Glial Cell Line-Derived Neurotrophic Factor: An Intraovarian Factor that Enhances Oocyte Developmental Competence in Vitro

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The success of early embryonic development depends on oocyte nuclear and cytoplasmic maturation. We have investigated whether glial cell line-derived neurotrophic factor (GDNF) affects the in vitro maturation (IVM) of porcine oocytes and their subsequent ability to sustain preimplantation embryo development. GDNF and both its coreceptors, GDNF family receptor α-1 (GFRα-1) and the rearranged during transformation (RET) receptor, were expressed in oocytes and their surrounding cumulus cells derived from small and large follicles. When included in IVM medium, GDNF significantly enhanced cumulus cell expansion of both small and large cumulus-oocyte complexes and increased the percentage of small follicle-derived oocytes maturing to the metaphase II stage, although nuclear maturation of large oocytes was not significantly affected. Examination of cyclin B1 protein expression as a measure of cytoplasmic maturation revealed that in the presence of GDNF, cyclin B1 levels were significantly increased in large follicle-derived oocytes, as well as in oocytes from small follicles to a level comparable to the untreated large group. After activation, a significantly higher percentage of both small and large oocytes that were matured in the presence of GDNF developed to the blastocyst stage compared with untreated controls. Indeed, GDNF enhanced the blastocyst rate of small oocytes to levels comparable to those obtained for large oocytes matured without GDNF. The effect of GDNF was specific; this was evident because its enhancement of nuclear maturation and embryo developmental potential was blocked by an antibody against GFRα-1. Our study provides the first functional evidence that GDNF affects oocyte maturation and preimplantation embryo developmental competence in a follicular stage-dependent manner. This finding may provide insights for improving the formulation of IVM culture systems, especially for oocytes from small follicles. (Endocrinology 148: 4292–4301, 2007)

**G**LIAL CELL LINE-DERIVED neurotrophic factor (GDNF) was first identified as promoting the survival, growth, and differentiation of several classes of neurons (1, 2). It has since become evident that GDNF is also expressed in tissues outside the central nervous system (3, 4) and that its overall expression is significantly higher in peripheral organs than in neuronal tissues (4). GDNF has been shown to regulate uterine branching during embryonic kidney development (5). In the testis, GDNF dose-dependently affects spermatogonial stem cells, triggering differentiation at low levels and stimulating self-renewal at higher doses (6).

The trophic effects of GDNF are mediated by a receptor complex consisting of two components, GDNF family receptor α-1 (GFRα-1) and the rearranged during transformation (RET) transmembrane tyrosine kinase receptor. GDNF interacts specifically with GFRα-1 (7, 8), a cell surface receptor linked to the membrane by a glycosyl-phosphatidylinositol lipid (9). As the high-affinity signaling component that couples to GFRα-1, RET may be recruited in response to GDNF and other related neurotrophic factors, including neurturin, artemin, and persephin (10).

Several reports have demonstrated the presence of GDNF mRNA in the adult murine ovary within the granular layer of the developing follicle (11, 12). Interestingly, a comparison of mRNA levels in 15 organs by in situ hybridization revealed that GDNF was most prominently expressed in the ovary (12). A recent report suggesting that GDNF may play a role in the onset of ovarian tumorigenesis in mutant mice carrying a disruption in the FSH receptor gene has shown that GDNF protein localizes to oocytes as well as to granulosa and stromal cells of the wild-type murine ovary (13). However, the role of GDNF in mediating ovarian function has, to date, not been investigated. Here we report that GDNF stimulates oocyte nuclear and cytoplasmic maturation and cumulus cell expansion within cumulus-oocyte complexes (COCs), also enhancing the developmental competence of porcine oocytes.

**Materials and Methods**

**COC collection**

Porcine ovaries were collected from gilts at a local abattoir, transported to the laboratory within 2 h while maintained at 37–39 C, and rinsed three times with sterile 1× PBS. COCs were aspirated with an 18-gauge needle from small (<3 mm in diameter; denoted as SM) and large (4–6 mm in diameter; denoted as LG) follicles. Using a narrow-bore glass pipette, COCs containing oocytes with a uniform ooplasm and cumulus cell mass were transferred into tissue culture medium TCM-199.
(Invitrogen, Hercules, CA) supplemented with 25 mM HEPES and washed three additional times in the same medium before use.

