O*-glycans, *N*-glycans or both (Shi *et al.* 2004, Williams *et al.* 2007, Williams & Stanley 2008).

The generation of both core 1 and 2 *O*-glycans (also referred to as complex *O*-glycans) and complex and hybrid *N*-glycans occurs on glycoproteins at specific motifs in a highly regulated manner (Fig. 1). The generation of core 1 and 2 *O*-glycans requires the glycosyltransferase T-synthase encoded by the *C1galt1* gene (previously known as *T-syn*). Core 1 *O*-glycans are synthesized by the addition of galactose to the *N*-acetylgalactosamine (GalNAc) attached via O-linkage to serine or threonine residues. Core 2 *O*-glycans are generated by the attachment of *N*-acetylglucosamine (GlcNAc) to the core 1 structure. Mice lacking *C1galt1* die during gestation at ~E14 due to defective angiogenesis (Xia *et al.* 2004, Williams *et al.* 2007). Mice lacking complex and hybrid *N*-glycans also die *in utero* at E9.5 (Ioffe & Stanley 1994, Metzler *et al.* 1994). Complex and hybrid *N*-glycans are generated by modification and extension of oligomannosyl structures which is initiated by the enzyme *N*-acetylglucosaminyltransferase 1 (GlcNAcT-1, encoded by the *Mgat1* gene). Therefore, to study biological functions of these glycans in the oocyte *Cre-loxP* technology has been used to eliminate the enzymes required for their generation. *Cre-loxP* technology allows a gene flanked by *loxP* sites to be deleted upon exposure to the enzyme *Cre* recombinase.

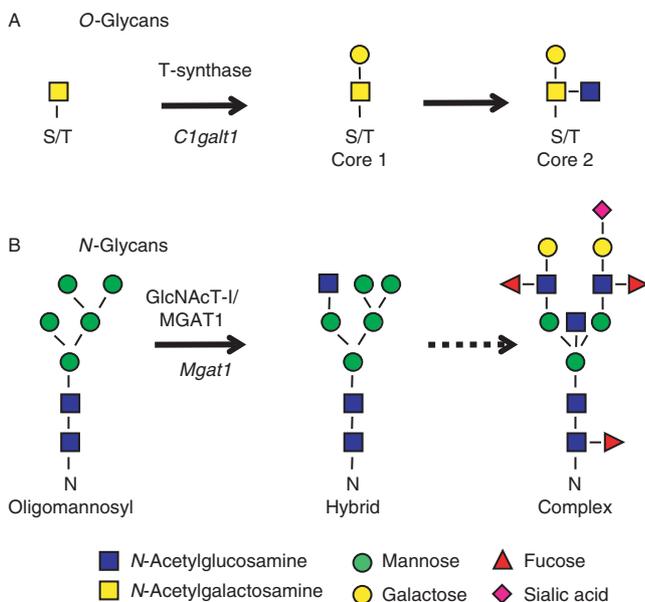


Figure 1 The generation of complex *O*- and *N*-glycans. (A) T-synthase (encoded by *C1galt1*) is required to initiate the synthesis of core 1 *O*-glycans by transferring galactose (Gal) to *N*-acetylgalactosamine (GalNAc) on serine/threonine residues (S/T). Core 2 *O*-glycans are generated by the addition of *N*-acetylglucosamine (GlcNAc) to the GalNAc of core 1 structure. (B) *N*-Acetylglucosaminyltransferase 1 (GlcNAcT-1, encoded by *Mgat1*) is required to generate complex and hybrid *N*-glycans from the oligomannosyl structure.

Therefore, by flanking the coding exons of *C1galt1* and/or *Mgat1* with *loxP* sites, and using a *Cre* recombinase transgene under the control of the oocyte-specific ZP3 promoter, mice can be generated which lack these enzymes specifically in the oocyte (Shi *et al.* 2004, Williams & Stanley 2008, 2011).

Females with oocyte-specific deletion of *C1galt1* generate oocytes lacking core 1 and 2 *O*-glycans and exhibit an increase in fertility, revealing a role for these glycans in the regulation of ovulation rate (Williams & Stanley 2008). Oocyte-specific deletion of *Mgat1* results in the generation of oocytes lacking complex and hybrid *N*-glycans. These glycans have a role in generating developmentally competent oocytes, and once fertilised, in embryo implantation and development (Shi *et al.* 2004). *Mgat1*^{F/F}:ZP3*Cre* females have reduced fertility producing smaller litters (Shi *et al.* 2004). Follicle development is also modified in these mice with considerable abnormalities evident in preovulatory follicles (Williams & Stanley 2009). Moreover, oocyte-specific deletion of both *Mgat1* and *C1galt1* in double mutant (DM) females results in ovarian dysfunction leading to an altered endocrine profile and premature ovarian failure (POF) by 3 months of age (Williams & Stanley 2011). However, embryonic development and fertility are normal for mice heterozygous for either *Mgat1* or *C1galt1* generated by crossing *Mgat1*^{del/+} or *C1galt1*^{del/+} mice (Ioffe & Stanley 1994, Xia *et al.* 2004). Furthermore, fertility is also normal in mice generating oocytes heterozygous for either *Mgat1* or *C1galt1* using oocyte-specific deletion (Williams *et al.* 2007). In addition, females generating oocytes heterozygous for both *Mgat1* and *C1galt1* have normal fertility (Williams *et al.* 2007).

The aim of this study was to determine the stage(s) at which the decrease in fertility occurs in DM females. We reveal that the decrease in fertility is not due to a reduced ovulation rate but due to modified embryo development.

Results

Ovulation rate

We have previously demonstrated that DM females mated at 6 weeks have decreased fertility producing only a single small litter of ~4 pups (Williams *et al.* 2007, Williams & Stanley 2011). To determine whether the smaller litter size was due to a decrease in the number of eggs ovulated, ovulation rate was assessed by analysis of the number of eggs and/or presumptive zygotes recovered at 0.5 days post coitum (dpc) in females joined with males, at either 6 weeks or 9 weeks of age (termed the '6-week group' and '9-week group' respectively) (Fig. 2).

