REPRODUCTION
RESEARCH

Ablation of TGFBR3 (betaglycan) in oocytes does not affect fertility in female mice

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Abstract

Ovarian follicle development is regulated by locally produced TGFβ superfamily members. The TGFβ type III receptor (TGFBR3, or betaglycan), which regulates the actions of diverse TGFβ ligands, including inhibins, is expressed in different ovarian cell types. However, its functional roles in the ovary have not been investigated *in vivo*. Here, we ablated *Tgfbr3* in murine oocytes using the Cre-loxP system. Oocyte-specific *Tgfbr3* knockout (cKO) females were fertile, producing litters of similar size and frequency as controls. Their ovarian weights and histology were also normal. Though we confirmed efficient recombination of the floxed alleles, we did not detect *Tgfbr3* mRNA in purified oocytes from superovulated cKO or control mice. These results challenge earlier observations of betaglycan protein expression in this cell type. Regardless, *Tgfbr3* in the murine oocyte is clearly dispensable for female fertility.

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Introduction

Mammalian ovarian folliculogenesis is dependent on multiple endocrine and intra-ovarian ligands and their downstream signaling pathways. Whereas the pituitary hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), play well-known roles in follicle development and hormone production, proper growth and differentiation also require bidirectional paracrine signaling between germ and somatic cells (Matzuk *et al.* 2002, Gilchrist *et al.* 2004, Kidder & Vanderhyden 2010). Indeed, disruption of the cross-talk between the oocyte and surrounding granulosa cells leads to failed folliculogenesis and infertility (Matzuk *et al.* 2002, Gilchrist *et al.* 2004, Kidder & Vanderhyden 2010).

Communication between the oocyte and somatic cells is highly dependent on ligands in the transforming growth factor beta (TGFβ) superfamily, including growth differentiation factor 9 (GDF9) (Massague 1998, Juengel & McNatty 2005, Myers & Pangas 2010, Knight *et al.* 2012). TGFβ ligands signal through complexes of type I and II serine-threonine kinase receptors that activate the homolog of *Drosophila* mothers against decapentaplegic (SMAD) family of signaling proteins to regulate gene expression (Massague 1998, Attisano & Wrana 2002). In addition, some TGFβ superfamily ligands, such as the TGFβs and inhibins, rely on so-called type III receptors to mediate their actions (Massague 1998).

The TGFβ type III receptor (TGFBR3, also known as betaglycan), which lacks kinase activity, binds the TGFβs and facilitates their interaction with the type II receptor, TGFBR2 (Bilandzic & Stenvers 2011). In addition to binding TGFβs, TGFBR3 also functions as an inhibin co-receptor (Lewis *et al.* 2000, Chapman *et al.* 2002, Wiater *et al.* 2009, Li *et al.* 2018). Inhibins A and B are heterodimers of the inhibin α subunit disulfide-linked to either the inhibin βA (inhibin A) or βB (inhibin B) subunits. Homo- and heterodimers of the inhibin β subunits form the activins (Ling *et al.* 1986*a*, Ling *et al.* 1986*b*). According to *in vitro* models, inhibins A and B antagonize the actions of activins by forming ternary complexes with activin type II receptors and TGFBR3. This sequesters the type II receptors, inhibiting activin binding and signaling (Lewis *et al.* 2000, Chapman *et al.* 2002). Inhibins can similarly inhibit BMP signaling by sequestering the relevant type II receptors (ACVR2A, ACVR2B, and the BMP type II receptor, BMPR2) in the presence of TGFBR3 (Wiater & Vale 2003, Vale *et al.* 2004, Farnworth *et al.* 2006). TGFBR3 can also potentiate the actions of some BMPs (Bilandzic & Stenvers 2011). Thus, TGFBR3 promotes or inhibits the signaling of several TGFβ superfamily ligands in a context-specific manner.