**RT and real-time PCR**

Cumulus cells were removed from 100 uncultured SM and LG oocytes by pipetting in 0.1% hyaluronidase (Fisher Scientific, Pittsburgh, PA). Denuded oocytes and their respective cumulus cells were washed once in 1× PBS, and total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). For each sample, 100 ng total RNA were treated with DNase I according to the manufacturer’s protocol (Invitrogen, Hercules, CA). RT reactions were carried out in a 30-μl final volume as described previously (14). A reaction in which the RT enzyme was omitted was included as negative control. Real-time PCR was performed in a 25-μl reaction consisting of 2.5 μl cDNA, 12.5 μl SYBR green mixture (Takara Bio USA, Madison, WI), and 0.3 μM forward (F) and reverse (R) primers. Reactions were run on a Smart Cycler (Cepheid, Sunnyvale, CA) for 40 total cycles. Intron-spanning primers for GDNF, GFRα-1, and RET were designed based on sequence homology between pig oocyte sequences and GenBank human and murine gene sequences. Primers and expected product sizes for each of the target genes are: for GFRα-1, F: 5′-GCTGGGCTGTCCTCTAC-3′ and R: 5′-CATGCCCTCCCCTACTTTG-3′, product size 150 bp; for GFRα-1 F: 5′-GTGCCGGTGTGCTCTTCA-3′ and R: 5′-GCTGACGACCTGACTGCG-3′, product size 138 bp; for RET, F: 5′-CGGTGCAGCTACTCATC-3′ and R: 5′-CTGCGCTGGCACTTCTT-3′, product size 188 bp; and for RPII (cellular reverse polymerase II gene), F: 5′-CAGGGATGATGCTCTGGGACAGC-3′, and R: 5′-CTGCGCTGTGGCACTTCTT-3′, product size 181 bp. To rule out potential contamination, cDNA was replaced with water in one reaction for each set of primers. The specificity of the melt curves was determined for all primer pairs, and products were verified by agarose gel electrophoresis. The identity of the products was confirmed using an inverted microscope. The percentage of oocytes at MI was calculated based on the number of live SM and LG oocytes in each treatment group. Experiments were repeated at least five times.

**Evaluation of the specificity of the GDNF effect on oocyte maturation and developmental competence**

The effect of receptor blocking on the GDNF response was evaluated using an anti-GFRα-1 antibody (R&D Systems, Minneapolis, MN), which was selected based on its ability to neutralize the receptor-ligand interaction for recombinant GDNF according to the manufacturer’s specifications. Groups of 50 COCs isolated from SM and LG follicles were cultured in the absence or presence of 50 ng/ml GDNF, or in the presence of 50 ng/ml GDNF with the addition of either 10 μg/ml GFRα-1 blocking antibody or 10 μg/ml purified goat IgG (Cedarlane, Burlington, NC) as negative control. The effect of receptor blocking was evaluated at the MI stage for SM oocytes by determining the percentage of first polar bodies in each treatment group, as well as at the two-cell, eight-cell, and blastocyst stages for activated LG oocytes.

**Evaluation of COC expansion**

COCs derived from SM and LG follicles were cultured in oocyte maturation medium in the absence and presence of 50 ng/ml GDNF for 24 h. Digitized images of COCs were analyzed using Openlab image analysis software (Improvision, Lexington, MA). The area occupied by a COC was calculated based on the following formula: area = length × width × 0.7854 (19), where the length and width corresponded to the distance between the two most widely and most closely spaced points, respectively.

**Immunohistochemistry**

Ovaries of gilts fixed in 4% paraformaldehyde for 24 h were processed for paraffin embedding. The 5-μm tissue sections mounted on glass slides were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, and treated with 0.2% sodium borohydride. After a 4-h block in 5% BSA supplemented with 2% fetal bovine serum, sections were incubated overnight at 4 C with polyclonal anti-GDNF antibody (1:50; Abcam Inc., Cambridge, MA) in humidified chambers. Sections were incubated for 2 h at room temperature with rabbit anti-goat IgG fluorescein isothiocyanate (1:300; Sigma) in the dark, counterstained with 1 μg/ml Hoechst nuclear stain, and mounted using fluorescent mounting medium (DakoCytomation, Glostrup, Denmark). Images were captured using an Olympus BX-UCB microscope and MetaMorph image analysis software (Universal Imaging Corp., West Chester, PA). Incubation of successive tissue sections with secondary antibody only served as negative control.