Most surprisingly, the ovulation rate did not differ between Control and DM females in the 6-week group (Control: 8.3 ± 0.2 , $n=9$; DM: 7.5 ± 0.8 , $n=8$), although ovulation rate of DM females was considerably more

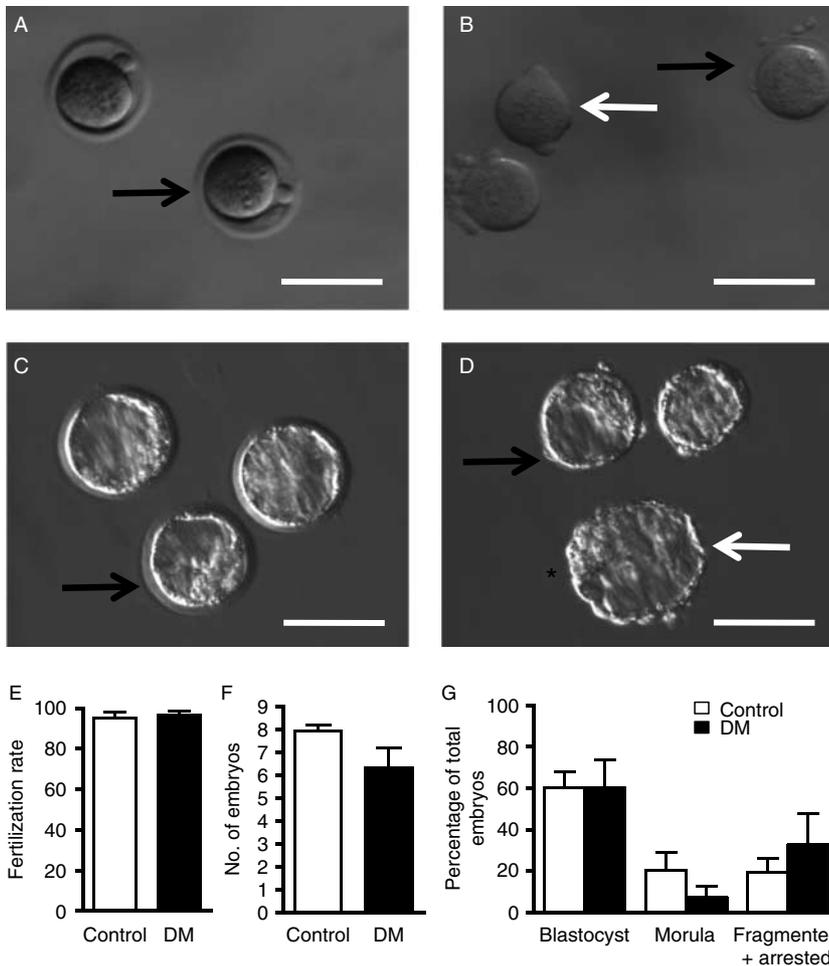


Figure 3 *In vitro* development of preimplantation embryos. Images of embryos collected and cultured *in vitro* from Control (A and C) and DM (B and D) 6-week group females. (A and B) Presumptive zygotes collected at 0.5 dpc. (C and D) Blastocyst after 4 days in culture. Black arrow indicates ZP; white arrows indicate DM embryos without ZP; asterisk indicates a bigger blastocyst that seems to have resulted from the fusion of two embryos because the number of embryos present in this dish was reduced by one. (E) Fertilisation rate of Control ($n=12$) and DM ($n=6$) females. (F) Total number of embryos at the end of the culture period from Control and DM females. (G) Percentage of blastocysts, morulae, fragmented and arrested embryos in Control and DM females at 4.5 dpc. Results are expressed as mean \pm S.E.M. Bar scale = 100 μ m.

The very high proportion of mated DM females that lack embryos at 3.5 dpc is most likely contributing significantly to the 70% of DM females that fail to produce a litter (Williams *et al.* 2007).

Of the DM females containing embryos, the number of embryos recovered was lower than Controls (Control: 7.0 ± 0.6 , DM: 3.2 ± 1.3 , $P=0.05$; Fig. 4H) and only 35% still possessed a ZP (Table 1). Moreover, DM females contained fewer blastocysts than Control females (Control: 5.8 ± 0.8 , DM: 1.2 ± 0.5 , $P<0.01$). When expressed as a proportion of total embryos, blastocysts and morulae were equally represented in the embryos from DM females whereas embryos from Control females were 80% blastocysts (Fig. 4I).

In light of these observations, *in vivo* and *in vitro* embryonic developments was compared revealing unexpected and striking differences in embryos collected from DM females (Table 2). The total number of *in vivo* developed embryos from DM females collected at 3.5 dpc was half of that obtained after culture ($P=0.08$). Furthermore, the number of blastocysts recovered *in vivo* from DM females was lower than the number obtained *in vitro* ($P=0.05$). Conversely, the

number of morulae collected from DM females at 3.5 dpc (1.4 ± 0.7) was almost three times higher than the number obtained in culture (0.5 ± 0.3) although this did not reach significance. In stark contrast to embryos developed *in vivo* from DM females, embryos developed *in vitro* from Control females collected at 3.5 dpc did not differ from those cultured. However, although the *in vitro* conditions were better for development of embryos from DM females, the number of fragmented and/or arrested embryos obtained from *in vitro* culture was three times higher than *in vivo* for embryos collected from both Control and DM females (although this did not reach significance; Table 2).

Implantation analysis

The high proportion of morulae collected from DM females at 3.5 dpc compared with Controls (Fig. 4I and Table 2 although significance was not reached) may indicate a delay in early development of embryos from DM females that could affect implantation. To evaluate whether these morulae are able to progress further and implant, the 6-week group of Control and DM females

Table 1 Number of presumptive zygotes and embryos lacking ZP in both *in vitro* and *in vivo* conditions.

	Collected (0.5 dpc)		<i>In vitro</i> (4.5 dpc)		<i>In vivo</i> (3.5 dpc)	
	No. of presumptive zygotes	No. of ZP-free presumptive zygotes (%)	No. of embryos	No. of ZP-free embryos (%)	No. of embryos	No. of ZP-free embryos (%)
Control	8.3 ± 0.2 (n=12)	0.2 ± 0.2 ^b (1.9 ± 1.9 [#]) (n=12)	7.9 ± 0.3 (n=12)	0.2 ± 0.2 ^a (3.3 ± 3.3 [*]) (n=12)	7.0 ± 0.6 (n=5)	0 (n=5)
DM	7.0 ± 1.3 (n=6)	1.7 ± 0.7 ^b (30.0 ± 12.0 [#]) (n=6)	6.3 ± 0.8 (n=6)	1.3 ± 0.5 ^a (29.0 ± 10.3 [*]) (n=6)	3.2 ± 1.3 (n=5)	1.2 ± 0.5 (65.0 ± 19.0) (n=5)

Mean ± S.E.M. Values with the same superscript are significantly different. $P=0.05$ (^a, ^{*}); $P<0.05$ (^b, [#]).

was mated and embryo development was analysed at 5.5 dpc. The number of implantation sites was unsurprisingly lower in DM females at 5.5 dpc (1.3 ± 0.4) than in Control females (7.3 ± 0.6 , $P<0.01$; Table 3). However, the number of implantation sites at 5.5 dpc in DM females was around half the number of embryos recovered at 3.5 dpc (3.5 dpc: 3.2 ± 1.3 , 5.5 dpc: 1.3 ± 0.4 , Tables 2 and 3). Furthermore, unfertilised eggs and non-implanted embryos were recovered in DM females in contrast to Control females, indicating that this process is aberrant in DM females.

