Obesity alters the ovarian DNA damage response and apoptotic proteins

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Abstract

In the overweight or obese female, reproductive complications include poor oocyte quality, decreased fecundity, gestational diabetes, and higher risk of reproductive cancers. Using lean and hyperphagia-induced obese female mice aged 10 weeks, we determined that the ovary from obese female mice had elevated (P < 0.10) levels of ataxia telangiectasia mutated (ATM) protein in oocytes of both small and large follicles. Phosphorylated ATM at serine 1981 was greater (P < 0.05) in large relative to small follicles with no additional impact of obesity. Obesity increased (P < 0.05) γH2AX in small follicles in obese relative to lean ovaries, while large follicles of both lean and obese mice had detectable levels of γH2AX. Cleaved caspase 3 was reduced (P < 0.05) in the small follicles of obese relative to lean ovaries. In large follicles of lean mice, cleaved caspase 3 was increased in large compared to small follicles (P < 0.05) but this pattern was absent in obese mice. Breast cancer type 1 susceptibility protein (BRCA1) or the phosphorylated BRCA1 proteins were observably altered by obesity. These data demonstrate that markers of DNA damage and repair have a follicle-dependent stage location and that obesity alters ATM and cleaved caspase 3 in a follicular stage dependent manner.

Introduction

Obesity is a preventable metabolic syndrome, associated with several interrelated disorders such as insulin resistance/diabetes (Colditz et al. 1995), cardiovascular disease (Lavie et al. 2009), dyslipidemia (Kang et al. 2016), chronic inflammation (Xu et al. 2003), and cancer (Cozzo et al. 2017). The prevalence of obesity in the population continues to rise, with an estimated 40% of adults and 19% of youth being obese/overweight in the United States, though these rates are higher for some ethnic groups (Hales 2017). A decline in reproductive potential is associated with obesity, and reproductive complications include poor oocyte quality (Brewer & Balen 2010), polycystic ovary syndrome (PCOS) (Pasquali & Casimirri 1993), decreased fecundity (Gesink Law et al. 2007), impaired pregnancy success using assisted reproductive technologies (Metwally et al. 2007), gestational diabetes (Chu et al. 2007), and increased risk of offspring health problems (Sheffield et al. 2002).

The ovary is the source of the female germ cell, the oocyte, and hormones necessary for female growth and development. Throughout the female lifespan, a finite pool of primordial germ cells will remain in diplotene stage of meiosis until they are primed for maturation toward ovulation and subsequent fertilization, or they undergo atresia (Hirshfield 1991). Once the primordial follicle pool is depleted, ovarian senescence occurs (Hirshfield 1991). Depletion of follicles prior to age 40 is termed primary ovarian insufficiency with often unknown etiology (Coulam et al. 1986). Cessation of or dysfunctional ovarian activity predisposes women toward at heightened risk for development of gynecological cancers (Nagle et al. 2015), osteoporosis (Compston 2001), cardiovascular disease (Mosca et al. 1997), and Alzheimer’s disease (Scheyer et al. 2018).

Accumulation of DNA damage is considered a contributing factor to obesity-related disorders due to the chronic low-grade inflammation and constant production of reactive oxygen species (ROS) that may induce endogenous DNA damage throughout the body (Cerda et al. 2014, Włodarczyk et al. 2018, Zaki et al. 2018). Obesity is also associated with ovarian DNA damage (Ganesan et al. 2014, 2015, 2017) and increased granulosa cell apoptosis (Walzem & Chen 2014). The cellular DNA damage response (DDR) prevents genomic damage by either repairing the damage or triggering the cell toward apoptosis if beyond repair. Central metabolic disturbances during obesity have adverse impacts on the DDR (Himbert et al. 2017, Azzara et al. 2016). We have previously demonstrated that progressive obesity alters ovarian folliculogenesis and inflammation (Nteeba et al. 2014b), the insulin responsive phosphatidylinositol 3-kinase (PI3K) pathway (Nteeba et al. 2013), steroid hormone biosynthesis (Nteeba et al. 2017), causes basal DNA damage and affects the response to genotoxicant