**Western blotting**

For cyn1 Bl immunoblotting, approximately 200 denuded oocytes and their corresponding cumulus cells derived from SM and LG, with or without 50 ng/ml GDNF, treatment groups were collected after 40–44
h of IVM and transferred into lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM NaF, 0.75 mM phenylmethylsulfonyl fluoride, 15% glycerol, and 10 μg/ml each of aprotinin, peptatin, and leupeptin) as described previously (20). Samples were boiled for 3 min and centrifuged for 5 min at 13,000 rpm, and the soluble protein fraction was electrophoresed under reducing conditions on 10% polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes, which were incubated overnight in 5% nonfat dry milk blocking buffer at 4°C. The blots were incubated in monoclonal anti-cyclin B1 antibody (1:1500; BD PharMingen, San Diego, CA; clone GNS-11) for 1 h at room temperature. Blots were incubated in monoclonal anti-cyclin B1 antibody (1:500; BD PharMingen, San Diego, CA; clone GNS-11) for 1 h at room temperature. Blots were stripped and reprobed with anti-GAPDH antibody as described. Densitometric analysis was carried out to determine the relative absorbance of band intensities. Four samples were analyzed in each experiment.

The mean fold changes in mRNA levels for GDNF, GFRα-1, and RET were calculated relative to SM oocytes. Real-time PCR data; the percentage of oocytes reaching MII, cleavage, eight-cell, and blastocyst stages; relative cyclin B1 and GFRα-1 protein levels; and the mean area of cumulus cell expansion were analyzed by one-way ANOVA using GraphPad analysis software. Data sets were further analyzed using the Tukey test for multiple comparisons to determine statistical differences between groups, which were denoted by different letter subscripts. Results were considered significant at P < 0.05. Experiments were repeated at least three times, and data represent the mean ± SEM of all repeats.

**Results**

**GDNF and its coreceptors GFRα-1 and RET are expressed in oocytes and cumulus cells derived from SM and LG follicles**

To determine whether GDNF and its coreceptors GFRα-1 and RET are present in the porcine ovary, the expression of the ligand and both receptors was examined in oocytes and cumulus cells. For GDNF, mRNA levels did not differ significantly in SM and LG oocytes. In contrast, statistically significant differences were observed for cumulus cells, with a 2-fold higher level of mRNA detected in the SM than the LG group (P < 0.05, Fig. 1A). The specificity of GDNF gene amplification in oocytes and cumulus cells was evaluated by verifying the expected size of real-time PCR products on an agarose gel (Fig. 1B) and sequencing of the PCR product (data not shown). Immunohistochemistry revealed that GDNF was present in oocytes and cumulus cells as well as in granulosa cells of antral follicles (Fig. 1C, panels II and VI). In addition, GDNF was found to be present in follicular fluid derived from SM and LG follicles by Western blotting. Under reducing conditions, porcine GDNF migrated at approximately 15 kDa (Fig. 1D), which was similar to the migration of recombinant human GDNF (data not shown). Although the levels of GDNF varied in the follicular fluid derived from seven independent collections, no overall difference between SM and LG follicles was observed after densitometric analysis of scanned images (P = 0.7, Fig. 1E), and Coomassie blue staining of the gels was similar across blots for each of the follicular fluid collections analyzed.