To determine whether there was a delay in implantation in DM females thus explaining the lower number of implantation sites at 5.5 dpc compared with the mean litter size of 4.0 ± 2.7 (Williams *et al.* 2007), we analysed implantation 24 h later at 6.5 dpc (Table 4). Interestingly, the number of implantation sites in DM females at 6.5 dpc (Table 4) was more than double that at 5.5 dpc (Table 3) and is consistent with the number of embryos at 3.5 dpc (Table 2).

Postimplantation embryo development

Analysis of embryos implanted at 5.5 and 6.5 dpc revealed a delay in implantation. To determine whether development of the implanted embryos was altered due to a difference in the timing of implantation, we analysed embryos at 9.5 dpc in females of the 6-week group. Uterine horns of DM females contained less deciduae and healthy embryos than those of Control females ($P<0.05$ and $P<0.001$ respectively; Table 5). The number of decidua in DM females (3.0 ± 0.6) did not differ from the number of embryos recovered at 3.5 dpc (3.2 ± 1.3), nor the number of implantations at 6.5 dpc (3.2 ± 1.0), and is consistent with a litter size of 4.0 ± 2.7 when DM females are mated at 6 weeks of age (Williams *et al.* 2007). However, not all deciduae in both, Control and DM females contained normal healthy embryos (Table 5), demonstrating that not all implanted embryos generated from either normal or mutant eggs are able to develop to term. Consistent with this, the number of healthy embryos in DM females at 9.5 dpc (2.0 ± 0.3)

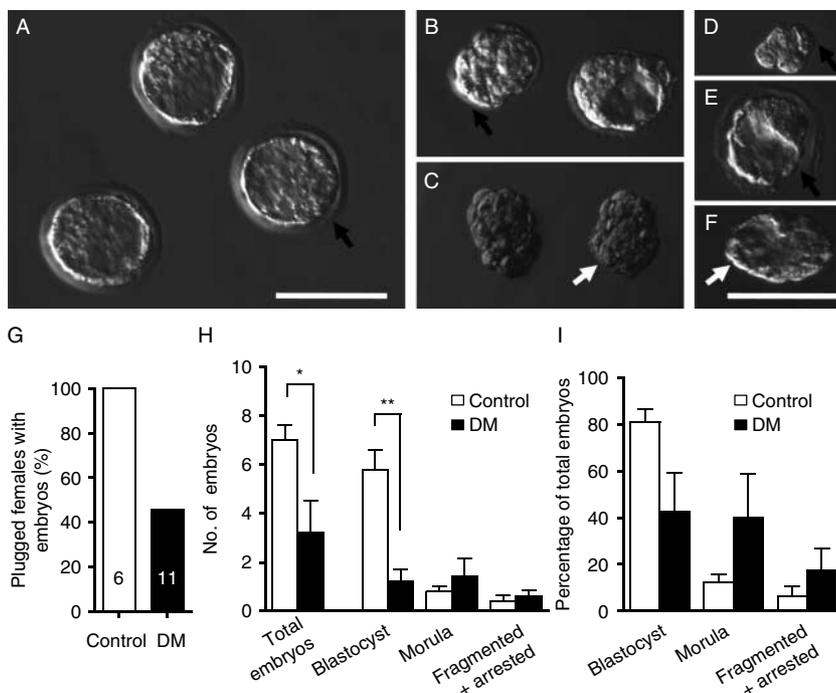


Figure 4 *In vivo* developed embryos at 3.5 dpc. Images of embryos from Control (A) and DM (B, C, D, E and F) females in the 6-week group. (A) Blastocysts, (B) blastocyst and morula, (C) morula, (D) arrested embryo at four-cell stage, (E) blastocyst with abnormal ZP and (F) blastocyst without ZP. Black arrow indicates ZP; white arrow indicates a DM morula without ZP. (G) Percentage of mated females, with embryos recovered after flushing. (H) Number of embryos and distribution of embryonic developmental stages in Control ($n=5$) and DM ($n=5$) females containing embryos. (I) Proportions of blastocysts, morulae and fragmented and arrested embryos as a percentage of the total number of embryos recovered at 3.5 dpc from Control and DM females. Results are expressed as mean ± S.E.M. Bar scale = 100 µm. * $P=0.05$; ** $P<0.01$.

Table 2 *In vitro* (4.5 dpc) and *in vivo* (3.5 dpc) development of embryos from 6-week group Control and DM females.

Total number	Control			DM		
	<i>In vitro</i>	<i>In vivo</i>	<i>In vivo:in vitro</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vivo:in vitro</i>
Females with embryos	12	5	–	6 ^a	5 ^b	–
Embryos per female	7.9±0.3	7.0±0.6	0.9	6.3±0.8	3.2±1.3	0.5
Blastocysts per female	4.8±0.7	5.8±0.8	1.2	3.8±0.9*	1.2±0.5*	0.3
Morulae per female	1.7±0.7	0.8±0.2	0.5	0.5±0.3	1.4±0.7	2.8
Fragmented+arrested embryos per female	1.5±0.5	0.4±0.2	0.3	2.0±0.8	0.6±0.2	0.3
Blastocysts+morulae per female	6.4±0.6	6.6±0.8	1.0	4.3±1.1	2.6±1.0	0.6

Mean±S.E.M. Values with the same superscript are significantly different, * $P=0.05$.

^aData are presented for females whose zygotes were cultured. ^bEleven DM females were evaluated; however, only five contained embryos.

was similar to the number of morphologically normal embryos (blastocyst+morula) recovered before implantation ($2.6±1.0$; Table 2).