Ataxia telangiectasia mutated (ATM) protein is a key DDR initiator, sensing DNA damage and transducing downstream targets including breast cancer type 1 susceptibility protein (BRCA1) for DNA repair (Gatei et al. 2000); serine/threonine-protein kinase Chk2 (CHK2) for cell cycle arrest (Falck et al. 2001); p53 for apoptosis (Meulmeester et al. 2005) and RAC-alpha serine/threonine-protein kinase (AKT) for cell survival (Liu et al. 2014). Despite hyperphagia-induced obese mice having an increase in ATM protein in response to a genotoxic chemical, we have previously demonstrated lack of an appropriate response in the proteins downstream of ATM (Ganesan et al. 2014, 2017). This finding provided the rationale for the hypothesis that ATM-mediated DDR and follicular atresia proteins are altered by obesity with impacts of follicle maturation stage. This study employed a mouse model of hyperphagia-induced obesity (Duhl et al. 1994, Michaud et al. 1994, Klebig et al. 1995) in which increased feed intake is a phenotype (Koegler et al. 1999, Bazhan et al. 2013). We chose this model based upon our previous characterization of the impact of obesity on a variety of ovarian endpoints, including DDR protein abundance (Nteeba et al. 2013, 2014a, b, 2017, Ganesan et al. 2014, 2017). In addition, the mice were 10 weeks of age, an intermediate point when there is no difference in body weight at 6 weeks of age and when the hyperphagia mice are heavier than their lean controls at 12 weeks of age (Yang et al. 2012). This approach facilitated the investigation of initiating events to induce an aberrant ovarian DDR.

Materials and methods

Animal procedure and tissue collection

All experiments were performed according to regulatory guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University. Female WT normal non-agouti (a/a; n=10; designated lean) and agouti lethal yellow (KK. Cg-Ay/J mice; n=10; designated obese) were purchased from Jackson laboratory (Bar Harbor) and maintained in an animal facility at Iowa State University under controlled room temperature (21°C–22°C) and lighting (12h light : 12h darkness) with access to food and water ad libitum. At 10 weeks of age, mice were euthanized in the proestrus phase of the estrous cycle and ovaries were fixed in 4% paraformaldehyde overnight at 4°C for histological analysis.

Estrous cycle monitoring

The estrous cycle was monitored by performing daily vaginal cytological analysis in the morning for 7 days to ensure mice were at the same stage of the estrous cycle upon euthanasia. Fresh, wet vaginal smears were collected by pipetting saline into the vagina, smearing onto histology slides and examining with a Nikon Optiphot microscope using a 10x objective. Classification of estrous cycle stages was determined as previously described (Byers et al. 2012). Briefly, pro-estrus was characterized by small, round nucleated epithelial cells, some cornified epithelial cells, and little to no leukocytes. Estrus was characterized by several cornified epithelial cells containing pyknotic nuclei. The presence of cornified cells and leukocytes indicated metestrus. In diestrus, nucleated epithelial cells reappeared in the vaginal smears in addition to the presence of polymorphonuclear leukocytes. Obese mice who displayed persistent diestrus (n=4) were subsequently excluded from further analysis.

Histology

After fixation, ovaries were passed through a 10, 20%/0.1M PBS gradient for 1–3 h each at room temperature, followed by 30% sucrose/0.1 M PBS overnight at 4°C and embedded in optimal cutting temperature (OCT; Fisher healthcare) compound before being flash frozen on dry ice. Ovaries were serially sectioned at 7 µm with a maximum of three sections per slide.

Immunofluorescence staining

Slides were warmed briefly on a 37°C slide warmer and tissue sections were encircled with a histology pap pen (Vector laboratories) to maintain staining solutions concentrated on the tissue during processing. Tissue was dehydrated and permeabilized in 0.1 M PBS with 0.1% Tween 20 (PBSTw) for 20 min, followed by blocking (0.1 M PBS/1% BSA/1% DMSO/5% goat serum) for 60 min at room temperature. Primary antibodies (Supplementary Table 1, see section on supplementary materials given at the end of this article) were diluted in fresh blocking solution, applied to the tissue sections and incubated in a humidified box at 4°C overnight. For antigen retrieval, 1% sodium dodecyl sulfate (SDS; Sigma Aldrich) was applied for 5 min after the rehydration step, followed by three washes of PBSTw for 5 min each and addition of blocking solution as described above. After primary antibody incubation, slides were washed three times in PBSTw for 10 min per wash. The appropriate secondary antibody (Supplementary Table 1) was added to fresh blocking solution, applied to tissue sections and incubated at room temperature for 60 min, followed by four washes in PBSTw for 10 min per wash. Slides were air dried, followed by addition of Vectashield with DAPI (H1200, Vector Labs) and stored overnight at 4°C. Negative technical controls to confirm specificity were performed using secondary antibodies alone (Supplementary Fig. 1). Images were captured on a Zeiss LSM700 confocal microscope equipped with an AxioCam MRc5 using a 5 or 20x objective.