For RET and GFRα-1, mRNA levels were similar in oocytes isolated at different stages of follicular development (Fig. 2, A and C, respectively). In contrast, expression levels for both receptors were significantly higher in the SM than in the LG follicle-derived cumulus cell group (~2-fold increase, P < 0.05, Fig. 2A for RET; 9-fold increase, P < 0.001, Fig. 2C for GFRα-1). The specificity of RET and GFRα-1 amplification in oocytes and cumulus cells was evaluated by verifying the expected size of real-time PCR products on agarose gels (Fig. 2, B for RET, and D for GFRα-1) and sequencing of each PCR product (data not shown). Comparisons of GDNF, GFRα-1, and RET mRNA levels in oocytes and cumulus cells derived from SM and LG follicles revealed that SM cumulus cells expressed the highest overall levels of GDNF and GFRα-1 (P < 0.05, Figs. 1A and 2C). RET expression levels did not fluctuate dramatically between SM and LG oocytes and SM cumulus cells, although LG cumulus cells expressed RET and GFRα-1 at significantly lower levels (P < 0.05, Fig. 2, A and C). Western blot analysis demonstrated the presence of porcine GFRα-1 protein in oocytes and cumulus cells derived from SM and LG follicles. The protein migrated at approximately 60 kDa (Fig. 2E), which was similar to the relative molecular mass reported for GFRα-1 in rat testis and human corneal epithelial cells (21, 22). Densitometric analysis revealed that, consistent with what was observed at the mRNA level, GFRα-1 protein was more highly expressed in SM cumulus cells compared with SM and LG oocytes or LG cumulus cells (P < 0.01, Fig. 2F).
human GDNF. After 40–44 h, denuded oocytes were activated and cultured in NCSU-23 without GDNF. Effects on preimplantation embryo development were evaluated at the two-cell, eight-cell, and blastocyst stages (Fig. 3, A–C). A significant increase in the percentage of embryos reaching the blastocyst stage was observed using 50 ng/ml GDNF ($P < 0.05$, Fig. 3C), and this dose was used in all subsequent experiments.

**GDNF stimulates oocyte cytoplasmic maturation during follicular development**

Because GDNF elicited a functional effect on LG follicle-derived oocytes, its ability to enhance the developmental potential of SM oocytes was evaluated. Most SM follicle-derived oocytes are known to be in their final growth phase, are generally less meiotically competent, and are unable to support preimplantation embryo development. Thus, the developmental competence of parthenogenetic embryos from SM and LG oocytes that were matured in the absence or presence of 50 ng/ml GDNF were monitored and compared. Although no significant changes in the cleavage rates were observed for either the SM +GDNF or LG +GDNF groups compared with untreated controls (Fig. 4A), a significantly higher percentage of SM +GDNF embryos reached the eight-cell stage compared with the SM control group (51 vs. 30%, $P < 0.05$, Fig. 4B). At the blastocyst stage, an approximate 9- and 2-fold increase in the blast rate was observed for the SM +GDNF and LG +GDNF groups, respectively, compared with each untreated control ($P < 0.05$ for both SM and LG groups, Fig. 4C). Interestingly, when matured in the presence of GDNF, the developmental competence of oocytes derived from SM follicles was enhanced at each stage to a level comparable to that of untreated LG follicle-derived oocytes (Fig. 4, A–C).

If the functional effect of GDNF on preimplantation embryo development is specific through interaction with the binding component of its receptor complex, the effect should be abrogated by blocking GFRα1 with a specific antibody. Figure 4D shows that 50 ng/ml GDNF failed to increase the developmental potential of oocytes that were matured in the presence of GFRα1 blocking antibody. Indeed, the presence of the antibody significantly reduced the percentage of control is a reaction carried out on a sample from which the RT enzyme was omitted during cDNA synthesis. C, Immunolocalization of GDNF in the adult porcine ovary. GDNF protein (green) was detected in the oocyte (a), cumulus cells (b), and granulosa cells (c) of antral follicles. Negative controls were included that probed successive tissue sections with secondary antibody only. Cells were counterstained with Hoechst dye to show nuclei (blue). Panels I (negative control) and II depict overlaid images at $\times100$ magnification with insets at a magnification of $\times400$. Arrows indicate enlargement of the right insets. Nonoverlaid images of a different follicle at $\times400$ magnification are shown in panels III and VI. Panels III and V represent nuclear staining, with their counterparts in panel IV (negative control) and panel VI. D, A representative Western blot of GDNF protein in follicular fluid derived from SM and LG follicles. Equal volumes of follicular fluid were analyzed by immunoblotting using a polyclonal anti-GDNF antibody. E, Results of densitometric analysis depicting the levels of GDNF in the follicular fluid derived from SM and LG follicles in terms of relative absorbance. Data represent the mean ± SEM of seven independent experiments.