Embryo and implantation site comparison at the five developmental stages

The number of preimplantation embryos, implanted embryos and deciduae at the five different developmental stages are compared in Fig. 5. At each stage of development, the number of implantations in DM females was markedly lower than that of Controls which was consistently between seven and eight. However, the number of presumptive zygotes recovered in DM at 0.5 dpc was significantly higher than the number of embryos found at any other stage. Therefore, the number of deciduae in DM females at 5.5 dpc is markedly lower than at any of the other time points analysed.

Corpora lutea observations

At all of the developmental stages studied, the number of corpora lutea (CL) was comparable with the number of deciduae in Controls, but in DM ovaries, the number of CL was significantly higher than the number of deciduae (Tables 3, 4 and 5). However, the CL in DM ovaries were not consistent in size or colour compared with Control CL. Some DM CL were smaller than control CL and pale (abnormal CL) compared with the bright red Control CL (normal CL). Despite the low number of implantations, the number of normal CL in DM ovaries was similar to the number of CL in Control females, consistent with a normal ovulation rate of DM females at 6 weeks (Tables 3, 4 and 5). However, at all stages of

development, the number of implantation sites or decidua in DM females was lower than the number of normal CL ($P<0.05$).

Discussion

DM females generating oocytes lacking complex N- and O-glycans produce only one small litter from mating at 6 weeks before undergoing POF by 3 months of age (Williams *et al.* 2007, Williams & Stanley 2011). To determine the basis of the small litter size we investigated ovulation rate and embryo development. Ovulation rate was unexpectedly normal in the DM females in the 6-week group, but decreased dramatically by 9 weeks, consistent with our previous findings (Williams *et al.* 2007, Williams & Stanley 2011). However, since ovulation rate was normal in the 6-week group, further analyses were required to reveal the basis for the decrease in litter size. Surprisingly, the number of embryos at 3.5 dpc was around half the ovulation rate 3 days earlier, revealing a considerable loss of embryos surviving during blastogenesis. Furthermore, embryo development in those embryos that did survive in DM females was delayed resulting in delayed implantation.

When mated at 6 weeks of age, DM females produce only a single small litter (Williams *et al.* 2007) yet, on average, they ovulate normal numbers of eggs, although the number of eggs ovulated per female is highly variable. This is consistent with the presence of morphologically normal follicles in 6-week DM ovaries, although ovaries also contain some follicular abnormalities such as oocytes lacking cumulus cells or empty follicles (Williams & Stanley 2011). Therefore, the most likely explanation for the precipitous decline in

Table 3 Implantation sites, CL, eggs and non-implanted embryos in Control and DM females at 5.5 dpc.

	No. of females (age plugged; weeks)	Implantation sites	Eggs	Non-implanted embryos	CL		
					Total	Normal	Abnormal
Control	8 (6.5±0.1)	7.3±0.6 ^b	0	0	8.3±0.3 ^c	8.1±0.3	0.1±0.1 ^a
DM	4 (6.4±0.0)	1.3±0.4 ^{b,*,*,§}	1.3±0.7	1.3±0.9	11.8±1.0 ^{c,§}	7.8±1.1*	4.0±0.9 ^{a,§}

Mean±S.E.M. Values with the same superscript are significantly different: $P<0.05$ (^a, [#], * and [§]); $P<0.01$ (^b and ^c).

Table 4 Implantation sites and CL in control and DM females at 6.5 dpc.

	No. of females (age plugged; weeks)	Implantation sites	CL		
			Total	Normal	Abnormal
Control	4 (6.7±0.2)	7.8±0.3 ^a	8.3±0.6	8.0±0.4	0.3±0.3
DM	5 (6.3±0.1)	3.2±1.0 ^{a,*,#}	11.5±1.6 [#]	9.3±0.7*	2.3±0.9

Mean ± S.E.M. Values with the same superscript are significantly different: $P < 0.05$ ([#] and *); $P < 0.01$ (^a).

ovulation rate in DM females from 6 to 9 weeks is defective follicle development that gets more severe with age, explaining the lack of a second litter in these females (Williams *et al.* 2007, Williams & Stanley 2011).

Despite the fact that ovulation rate is normal at 6 weeks in DM females, only 45% of mated DM females contained embryos at 3.5 dpc. The very high proportion of mated DM females that lack embryos at 3.5 dpc is most likely contributing significantly to the 70% of DM females that fail to produce a litter (Williams *et al.* 2007). Furthermore, in those DM females that did contain embryos, the number was significantly lower than Controls. This was in stark contrast to just 3 days earlier when ovulation rate in DM females was normal and eggs were fertilised equivalently to those from Control females. These data indicate that the lack of complex *N*- and *O*-glycans during oocyte development results in a substantial decrease in embryo viability *in vivo* with an associated decrease in female fertility. In addition, around half of the morphologically normal embryos recovered from DM females at 3.5 dpc were morulae compared with ~11% in Controls, indicating a delay in development. Delayed development of embryos from DM females was not unexpected as embryos generated from oocytes lacking only complex *N*-glycans are also retarded (Shi *et al.* 2004). However, the development of embryos from DM females was supported *in vitro*, with the same proportion of embryos developing to the blastocyst stage as embryos from Controls. This demonstrates that *in vivo* development of embryos from DM females is affected by factors not present *in vitro*. Furthermore, these factors can either be intrinsic to the developing embryo or result from a modified environment within DM females as embryo development is affected by both oocyte quality and the environment in which the embryo develops (Mann & Lamming 2001, Van der Auwera & D'Hooghe 2001, Kidder & Vanderhyden 2010).

Focusing first on the embryo, as a consequence of the lack of *N*- and *O*-glycans, the ZP of DM oocytes is

thinner and more fragile (Williams *et al.* 2007, Williams & Stanley 2009). During the transit through the oviduct, embryos develop within the protective coat of the ZP undergoing cleavage, compaction and cavitation before developing into a blastocyst. Blastocysts hatch from the ZP and implant in the endometrial lining of the uterus (Nagy *et al.* 2003). We have observed that eggs and preimplantation embryos from DM females often lack a ZP which is most likely removed either during ovulation or whilst in transit from the oviduct to the uterus. Analysis of ZP-free eggs and embryos that were reintroduced to the oviduct illustrated a critical role for the ZP in preventing adherence of developing embryos to the oviduct (Modlinski 1970). These data indicate that the dramatic reduction in DM embryos flushed from 3.5 dpc as opposed to the number found at 0.5 dpc may be attributed to adherence of naked DM pre-blastocyst embryos to the oviduct. Furthermore, embryos with the ZP removed early in development have reduced developmental potential (Bronson & McLaren 1970). In addition, embryo lethality induced by shear stress (caused by the movement of fluid over the surface of cells) is hastened by ZP removal (Xie *et al.* 2006) which may also contribute to embryo loss in DM females. The ZP has also a role maintaining embryo morphology including the connections between blastomeres which can influence embryo development in mice (Suzuki *et al.* 1995, Ribas *et al.* 2005). Moreover, removal of the ZP in mice results in reduced DNA methylation at the two-cell and four-cell stages (Ribas *et al.* 2006). In addition, the thickness of the ZP or its variation has been used as a marker of successful implantation of human embryos (Garside *et al.* 1997, Gabrielsen *et al.* 2001, Shen *et al.* 2005). Based on these observations it is likely that the altered thickness or absence of a functional ZP in DM embryos has a role in the aberrant early embryogenesis in embryos from DM females that is not observed *in vitro*.