TGFBR3 protein and *Tgfbr3* mRNA have been detected in murine oocytes, granulosa cells, and theca cells, but the protein's roles therein have not yet been determined *in vivo* (Lewis *et al.* 2000, Drummond *et al.* 2002, MacConell *et al.* 2002). In theca cell cultures, inhibin A upregulates androgen production and antagonizes BMP4, 6, 7, and activin A signalling, perhaps via TGFBR3 (Hillier *et al.* 1991, Glister *et al.*

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2010). Complexes of inhibin A bound to TGFBR3 and ACVR2A can be immunoprecipitated from KK-1 cells (a murine granulosa cell line), suggesting that TGFBR3 may mediate inhibin actions in granulosa cells (Lewis *et al.* 2000). In cows, inhibin A inhibits oocyte maturation and developmental competence (O *et al.* 1989, Silva *et al.* 1999). Activin A, in contrast, has the opposite effects in both cows and rodents (Sadatsuki *et al.* 1993, Silva & Knight 1998). Whether inhibins act via TGFBR3 in oocytes or somatic cells to produce these effects is unclear. Here, we generated oocyte-specific *Tgfbr3* knockout mice to investigate potential roles of the TGFBR3 protein in germ cell function and female fertility.

Materials and methods

Generation of conditional knockout mice

Tgfbr3$^{fx/fx}$ mice (on a mixed C57BL6 and 129SvEv background) were previously described (Li *et al.* 2018). *Tgfbr3*$^{fx/fx}$ females were crossed with *Gdf9*-iCre males (011062; The Jackson Laboratory) to generate *Tgfbr3*$^{fx/+}$;*Gdf9*-iCre/+ progeny (Lan *et al.* 2004). *Tgfbr3*$^{fx/+}$;*Gdf9*-iCre/+ males were then crossed with *Tgfbr3*$^{fx/fx}$ females to obtain *Tgfbr3*$^{fx/fx}$;*Gdf9*-iCre/+ offspring. Finally, *Tgfbr3*$^{fx/fx}$;*Gdf9*-iCre/+ males were crossed with *Tgfbr3*$^{fx/fx}$ females to generate *Tgfbr3*$^{fx/fx}$ (control) and conditional knockout *Tgfbr3*$^{fx/fx}$;*Gdf9*-iCre/+ (cKO) littermates. As *Gdf9*-iCre is expressed in female (but not male) germ cells, this allele was contributed by the male in our crosses. PCR primers used for genotyping and to assess genomic recombination are listed in Table 1. Animals were housed on a 12 h light:12 h darkness cycle and were given access to food and water *ad libitum*. All animal work was performed in accordance with institutional and federal guidelines and approved by the McGill University and Goodman Cancer Centre Facility Animal Care Committee (protocol 5204).

Table 1 Genotyping and qPCR primers.

Genotyping	
Tgfbr3	
Forward	TGATCTTAGTGGTAACCTCGCC
Reverse	CTAGCATGACAGGAATGTAC
Recombined	TTAGGTCGGTGCTGTCCTTGTT
Gdf9-iCre	
Forward	TCTGATGAAGTCAGGAAGAACC
Reverse	GAGATGTCCTTCACTCTGATTC
qPCR	
Rpl19	
Forward	CGGGAATCCAAGAAGATTGA
Reverse	TTCAGCTTGTGGATGTGCTC
Tgfbr3	
Forward	TGTTGGAGAGATGGCAGTGA
Reverse	TGGACTGGATGAGAGGCACT
Foxl2	
Forward	ACAACACCGGAGAAACCAGAC
Reverse	CGTAGAACGGGAACTTGGCTA
Gdf9	
Forward	TCTTAGTAGCCTTAGCTCTCAGG
Reverse	TGTCAGTCCCATCTACAGGCA

Assessment of puberty onset, estrous cyclicity, and fertility in females

Puberty onset was assessed by monitoring vaginal opening daily following weaning at postnatal day 21. From 7 weeks of age, females were swabbed daily at 10:00 h to assess estrous cyclicity, for a total of 3 weeks. Vaginal cytology was analyzed using 0.1% methylene blue, as previously described (Caligioni 2009).

To assess fertility, 10-week-old female control and cKO mice were paired with WT C57BL/6 males (000664, Charles River Senneville, Quebec, Canada) for a period of 6 months. Starting from 20 days after pairing, the cages were inspected daily for the presence of newborn mice. As soon as a litter was present, pups were carefully counted and put back into the cage. Pups were separated from the mother at postnatal day 15.