Fig. 1. Detection of GDNF in SM and LG ovarian follicles. A, Relative mRNA expression levels of GDNF in SM and LG oocytes (OO) and cumulus cells (CC) relative to SM follicle-derived oocytes. Data represent the mean ± SEM of three independent experiments. Different letters denote statistical differences between groups ($P < 0.05$). B, Representative agarose gel image depicting the size of the GDNF real-time PCR product (150 bp). The negative (-ve) real-time PCR
blastocysts to a level below that of the untreated control group \((P < 0.05, \text{Fig. 4D})\). The antibody effect was specific to GFRα-1; this was evident because no inhibitory effect on GDNF action was observed when the same concentration of goat IgG was present in the maturation medium (Fig. 4D).

In addition to preimplantation developmental competence, cyclin B1 protein levels also have been used as an indicator of oocyte cytoplasmic maturation (18, 24, 25). Increases in cyclin B1 protein have been directly correlated with the activity of M-phase promoting factor (MPF), which plays an important role during oocyte maturation (26, 27). Western blotting was performed to investigate whether GDNF influences oocyte cyclin B1 expression during follicular development. After 40–44 h of IVM, cell lysates collected from SM and LG oocytes cultured in the absence and presence of 50 ng/ml GDNF were analyzed using an antibody against cyclin B1. A 62-kDa protein corresponding to cyclin B1 was detected for all samples (Fig. 5A). Densitometric analysis of the blots revealed that the level of cyclin B1 was significantly higher in LG GDNF oocytes compared with untreated controls (1.5-fold increase, \(P < 0.05, \text{Fig. 5B})\). In addition, the level of cyclin B1 in the SM GDNF group, although not significantly different from the SM untreated control, was also increased to a level comparable to that of untreated LG oocytes (Fig. 5B).

GDNF elicits an effect on oocyte nuclear maturation and cumulus cell expansion

To investigate whether GDNF influences the nuclear maturation of oocytes, which may have contributed to the stage-dependent effects observed during embryo culture, oocytes cultured in the absence and presence of 50 ng/ml GDNF were evaluated after 40–44 h of IVM. At this stage, which corresponds to MII, it is possible to examine the extrusion of the first polar body as an index of oocyte nuclear maturation. As shown in Fig. 6A, the percentage of oocytes that progressed to MII was significantly higher in the SM GDNF group compared with SM controls (58 vs. 44\%, respectively, \(P < 0.01\)), again reaching a level comparable to the untreated LG group (67\%). No significant differences were observed between the LG control and LG + GDNF groups. To determine whether the GDNF effect observed at MII in SM follicle-derived oocytes was specific through its binding receptor, a GFRα-1 blocking antibody was included in the IVM medium. The antibody significantly reduced the percentage of oocytes

\(\text{OO}\) and cumulus cells (CC) relative to SM follicle-derived oocytes. Data represent the mean ± SEM of three independent experiments. \textit{Different letters} denote statistical differences between groups \((P < 0.05 \text{ for RET}; P < 0.01 \text{ for GFRα-1})\). B and D, Representative agarose gel images depicting the size of real-time PCR products for RET (188 bp) (B) and GFRα-1 (138 bp) (D) in oocytes and cumulus cells. The negative (-ve) real-time PCR control is a reaction carried out on a sample from which the RT enzyme was omitted during reverse transcription. E, A representative Western blot of uncultured SM and LG oocytes and cumulus cell lysates depicting the migration of porcine GFRα-1 protein at approximately 60 kDa and GAPDH protein at 36 kDa. F, Results of the densitometric analysis depict relative expression levels of GFRα-1 protein normalized by GAPDH as the fold increase of the SM oocytes group. Data represent the mean ± SEM of three independent experiments. \textit{Different letters} denote statistical differences between groups \((P < 0.01)\).
extruding a first polar body in the +GDNF +antibody treatment group compared with either the +GDNF or +GDNF +goat IgG controls (33.5 vs. 50.5 and 51%, respectively, $P < 0.001$, Fig. 6B). Interestingly, the blocking antibody also significantly reduced the number of SM oocytes progressing to MII to a level below that of the control group, in which no GDNF had been added to the IVM medium (33.5 vs. 40%, respectively, $P < 0.05$, Fig. 6B).