In vitro analysis revealed one unexpected occurrence of embryo fusion in embryos from DM female. Observations of *in vitro* cultured embryos revealed that

Table 5 Number of decidua, embryos and CL in Control and DM females at 9.5 dpc.

	No. of females (age plugged; weeks)	Unhealthy embryos and reabsorptions			CL		
		Decidua	Healthy embryos	Unhealthy embryos and reabsorptions	Total	Normal	Abnormal
Control	5 (6.6±0.3)	7.6±0.5 ^a	7.2±0.4 ^d	0.6±0.2	8.2±0.6 ^c	7.6±0.5	0.6±0.4 ^b
DM	6 (6.3±0.1)	3.0±0.6 ^{a,*,#}	2.0±0.3 ^d	1.0±0.5	11.3±0.6 ^{c, #}	8.3±0.5*	3.0±0.7 ^b

Mean ± S.E.M. Values with same superscript are significantly different: $P < 0.05$ (^a and ^b); $P < 0.01$ (^c, [#] and *); $P < 0.001$ (^d).

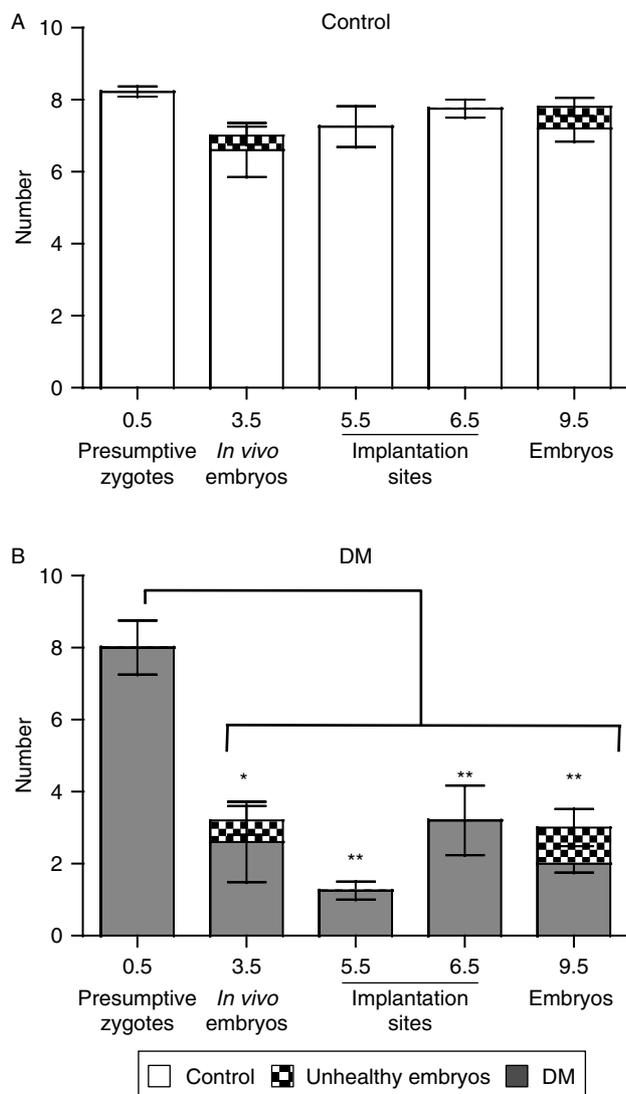


Figure 5 Numbers of eggs ovulated and pre- and post-implantation embryos in Control and DM females at four developmental stages. The graph shows the number of eggs ovulated (0.5 dpc; presumptive zygotes), preimplantation embryos (3.5 dpc) and implanted embryos (5.5, 6.5 and 9.5 dpc) in Control (A) and DM (B) females. * $P < 0.05$; ** $P < 0.01$, with regard to the zygotes collected at 0.5 dpc. Results are expressed as mean \pm S.E.M.

although five DM zygotes were present at the beginning of the culture, at the end, only four embryos were present including one abnormally large blastocyst (Fig. 3D). Fusion of two of the ZP-free embryos is the only logical conclusion in the absence of a degenerating embryo. The formation of natural chimeras has been described in mammals, including in humans (Strain *et al.* 1998). Although chimeric embryos can be generated by injection of stem cells into blastocysts, they can also be generated by culture of embryonic stem cells which aggregate with denuded morulae *in vitro* which develop normally upon transfer to a pseudo-pregnant recipient (Prather *et al.* 1989, Eakin & Hadjantonakis 2006).

Although our data indicate that this phenomena can occur in ZP-free DM embryos *in vitro*, its occurrence *in vivo* is unknown. However, it is unlikely to be involved in decreasing the number of embryos and pups as none of the embryos collected at 3.5 dpc were abnormally large.