Quantification of protein abundance

For quantification of ATM (n=6 ovaries per treatment; six sections per ovary), and pATM (n=6 ovaries per treatment; six sections per ovary), the threshold percentage of the total image area was limited to the oocyte using ImageJ software (Schneider et al. 2012). For γH2AX (six ovaries per treatment; six sections per ovary) and cleaved CASP3 (six ovaries per
treatment; six sections per ovary), immunopositive cells were manually counted in the granulosa cells of primary, secondary and antral follicles using the cell counter module of ImageJ software (Schneider et al. 2012). Briefly, primary follicles were identified as containing an oocyte surrounded by a single layer of cuboidal granulosa cells; secondary follicles contained an oocyte surrounded by multiple layers of granulosa cells and antral follicles were identified by the presence of an oocyte enclosed by several layers of granulosa cells and concurrent presence of a fluid-filled antral space. For comparisons, small follicles are considered primary and early secondary follicles, while large follicles are considered late secondary follicles and antral follicles.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 7.0 unpaired t-test function. A P-value ≤ 0.05 was considered statistically significant and a trend for a statistical difference was considered at P < 0.1. Values are expressed as mean ± s.e.m.

**Results**

**Body weight is increased in female lethal yellow mice at 10 weeks of age**

Body weight was higher in agouti lethal yellow mice (henceforth designated obese) relative to WT controls (henceforth designated lean; P < 0.05; Fig. 1A) at the onset of the experiment. The weights of the heart (Fig. 1B), spleen (Fig. 1D) and the uterus (Fig. 1F) from the obese females were reduced (P < 0.05) in comparison to lean controls, while the liver weight was increased (P < 0.05; Fig. 1C) in obese females. There were no differences (P > 0.05) in the weights of the kidneys (Fig. 1E) or ovaries (Fig. 1G) between lean and obese mice.

**Obesity increases ovarian ATM protein abundance**

In order to establish the basal level of ovarian DDR proteins due to progressive obesity, both basal and phosphorylated levels of ATM protein were examined. Basal levels of ATM immunofluorescence were localized in the oocyte of all follicular stages with no difference in localization patterns between lean or obese mice (Fig. 2A, B, C and D). ATM oocyte immunoreactivity was elevated (P < 0.05) in both small and large follicles in the obese (Fig. 2I and K) relative to the lean (Fig. 2J and L) ovary.

Phosphorylation of ATM at serine 1981 was present in 1981 was present in the oocyte and granulosa cells of all follicular stages (Fig. 2E, F, G and H). There were greater levels of pATM\textsuperscript{Ser1981} in small follicles of the obese, relative to lean ovary (Fig. 2M), but this was not the case in large follicles (Fig. 2O). In the lean ovary, pATM\textsuperscript{Ser1981} was higher in large relative to small follicles (Fig. 2N) but this pattern was not observed in the obese ovary (Fig. 2P).

**Obesity increases ovarian γH2AX**

To determine if obesity leads to ovarian DNA double strand breaks (DSB), ovary sections were analyzed for localization of γH2AX and positive foci were quantified (Fig. 3). The number of ovarian cells (granulosa cells and/oocytes) that contained positive γH2AX foci tended (P < 0.1) to be greater in total (Fig. 3E), as well as in both small (P < 0.05) and large (P < 0.1) follicles (Fig. 3F and G) in the obese relative to lean ovary. In both the lean and obese ovaries, larger follicles had more (P < 0.05) γH2AX positive foci than in smaller follicles (Fig. 3H and I).