Reproductive organ analyses and ovarian histology

Ovaries were collected from 10-week-old females on the afternoon of metestrus/diestrus and weighed on a precision balance. Ovarian histology was performed as previously described (Li *et al.* 2018) at the McGill Centre for Bone and Periodontal Research. Images were acquired with a Leica DFC310 FX 1.4-megapixel digital color camera with a Leica DM1000 LED light microscope using Leica Application Suite Version 4.0.0 software or with a Zeiss Axio Imager M2 microscope.

Oocyte and cumulus cell purification

Superovulation was performed in adult females as described previously (Li *et al.* 2017). Briefly, control and cKO females were i.p. injected with 5 IU of pregnant mare's serum gonadotropin (eCG; G4877; Sigma). Forty-eight hours later, they were injected intraperitoneally with 5 IU of human chorionic gonadotropin (hCG; C1063; Sigma). Sixteen hours following the second injection, mice were euthanized and cumulus-oocyte complexes (COCs) were harvested in M199 culture medium (31100-035, Invitrogen) supplement with 10% FBS (098150, Wisent, St-Bruno, Quebec, Canada). COCs were incubated in 10 µL/mL hyaluronidase (H3884, Millipore Sigma) for 10 min at 37°C. Oocytes were then manually purified by mouth pipet. RNA was extracted from oocytes and cumulus cells using TRIzol reagent following the manufacturer's guidelines (15596018, ThermoFisher Scientific).

RT-qPCR

RNA concentration was determined by NanoDrop. cDNA was synthesized as previously described (Li *et al.* 2018). In brief, 1 µg of RNA per sample were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (172807, Promega) and random hexamer primers (184865, Promega) in a final volume of 40 µL. Two microliters of cDNA were used for qPCR analysis on a Corbett Rotorgene 600 instrument (Corbett Life Science) using EvaGreen reagent (ABMMmix-S-XL, Diamed, Mississauga, Ontario, Canada) and primers listed in Table 1. Relative gene expression was normalized to the housekeeping gene, ribosomal protein L19 (*Rpl19*). All oligos were synthesized

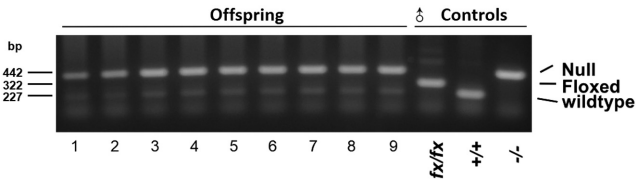

Figure 1 Recombination of the floxed *Tgfbr3* allele in cKO mice. *Tgfbr3*fx/fx;*Gdf9*-iCre/+ (cKO) females were mated with WT (+/+) males. Genomic DNA was extracted from the tails of the offspring (lanes 1–9). PCR was performed to detect the presence of the WT, floxed, and null (recombined) *Tgfbr3* alleles. Genomic DNA extracted from a *Tgfbr3* global knockout embryo was used as the null allele control.

by Integrated DNA Technologies (IDT, Coralville, IA). All primer sets were validated for efficiency and specificity.

Statistical analysis

All data were analyzed on GraphPad Prism 8 using either Student *t*-tests or two-way ANOVA. Results were considered statistically significant if $P < 0.05$.

Results

Generation of oocyte-specific cKO mice

To generate oocyte-specific *Tgfbr3* knockout mice, we crossed floxed *Tgfbr3* females to *Tgfbr3*$^{fx/fx}$;*Gdf9*-iCre males. To assess recombination in oocytes, we mated conditional knockout (cKO; *Tgfbr3*$^{fx/fx}$;*Gdf9*-iCre/+) females with WT males. PCR analysis of genomic DNA from the offspring showed one WT *Tgfbr3* allele (from the father) and one null allele in all offspring, indicating that the floxed *Tgfbr3* allele had been efficiently recombined in oocytes (Fig. 1).

cKO females have normal fertility

Female cKO mice entered puberty (defined by day of vaginal opening) at the same age as control mice (Fig. 2A). Moreover, there were no differences in the amount of time control and cKO mice spent in each stage of the estrous cycle (Fig. 2B).