The delay in *in vivo* embryo development in DM females, revealed by the high proportion of embryos at the morula stage, was reflected in a delay in implantation. At 5.5 dpc, only around a third of the number of the preimplantation embryos recovered at 3.5 dpc were implanted (Fig. 5). However, 1 day later at 6.5 dpc, the number of embryos implanted was equivalent to the number found at 3.5 dpc (Fig. 5). In addition, the number of deciduae found at 9.5 dpc in DM females was similar to the number of embryos recovered at 3.5 dpc and to the number of implantations at 6.5 dpc. However, not all deciduae in DM females contained normal healthy embryos with a number of healthy embryos equal to the number of morphologically normal embryos recovered before implantation. The difference between the number of healthy embryos found in this study and the litter size previously published (4.2 ± 2.7) (Williams *et al.* 2007), could be explained by the low sample size (only three of ten mated produced a litter) and the high variability of the fertility data in DM females. Recent studies reveal an active role of the embryo in regulating the expression of uterine genes involved in implantation and decidualization (Kashiwagi *et al.* 2007, Park & Dufort 2011). It is possible that embryos generated from oocytes lacking complex N- and O-glycans do not induce the appropriate expression of required genes for timely implantation. Implantation is a complex process, ovarian steroid-dependant and only possible during a limited period in which the uterus is receptive to the competent embryo. This process requires complex interactions between the blastocyst and the receptive uterus in many species including mice and humans (Simon *et al.* 2000, Dey *et al.* 2004). Interestingly, the delay in embryo development and implantation was not uniformly deleterious to subsequent development of embryos as some embryos that implanted at 6.5 dpc remained healthy at 9.5 dpc (Fig. 5). Transferring embryos at different stages of development (zygotes and eight-cell embryos) into the uterus of pseudo-pregnant mice revealed that the 'window of implantation' could be expanded (Ueda *et al.* 2003). This may explain why all the blastocysts and morulae found at 3.5 dpc in DM females are able to implant over an extended period.

In addition to modified embryo competence, the maternal endocrine environment may also have a role in modifying embryo development. Maternal ovarian steroids including estradiol and progesterone regulate embryo development and uterus receptivity to implantation (Dey *et al.* 2004, Mohamed *et al.* 2004, 2005). However, although the endocrine profile in DM females is modified by 3 months of age with mice exhibiting elevated FSH, and decreased Inhibin A and testosterone,

this is in association with a severe lack of ovarian follicles and ovarian dysfunction (Williams & Stanley 2011). Most importantly, estradiol and progesterone concentrations were normal in DM females at 3 months (Williams & Stanley 2011) and therefore, it is likely that estradiol and progesterone concentrations are also normal in the 6-week old mice used in these studies. We therefore conclude that modified maternal estradiol and progesterone are unlikely to be involved in modifying embryo development in DM females.

The increased number of CL in DM females was unexpected. We have previously shown in DM females that at 3 weeks follicle development is vastly normal; however, by 6 weeks, ovaries contained fewer follicles and more follicular abnormalities (Williams & Stanley 2011). CL in postpubertal females (6 weeks and 3 months) were not counted or measured in these analyses and therefore the increased number of CL was not revealed. Interestingly, the number of normal CL in DM females corresponds to the ovulation rate of DM (and Control females) at 6 weeks. Therefore, the abnormal CL may be present due to abnormal CL regression as DM females do not exhibit an increased ovulation rate. However, how oocyte-specific deletion of glycosyltransferases results in modified CL regression is intriguing and is under further investigation.

Female mice heterozygous for both *Mgat1* and *C1galt1* exhibit normal fertility (Williams *et al.* 2007), demonstrating that aberrant embryo development in DM females results from oocyte glycoproteins lacking complex O- and N-glycans during oocyte development and early embryogenesis before activation of the paternal genome. Furthermore, it is clear that activation of the paternal genome during preimplantation embryo development (De Vries *et al.* 2004) cannot rescue later development as the proportion of embryos dying from 6.5 to 9.5 dpc is considerably higher in DM females than in Controls.

In summary, our results reveal that a lack of complex N- and O-glycans during oocyte development impairs early embryo development and viability *in vivo*, which could lead to delayed implantation and reduced final litter size. Moreover, these data demonstrate that oocyte glycoproteins have a role in early embryo development and the interaction of the embryo with the maternal environment before implantation.

Materials 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 because the floxed alleles function as wild-type genes (Shi *et al.* 2004, Williams *et al.* 2007). Mice were kept under controlled conditions in

12 h light:12 h darkness cycles. All the experiments involving mice were done with permission from the Home Office and the Local Ethical Review Committee.

Timed mating

Control and DM female mice aged either 6 or 9 weeks were joined with fertile males (*C1galt1^{F/F}Mgat1^{F/F}±ZP3Cre*) for mating for up to a maximum of 2 weeks. Embryos generated from *C1galt1^{F/F}Mgat1^{F/F}* females will be *C1galt1^{F/F}Mgat1^{F/F}±ZP3Cre* and embryos generated from *C1galt1^{F/F}Mgat1^{F/F}:ZP3Cre* females will be *Mgat1^{del/F}C1galt1^{del/F}±ZP3Cre* with the deleted genes (del) originating from oocyte-specific deletion in the mother and the floxed genes from the sperm of the father. Those joined with males at 6 weeks are herein termed the 6-week group, and those joined with males at 9 weeks are herein termed the 9-week group. Females were examined in the morning daily for evidence of mating by the presence of a vaginal plug and if present, removed from the cage. Midday of the day a plug was visible was deemed 0.5 dpc.

Ovulated egg, presumptive zygote and embryo analysis

For collection of eggs and presumptive zygote, females joined with males at 6 and 9 weeks of age were killed at 0.5 dpc and oviducts were collected in warmed M2 medium (Sigma–Aldrich) and examined. Eggs collected from Control and DM females at 0.5 dpc were assumed to have been fertilised and were termed ‘presumptive zygotes’. If they were visible in the oviduct, the oviducts were ripped open to release the presumptive zygotes. Cumulus cells were disaggregated with 0.3 mg/ml of hyaluronidase (Sigma–Aldrich) in M2 media for up to 3 min and the number of presumptive zygotes was recorded. If no presumptive zygotes were visible, the oviducts were flushed with M2 media to collect them and the number was recorded.

In vivo preimplantation embryo development was analysed at 3.5 dpc from female mice joined with males at 6 weeks. Females were killed and the uterus and oviduct collected and placed in warmed M2 media. Embryos were flushed from the oviduct and uterus with warmed M2 media and classified as blastocysts, morulae, and arrested or fragmented embryos, based on morphology.

In vitro embryo development

Presumptive zygotes obtained as described above at 0.5 dpc from the 6-week female group were cultured for 4 days in 50 µl drops of KSOM media (Millipore, Billerica, MA, USA) supplemented with essential and non-essential amino acids (Gibco), covered with mineral oil (Sigma–Aldrich) and incubated at 37 °C in air with 5% CO₂. The fertilisation rate was determined after 24 h of culture by analysing the number that had developed into two-cell embryos. Development was analysed and images were collected using a Leica M125 microscope (Leica Microsystems, Germany) and MicroPublisher 5.0 RTV camera (Qimaging, London, UK) every 24 h during the 4-day culture period until 4.5 dpc. *In vitro* embryo culture was for 1 day longer than *in vivo* because embryo development *in vitro* takes longer than *in vivo* (Van der Auwera

& D'Hooghe 2001). Embryos were classified as either blastocysts, morulae, fragmented or arrested based on morphology.