**Apoptosis is not elevated in the obese ovary though a shift between follicle sizes occurs**

To determine if there is an increase in follicular atresia in the obese ovary, tissue sections were analyzed for cleaved CASP3 via immunofluorescence (Fig. 4A, B, C and D) and positive foci were quantified. The number of
total cleaved CASP3 positive granulosa cells were not different ($P > 0.05$) between the lean and obese mice (Fig. 4E). However, there were more ($P < 0.05$) CASP3 positive foci in smaller follicles of the lean versus the obese ovary (Fig. 4F) and more ($P < 0.05$) positive foci in the larger follicles relative to the smaller follicles in the obese ovary (Fig. 4I). There were no differences ($P > 0.05$) in numbers of positive CASP3 cells between the large follicles in lean and obese or between the follicle sizes in the lean ovaries alone (Fig. 4G and H).

Figure 2 Impacts of obesity on ATM and pATMser1981. ATM protein positive immunofluorescent staining in (A) small follicles of lean mice; (B) large follicles of lean mice; (C) small follicles of obese mice; (D) large follicles of obese mice, and pATMser1981; (E) small follicles of lean mice; (F) large follicles of lean mice; (G) small follicles of obese mice; (H) large follicles of obese mice. Green stain indicates ATM and red stain indicates pATMser1981 while cellular DNA is stained in blue; scale bar = 50 μm (25 μm for insets). Asterisk indicates oocyte. Bar charts present mean fluorescence intensity ± s.e.m. of ATM in (I) small follicles; (J) lean small/large follicles; (K) large follicles; (L) obese small/large follicles and of pATMser1981; (M) small follicles; (N) lean small/large follicles; (O) large follicles; (P) obese small/large follicles *$P < 0.05$, †$P < 0.1$.

Figure 3 Effect of progressive obesity on ovarian γH2AX abundance. Positive immunolocalization for γH2AX in (A) small follicles of lean mice; (B) large follicles of lean mice; (C) small follicles of obese mice and (D) large follicles of obese mice. Green punctate stain indicates γH2AX while cellular DNA is stained in blue; scale bar = 50 μm (25 μm for insets). Asterisk indicates oocyte. Bar charts present mean number of positive γH2AX foci ± s.e.m. in (E) all follicles; (F) small follicles; (G) large follicles; (H) lean small/large follicles; (I) obese small/large follicles; *$P < 0.05$, †$P < 0.1$.
Ovarian BRCA1 levels are not impacted by obesity

BRCA1 protein abundance was detected in oocytes of all follicle stages, as well as theca cells, stromal cells, and there was some modest staining in the granulosa cells of larger follicles (Fig. 5A, B, C and D). Staining intensity visibly increased in the theca cells as follicle size increased. There was no observable effect of obesity on ovarian BRCA1 protein.

Localization of pBRCA1<sub>Ser1423</sub> in the ovary was observed in the granulosa cells and oocyte nucleus (Fig. 5E, F, G and H). Similarly, BRCA1<sub>Ser1524</sub> immunoreactivity was localized to granulosa cells and the cytoplasm of the oocyte (Fig. 5I, J, K and L). Obesity did not observably alter phosphorylation of pBRCA1<sub>Ser1423</sub> or BRCA1<sub>Ser1524</sub>.

Discussion

The impacts of obesity on health have been well documented, though the percentage of the population, including both adults and children, affected by obesity and obesity-related disorders continues to rise. Reproductive outcomes for obese or overweight women include impaired fertility due to poor oocyte quality (Metwally et al. 2007), disturbances in the menstrual cycle (Mustaqeem et al. 2015), PCOS (Dravecka et al. 2003), and heightened risk of developing ovarian cancer (Olsen et al. 2013). The impacts of obesity may also not be limited to the mother, as offspring exposed to maternal obesity in utero have increased risk for neural tube defects (Huang et al. 2017), glucose intolerance (Hanafi et al. 2016), intrauterine growth restriction (IUGR) (Radulescu et al. 2013), and increased circulating cholesterol and body fat (Desai et al. 2014). Female offspring experiencing the metabolic effects of obesity in utero also exhibit a diminution of the ovarian follicular reserve (Aiken et al. 2016a, b, Clark et al. 2019) and decreases in ovarian vascularity (Chan et al. 2015).