To assess fertility, we paired control and cKO female littermates with WT C57BL/6 males for 6 months. There were no differences in the number of pups per litter or in the frequency of litters between control and cKO females (Fig. 2C and D).

cKO females have apparently normal gonadal development

Next, we assessed whether loss *of Tgfbr3* in oocytes affected gonadal development. There was no difference in ovarian weights between adult control and cKO mice

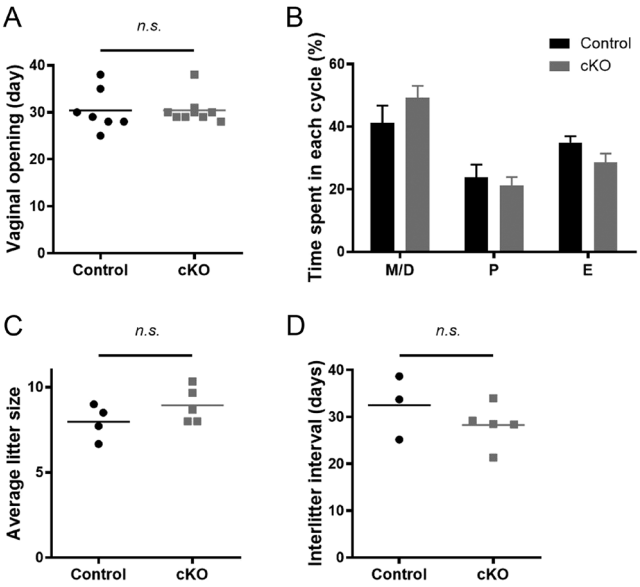

Figure 2 Normal fertility in cKO mice. (A) Puberty onset as assessed by day of vaginal opening in control ($n = 7$) and cKO ($n = 9$) females. (B) Percent of days in each stage of the estrous cycle in control ($n = 6$) and cKO ($n = 9$) females. Estrous cyclicity was assessed by vaginal cytology. Stages are indicated as estrus (E), proestrus (P), and metestrus/diestrus (M/D). (C and D) Female control and cKO littermates were paired with WT C57BL/6 males for 6 months. (C) Average number of pups per litter in control ($n = 4$) and cKO ($n = 5$) females. (D) Average number of days between litters. *t*-tests were used for statistical analyses of puberty onset, litter size, and litter frequency. Two-way ANOVA was used for statistical analysis of the estrous cycle. NS, not significantly different ($P > 0.05$).

(Fig. 3A). Additionally, ovarian histology was similar between genotypes, with follicles at all developmental stages observed (Fig. 3B). Although there were no obvious differences in follicle development between control and cKO mice, we did not systematically count the number of follicles.

Tgfbr3 is expressed at low levels in the oocyte

Though the floxed *Tgfbr3* allele was efficiently recombined in oocytes (Fig. 1), we next examined the effects of the deletion on gene expression. We collected cumulus-oocyte complexes (COCs) from superovulated adult control and cKO females. The oocyte and granulosa cells were manually dissociated and purified. *Gdf9* (Fig. 4A) and *Foxl2* (Fig. 4B) mRNAs were exclusively detected in oocytes and cumulus cells, respectively, demonstrating the purity of the cell preparations. There were no differences in the expression of either gene between genotypes. Similarly, *Tgfbr3* mRNA levels were not altered in cumulus cells of cKO mice relative to controls (Fig. 4C). Unexpectedly, *Tgfbr3* mRNA expression was very low in oocytes of both genotypes. Indeed, in the control animals, *Tgfbr3* expression in the oocyte was 0.09% of the level observed in cumulus cells.