Implantation analysis

Implantation was evaluated in females in the 6-week group at 5.5 and 6.5 dpc. To assist visualisation of implantation sites at 5.5 dpc, females were injected intravenously under anaesthesia with 100 µl of 1% Chicago Sky Blue (Sigma–Aldrich) in ddH₂O, and killed 5 min later. The uterine horns were removed and the number of implantation sites visible as blue dots recorded. Uterine horns were also flushed at 5.5 dpc to recover any non-implanted embryos. Implantation sites at 6.5 dpc were easily identified. At both time points, ovaries were dissected to determine the number of CL.

Post-implantation embryo development

Post-implantation embryo development was assessed in the 6-week group. Plugged females were killed at 9.5 dpc and the number of deciduae (each developing around an implanted embryo) determined. To analyse embryonic development, the deciduae were dissected, embryos extracted and morphology evaluated. Embryos classified as healthy had normal gross morphology and were alive determined by the presence of a beating heart. Ovaries were collected and dissected to determine the number of CL.

Statistical analysis

Results are presented as mean ± s.e.m. The Mann–Whitney *U* test was performed for all statistical analyses with the exception of the Pearson test, which was used to assess the correlation between the age and the number of eggs ovulated. All analyses were performed using Prism software version 4.0b (GraphPad Software, Inc., version 4.0b, 2004. La Jolla, CA, USA).

Declaration of interest

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

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References

- Bleil JD & Wassarman PM** 1980a Mammalian sperm–egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell* **20** 873–882. (doi:10.1016/0092-8674(80)90334-7)
- Bleil JD & Wassarman PM** 1980b Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Developmental Biology* **76** 185–202. (doi:10.1016/0012-1606(80)90371-1)
- Bronson RA & McLaren A** 1970 Transfer to the mouse oviduct of eggs with and without the zona pellucida. *Journal of Reproduction and Fertility* **22** 129–137. (doi:10.1530/jrf.0.0220129)
- De Vries WN, Evisikov AV, Haac BE, Fancher KS, Holbrook AE, Kemler R, Solter D & Knowles BB** 2004 Maternal β-catenin and E-cadherin in mouse development. *Development* **131** 4435–4445. (doi:10.1242/dev.01316)
- Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T & Wang H** 2004 Molecular cues to implantation. *Endocrine Reviews* **25** 341–373. (doi:10.1210/er.2003-0020)
- Diaz FJ, Wigglesworth K & Eppig JJ** 2007 Oocytes are required for the preantral granulosa cell to cumulus cell transition in mice. *Developmental Biology* **305** 300–311. (doi:10.1016/j.ydbio.2007.02.019)
- Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N & Matzuk MM** 1996 Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383** 531–535. (doi:10.1038/383531a0)
- van Duin M, Polman JE, De Breet IT, van Ginneken K, Bunschoten H, Grootenhuis A, Brindle J & Aitken RJ** 1994 Recombinant human zona pellucida protein ZP3 produced by chinese hamster ovary cells induces the human sperm acrosome reaction and promotes sperm–egg fusion. *Biology of Reproduction* **51** 607–617. (doi:10.1095/biolreprod51.4.607)
- Eakin GS & Hadjantonakis AK** 2006 Production of chimeras by aggregation of embryonic stem cells with diploid or tetraploid mouse embryos. *Nature Protocols* **1** 1145–1153. (doi:10.1038/nprot.2006.173)
- Gabrielsen A, Lindenberg S & Petersen K** 2001 The impact of the zona pellucida thickness variation of human embryos on pregnancy outcome in relation to suboptimal embryo development. A prospective randomized controlled study. *Human Reproduction* **16** 2166–2170. (doi:10.1093/humrep/16.10.2166)
- Garside WT, Loret de Mola JR, Bucci JA, Tureck RW & Heyner S** 1997 Sequential analysis of zona thickness during *in vitro* culture of human zygotes: correlation with embryo quality, age, and implantation. *Molecular Reproduction and Development* **47** 99–104. (doi:10.1002/(SICI)1098-2795(199705)47:1<99::AID-MRD13>3.0.CO;2-V)
- Gilchrist RB, Lane M & Thompson JG** 2008 Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Human Reproduction Update* **14** 159–177. (doi:10.1093/humupd/dmm040)
- Han L, Monne M, Okumura H, Schwend T, Cherry AL, Flot D, Matsuda T & Jovine L** 2010 Insights into egg coat assembly and egg–sperm interaction from the X-ray structure of full-length ZP3. *Cell* **143** 404–415. (doi:10.1016/j.cell.2010.09.041)
- Hutt KJ & Albertini DF** 2007 An oocentric view of folliculogenesis and embryogenesis. *Reproductive BioMedicine Online* **14** 758–764. (doi:10.1016/S1472-6483(10)60679-7)
- Ioffe E & Stanley P** 1994 Mice lacking *N*-acetylglucosaminyltransferase I activity die at mid-gestation, revealing an essential role for complex or hybrid *N*-linked carbohydrates. *PNAS* **91** 728–732. (doi:10.1073/pnas.91.2.728)
- Kashiwagi A, DiGirolamo CM, Kanda Y, Niikura Y, Esmon CT, Hansen TR, Shioda T & Pru JK** 2007 The postimplantation embryo differentially regulates endometrial gene expression and decidualization. *Endocrinology* **148** 4173–4184. (doi:10.1210/en.2007-0268)
- Kidder GM & Vanderhyden BC** 2010 Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence. *Canadian Journal of Physiology and Pharmacology* **88** 399–413. (doi:10.1139/Y10-009)
- Litscher ES, Williams Z & Wassarman PM** 2009 Zona pellucida glycoprotein ZP3 and fertilization in mammals. *Molecular Reproduction and Development* **76** 933–941. (doi:10.1002/mrd.21046)