The agouti lethal yellow mouse (KK.Cg-Ay/J) phenotype arises from a mutation in the normal WT non-agouti (a/a) background that results in ectopic agouti expression (Duhl et al. 1994, Michaud et al. 1994, Klebig et al. 1995). Within the hypothalamus, this increased agouti abundance causes hyperphagia due to melanocyte stimulating hormone receptor inhibition and subsequent dysregulation of alpha-melanocyte-stimulating hormone and cocaine- and amphetamine-regulated transcript (Lu et al. 1994). At 6 weeks of age, there is no difference in body weight or circulating insulin level between the lethal yellow agouti mice and their lean counterparts, with an observable increase in both body weight and insulin at 12 weeks of age (Yang et al. 2012). We have demonstrated using this model that hyperphagia-induced obese mice have basal levels of DNA damage in the ovary and a blunted DDR after genotoxicant exposure (Ganesan et al. 2014, 2017, Nteeba et al. 2014a, 2017). Importantly, those observations were made in mice that were 14 weeks of age and older. This study elected to use mice that were younger, 10 weeks of age, in order to avoid the confounding effect of reduced primordial follicle number which we previously noted from 12 weeks of age onwards (Nteeba et al. 2014b) and to facilitate observance of initiating events within the ovary that alter the DDR in the ovary of an obese female.

ATM is first activated by the MRE11, RAD50, and NBS1 (MRN) complex and stabilized at the location of the DNA DSBs by autophosphorylation at serine 1981 (So et al. 2009). Ablation of this autophosphorylation site results in cessation of ATM’s ability to phosphorylate any downstream targets (So et al. 2009). Further, mutations
in the Atm gene elicit an inadequate response to DNA damage, increasing both radiation sensitivity and cancer predisposition as a result of faulty DNA repair (Boder & Sedgwick 1958). We discovered more basal ATM in the ovary of an obese female, though no measurable differences in the amount of total phosphorylated ATM$^{\text{Ser1981}}$ were observed. Despite the lack of an effect on total pATM$^{\text{Ser1981}}$, pATM$^{\text{Ser1981}}$ abundance was elevated in smaller compared to larger follicles in the obese mice, suggesting both dysfunction in ATM phosphorylation in a follicle maturation stage dependent manner. This is consistent with our previous findings that ATM acts as a sensor for ovarian DNA damage (Ganesan et al. 2014, 2017, Ganesan & Keating 2015, 2016), and that there is a disconnect between elevated ATM and the subsequent activation of ATM downstream proteins in the ovary of an obese female (Ganesan et al. 2014, 2017).

The presence of γH2AX is considered the gold standard for double strand break localization (Fernandez-Capetillo et al. 2004), and H2AX is activated upon phosphorylation by ATM at the DNA break site (Meier et al. 2007, Savic et al. 2009). In support of our previous findings of increased ATM and γH2AX in the obese ovary both basally and in response to a genotoxicant exposure (Ganesan et al. 2014, 2017), there was increased abundance of γH2AX positive foci in the ovaries from the obese mouse in both small and large follicles. Despite a follicular stage dependent pattern of elevated ATM and follicle-stage increased pATM$^{\text{Ser1981}}$ in the obese ovaries, γH2AX was increased in all stages analyzed due to obesity. This could indicate a disconnect between ATM/ pATM$^{\text{Ser1981}}$ and γH2AX in the obese ovary.

A higher BMI contributes to the development of DNA lesions (Wlodarczyk et al. 2018), but the origins of the elevated amounts of DNA DSBs remain to be elucidated. It is presumable that these lesions are occurring due to the increased inflammation and enhanced ROS...
production that are present during obesity (Igosheva et al. 2010, Donmez-Altuntas et al. 2014, Setayesh et al. 2018). Proinflammatory cytokines TNFα, interleukin family members, and NF-kB are implicated in ovarian pathologies such as endometriosis (Carlberg et al. 2000), ovarian hyperstimulation (Ma et al. 2010), and cancer (DiDonato et al. 2012). Increased amounts of DNA damage in the ovary, particularly in the oocyte, may impair oocyte developmental competence and result in aberrant implantation or trophoblast invasion (Robker 2008). Obesity also disrupts ovarian function via the alteration of the estrous cycle in rodents (Nteeba et al. 2014b, Bazzano et al. 2015), and depleting the primordial follicle pool (Nteeba et al. 2014b, Wang et al. 2014) concurrent with the increase of follicle activation and follicular atresia (Nteeba et al. 2014b, Wang et al. 2014, Bazzano et al. 2015, Skaznik-Wikiel et al. 2016). Thus, our findings recapitulate our previous observations (Ganesan et al. 2014, 2017) and support that basal DNA damage is present in the ovary of obese females.