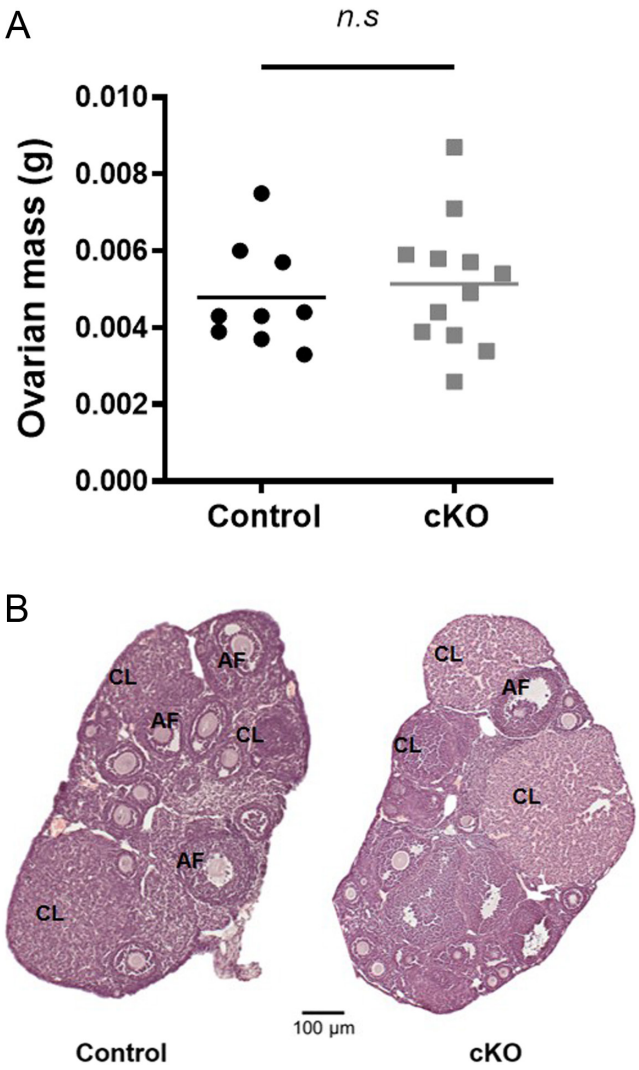

Figure 3 Normal ovarian weight and histology in cKO mice. (A) Ovarian weights were measured in 10-week-old female control (n=9) and cKO (n=12) mice. t-tests were used for statistical analyses. NS, not significantly different ($P > 0.05$). (B) H&E-stained ovarian sections from representative 10-week-old control and cKO females.

Discussion

To assess potential roles of TGFBR3 in oocytes, we generated oocyte-specific *Tgfbr3* knockout mice. The cKO females had normal ovarian histology and fecundity compared to littermate controls. The lack of a discernible phenotype is unlikely to be explained by incomplete Cre-mediated recombination, as cKO females passed the modified allele to all of their progeny. Rather, we suspect that, contrary to previous reports (Lewis *et al.* 2000, Drummond *et al.* 2002, Sarraj *et al.* 2007), it is absence of tangible *Tgfbr3* expression in the murine oocyte that likely explains our results.

While several studies demonstrated strong immunostaining for TGFBR3 protein in both rat and murine oocytes (Lewis *et al.* 2000, Drummond *et al.*