- Mann GE & Lamming GE 2001 Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction* **121** 175–180. (doi:10.1530/rep.0.1210175)
- Matzuk MM, Burns KH, Viveiros MM & Eppig JJ 2002 Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science* **296** 2178–2180. (doi:10.1126/science.1071965)
- Metzler M, Gertz A, Sarkar M, Schachter H, Schrader JW & Marth JD 1994 Complex asparagine-linked oligosaccharides are required for morphogenic events during post-implantation development. *EMBO Journal* **13** 2056–2065.
- Modlinski JA 1970 The role of the zona pellucida in the development of mouse eggs *in vivo*. *Journal of Embryology and Experimental Morphology* **23** 539–547.
- Mohamed OA, Dufort D & Clarke HJ 2004 Expression and estradiol regulation of Wnt genes in the mouse blastocyst identify a candidate pathway for embryo-maternal signaling at implantation. *Biology of Reproduction* **71** 417–424. (doi:10.1095/biolreprod.103.025692)
- Mohamed OA, Jonnaert M, Labelle-Dumais C, Kuroda K, Clarke HJ & Dufort D 2005 Uterine Wnt/ β -catenin signaling is required for implantation. *PNAS* **102** 8579–8584. (doi:10.1073/pnas.0500612102)
- Nagy A, Gertsenstein M, Vintersten K & Behringer R 2003 *Manipulating the Mouse Embryo. A Laboratory Manual*. 3rd Edn. New York, NY, USA: Cold Spring Harbor Laboratory Press.
- Park CB & Dufort D 2011 Nodal expression in the uterus of the mouse is regulated by the embryo and correlates with implantation. *Biology of Reproduction* **84** 1103–1110. (doi:10.1095/biolreprod.110.087239)
- Philpott CC, Ringuette MJ & Dean J 1987 Oocyte-specific expression and developmental regulation of ZP3, the sperm receptor of the mouse zona pellucida. *Developmental Biology* **121** 568–575. (doi:10.1016/0012-1606(87)90192-8)
- Prather RS, Hagemann LJ & First NL 1989 Preimplantation mammalian aggregation and injection chimeras. *Gamete Research* **22** 233–247. (doi:10.1002/mrd.1120220210)
- Ribas R, Oback B, Ritchie W, Chebotareva T, Ferrier P, Clarke C, Taylor J, Gallagher EJ, Mauricio AC, Sousa M *et al.* 2005 Development of a zona-free method of nuclear transfer in the mouse. *Cloning and Stem Cells* **7** 126–138. (doi:10.1089/clo.2005.7.126)
- Ribas RC, Taylor JE, McCorquodale C, Mauricio AC, Sousa M & Wilmut I 2006 Effect of zona pellucida removal on DNA methylation in early mouse embryos. *Biology of Reproduction* **74** 307–313. (doi:10.1095/biolreprod.105.046284)
- Shen Y, Stalf T, Mehnert C, Eichenlaub-Ritter U & Tinneberg HR 2005 High magnitude of light retardation by the zona pellucida is associated with conception cycles. *Human Reproduction* **20** 1596–1606. (doi:10.1093/humrep/deh811)
- Shi S, Williams SA, Seppo A, Kurniawan H, Chen W, Ye Z, Marth JD & Stanley P 2004 Inactivation of the Mgat1 gene in oocytes impairs oogenesis, but embryos lacking complex and hybrid N-glycans develop and implant. *Molecular and Cellular Biology* **24** 9920–9929. (doi:10.1128/MCB.24.22.9920-9929.2004)
- Simon C, Martin JC & Pellicer A 2000 Paracrine regulators of implantation. *Baillière's Best Practice & Research. Clinical Obstetrics & Gynaecology* **14** 815–826. (doi:10.1053/beog.2000.0121)
- Strain L, Dean JC, Hamilton MP & Bonthron DT 1998 A true hermaphrodite chimera resulting from embryo amalgamation after *in vitro* fertilization. *New England Journal of Medicine* **338** 166–169. (doi:10.1056/NEJM199801153380305)
- Sugiura K, Su YQ, Li Q, Wigglesworth K, Matzuk MM & Eppig JJ 2010 Estrogen promotes the development of mouse cumulus cells in coordination with oocyte-derived GDF9 and BMP15. *Molecular Endocrinology* **24** 2303–2314. (doi:10.1210/me.2010-0260)
- Suzuki H, Togashi M, Adachi J & Toyoda Y 1995 Developmental ability of zona-free mouse embryos is influenced by cell association at the 4-cell stage. *Biology of Reproduction* **53** 78–83. (doi:10.1095/biolreprod53.1.78)
- Ueda O, Yorozu K, Kamada N, Jishage K, Kawase Y, Toyoda Y & Suzuki H 2003 Possible expansion of "Window of Implantation" in pseudopregnant mice: time of implantation of embryos at different stages of development transferred into the same recipient. *Biology of Reproduction* **69** 1085–1090. (doi:10.1095/biolreprod.103.017608)
- Van der Auwera I & D'Hooghe T 2001 Superovulation of female mice delays embryonic and fetal development. *Human Reproduction* **16** 1237–1243. (doi:10.1093/humrep/16.6.1237)
- Wassarman PM 2005 Contribution of mouse egg zona pellucida glycoproteins to gamete recognition during fertilization. *Journal of Cellular Physiology* **204** 388–391. (doi:10.1002/jcp.20389)
- Williams SA & Stanley P 2008 Mouse fertility is enhanced by oocyte-specific loss of core 1-derived O-glycans. *FASEB Journal* **22** 2273–2284. (doi:10.1096/fj.07-101709)
- Williams SA & 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. (doi:10.1530/REP-07-0469)
- Williams SA & Stanley P 2011 Premature ovarian failure in mice with oocytes lacking core 1-derived O-glycans and complex N-glycans. *Endocrinology* **152** 1057–1066. (doi:10.1210/en.2010-0917)
- Williams SA, Xia L, Cummings RD, McEver RP & Stanley P 2007 Fertilization in mouse does not require terminal galactose or N-acetylglucosamine on the zona pellucida glycans. *Journal of Cell Science* **120** 1341–1349. (doi:10.1242/jcs.004291)
- Xia L, Ju T, Westmuckett A, An G, Ivanciu L, McDaniel JM, Lupu F, Cummings RD & McEver RP 2004 Defective angiogenesis and fatal embryonic hemorrhage in mice lacking core 1-derived O-glycans. *Journal of Cell Biology* **164** 451–459. (doi:10.1083/jcb.200311112)
- Xie Y, Wang F, Zhong W, Puscheck E, Shen H & Rappolee DA 2006 Shear stress induces preimplantation embryo death that is delayed by the zona pellucida and associated with stress-activated protein kinase-mediated apoptosis. *Biology of Reproduction* **75** 45–55. (doi:10.1095/biolreprod.105.049791)

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