If the DNA damage within a cell cannot be repaired, the cell may be shunted toward apoptosis. Apoptosis is coordinated by the activation of caspases, which then proteolytically process various cellular targets through the amplification of apoptotic signals (Cohen 1997). Caspase 3 (CASP3) is an apoptotic protease involved in ovarian granulosa (Matikainen et al. 2001) and luteal (Carambula et al. 2002) cell death, though is not required for oocyte death (Matikainen et al. 2001). Further, increased granulosa cell apoptosis has been reported with obesity (Walzem & Chen 2014). We and others have shown a dramatic increase in ovarian CASP3 positive cells after a genotoxicant exposure (Plowchalk & Mattison 1991, Ganesan & Keating 2015, Nguyen et al. 2018, Clark & Keating 2019, Luan et al. 2019). There was no increase in total ovarian CASP3 positive foci due to obesity, however, an interesting shift occurred in that the obese ovary had less CASP3 in small compared to large follicles, compared to the lean ovary in which the pattern was switched. Thus, similar to the observations with ATMSer1981, there may be follicle type-specific alterations due to obesity that could alter fertility in females. The accumulation of DNA DSBs in a transcriptionally quiescent cell like the meiotically arrested oocyte can either induce oocyte death or stimulate DNA repair in order to recover oocyte integrity and promote survival (Carroll & Marangos 2013, Collins & Jones 2016). It is also possible that basal DNA damage isn’t being repaired adequately, and that the damaged follicles are not being shunted toward demise, contributing to poor oocyte quality. This finding is consistent with our findings of reduced CASP3 and cytochrome C in Atm−/− mice exposed to the genotoxicant phosphoramid mustard in which ATM deficiency resulted in fewer ovarian follicles being depleted by the toxicant exposure (Clark & Keating 2019), supporting that ATM plays a role in mediating follicular atresia.

Another critical protein required for efficient DNA repair is BRCA1. Phosphorylation of BRCA1 at Ser1423 and Ser1524 is ATM-dependent in the event of DNA damage (Cortez et al. 1999). Women with a mutated allele of the Brca1 gene have increased risk of tumor development, specifically breast and ovarian cancers, as a result of defective DNA repair (Robson et al. 1998). We have previously demonstrated that progressive obesity reduced ovarian BRCA1 protein in mice aged 18 and 24 weeks (Ganesan et al. 2014, 2017). At the earlier stage of obesity examined in this study (10 weeks of age), there were no observable differences in basal BRCA1 abundance or in the amount of phosphorylated BRCA1Ser1423 or BRCA1Ser1524 relative to lean ovaries. Interestingly, DNA repair gene and protein activity in the ovary decrease with age (Titus et al. 2013, Govindaraj et al. 2015), emphasizing the importance of the DDR in ovarian ageing and fertility. Thus, the obesity-induced decline in BRCA1 that we previously noted may be influenced by either attainment of a certain threshold of obesity or by ovarian age.

Conclusions

Taken together, this study confirmed that there is elevated basal DNA damage in the ovaries of obese mice. In addition, the data confirms greater ATM protein abundance in large compared to small follicles (both lean and obese) and that there is dysfunction in ATM phosphorylation due to obesity. Further, we also discovered a reduction in CASP3 protein in small follicles in the obese relative to lean females. The model used eliminates the confounding effects of different diets since the obese mice overeat the same diet as the lean mice. We did not note any effect on BRCA1 at this stage of obesity, potentially suggesting that there is either a threshold of obesity that must be attained before reduced BRCA1 is noted, or that ovarian aging coupled with obesity has an additive effect on BRCA1 abundance. While obesity is not a complete barrier to healthy fertility, the evidence that obesity has negative effects on ovarian function and oocyte quality is unequivocal. Estimation of ovarian DNA damage and the resulting alterations in DDR during obesity could potentially aid in diagnosis and prevention of obesity-related reproductive disorders and consideration of DDR-initiating proteins as markers of ovarian health have merit.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/REP-20-0070.
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.

Funding

This work was funded by the Bailey Career Development Award from Iowa State University (A F K).

Author contribution statement

K L C and A F K contributed to experimental conception and design. K L C and C M R performed the experiments and K L C analyzed the data. K L C wrote the first draft of the paper. All authors reviewed and approved the final manuscript.

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Reproduction (2020) 160 751–760

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