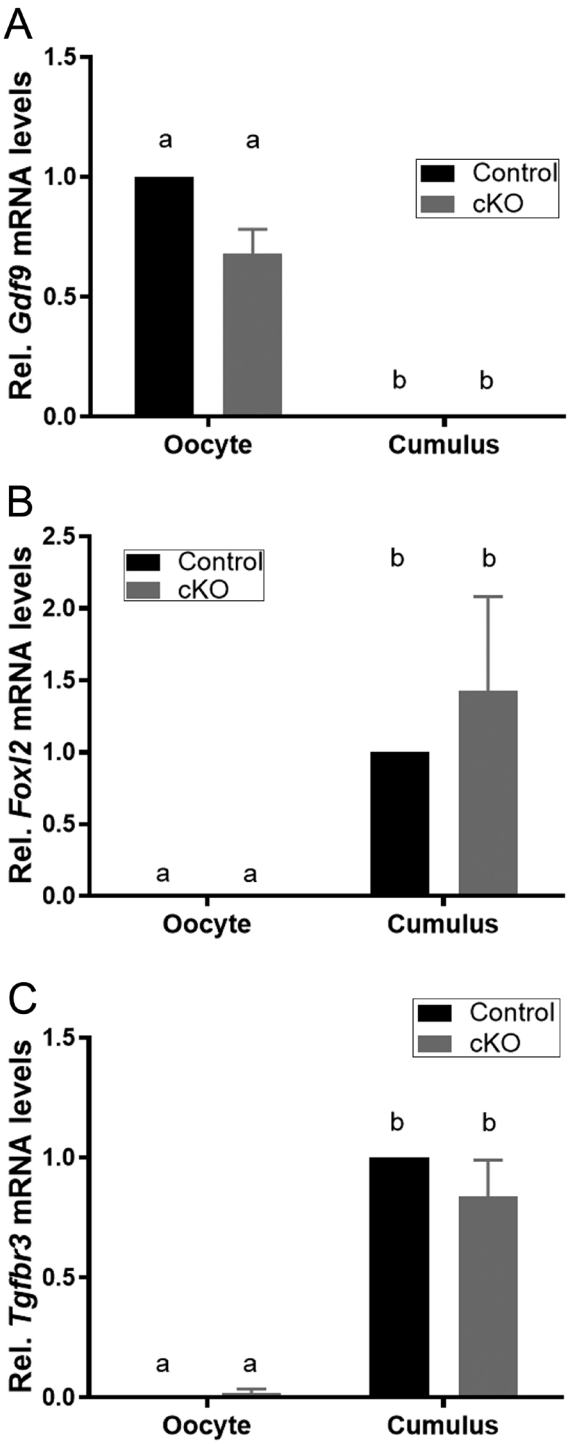

Figure 4 *Tgfbr3* is expressed in cumulus cells but not oocytes. Cumulus-oocyte complexes were collected from superovulated control (n = 3) and cKO (n = 3) adult females. Complexes were dissociated and RNA was extracted separately from purified oocytes and cumulus cells. mRNA levels of *Gdf9* (A), *Foxl2* (B), and *Tgfbr3* (C) were assessed by quantitative real-time PCR. In panel A, values for control oocytes were set to 1. In panels B and C, values for control cumulus cells were set to 1. For all graphs, data are mean ± S.E.M. Two-way ANOVAs were used for statistical analyses. Different letters indicate statistically significant differences ($P < 0.05$).

2002, Sarraj *et al.* 2007), little to no expression of *Tgfbr3* mRNA was observed in rat oocytes as assessed by *in situ* hybridization (MacConell *et al.* 2002). Our data from purified superovulated murine oocytes are consistent with this latter observation. We should note, however, that *Tgfbr3* mRNA expression is down-regulated in cumulus cells after hCG treatment (Watson *et al.* 2012). Therefore, we cannot exclude the possibility that levels of *Tgfbr3* mRNA we observed in superovulated oocytes were lower than might be observed *in vivo*. Nevertheless, we had no difficulty in detecting *Tgfbr3* mRNA in cumulus cells in the same experiment. As a result, we would argue that the discrepancies between the protein and mRNA data suggest that the immunostaining in previous publications was likely non-specific and that TGFBR3 protein is not actually expressed in murine oocytes. Indeed, we did not observe differences in TGFBR3 immunoreactivity in oocytes of control vs cKO mice, despite the completeness of the knockout (data not shown).

These results also comport with earlier observations that TGFβ family signaling may be more prominent in somatic cells than in oocytes. While TGFβ ligands, such as GDF9, are produced in the oocyte and signal in adjacent cumulus cells, it is possible that autocrine/paracrine TGFβ signaling in oocytes is negligible (Dong *et al.* 1996, Knight & Glister 2006). This idea is supported by the minimal fertility defects observed in oocyte-specific *Smad4* knockout mice (Li *et al.* 2012). SMAD4, the co-SMAD, mediates signaling by most TGFβ ligands. Whereas *Smad4* deletion in oocytes is associated with only a small reduction in fertility, granulosa cell-specific *Smad4* knockouts show impaired steroidogenesis, premature luteinization of granulosa cells, and premature ovarian failure (Pangas *et al.* 2006). These data suggest that TGFβ ligands may principally regulate fertility in female mice via their actions in granulosa cells rather than in oocytes.

In conclusion, *Tgfbr3* mRNA expression is low in superovulated murine oocytes, likely explaining why the inactivation of the *Tgfbr3* gene in these cells does not result in any fertility defects. In light of these data, we suggest that future studies of intraovarian functions of TGFBR3 focus on the protein's role in somatic cells.

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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Author contribution statement

Y L, H J C, and D J B designed the experiments. Y L and X Z performed the experiments. Y L and D J B analyzed the data. Y L, Y F L, and D J B wrote the manuscript, which was edited and approved by all co-authors.

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