An additional effect of GDNF observed during IVM was the enhancement of cumulus cell expansion in SM and LG COCs cultured in the presence of 50 ng/ml GDNF (Fig. 7, A and B). After 24 h, the mean area of COCs was significantly increased from 0.075 to 0.113 mm$^2$ for the SM +GDNF group ($P < 0.05$), reaching a level comparable to the untreated LG control group (0.117 mm$^2$, Fig. 7C). GDNF also significantly increased LG COC expansion (to a mean area of 0.213 mm$^2$, $P < 0.001$, Fig. 7C).

**Discussion**

Although GDNF is known to promote diverse functions in several different tissues, its potential role in the ovary has remained unexplored. The current investigation is the first to report that GDNF acts as an important autocrine/paracrine regulator during ovarian follicular development. Our findings are supported by the following observations. 1) GDNF and both its coreceptors, GFRα-1 and RET, are expressed in COCs during folliculogenesis. The ligand is therefore spatially available to elicit its function(s), with both oocytes and cumulus cells able to respond to GDNF stimulation. 2) GDNF stimulates in vitro cumulus cell expansion, promotes meiotic progression to MII, increases oocyte cyclin B1 expression levels, and enhances oocyte developmental competence during follicular development.

Our expression data are consistent with previous reports describing the presence of mRNA transcripts for GDNF and its coreceptors in follicles of the adult murine ovary (11, 12, 28). A comparison of the relative expression levels of GDNF, GFRα-1, and RET transcripts in SM and LG follicle-derived COCs revealed that GDNF and GFRα-1 mRNA levels are highest in SM cumulus cells. In contrast, the lowest levels of both GFRα-1 and RET mRNA were observed in LG cumulus cells. GDNF may play a particularly important role during the later phases of follicular development, which are critical for the maturation and developmental competence of oocytes derived from SM follicles, a notion supported by our functional data. Interestingly, mRNA levels for the ligand and both its receptors remained relatively constant in SM and LG follicle-derived oocytes. Thus, the current study demonstrates that the expression of GFRα-1 and RET may change in a stage-dependent manner in cumulus cells during folliculogenesis but not in oocytes. In addition to the presence of these transcripts, GDNF protein was shown to localize to SM antral follicles. GDNF protein was also present at similar levels in the follicular fluid derived from SM and LG follicles. GFRα-1, the ligand-binding component of the GDNF recep-

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**Fig. 3.** Preimplantation development of parthenogenetically activated oocytes matured *in vitro* in the presence of different doses of GDNF. COC derived from LG follicles were *in vitro* matured in 0, 25, 50, or 75 ng/ml recombinant human GDNF for 40–44 h. Parthenogenetic embryos were generated by electroactivation and cultured for 7 d. A, Cleavage rate (number of embryos at the two-cell stage/number of activated MII oocytes) 24 h after activation; B, percentage of embryos reaching the eight-cell stage (number of embryos at the eight-cell stage/number of two-cell-stage embryos) 72 h after activation; C, percentage of embryos developing to the blastocyst stage (number of embryos at the blastocyst stage/number of two-cell-stage embryos) after 7 d of culture. Data represent the mean ± SEM of four independent experiments; 200 embryos were analyzed in each treatment group. *b*, Statistical differences between groups for embryos reaching the blastocyst stage after IVM in 50 ng/ml GDNF ($P < 0.05$).
In the current study, the medium adapted for all IVM experiments included 10% porcine follicular fluid, which our laboratory has routinely included for the successful culture of oocytes (18, 25), because it is known to be important for oocyte in vitro competence to support preimplantation embryo development in pigs (30, 31). As shown in the current study, GDNF protein is present in porcine follicular fluid. Thus, the effects of exogenous GDNF on oocyte developmental competence were evaluated beyond the potential role played by the addition of 10% porcine follicular fluid. Al-

**Fig. 4.** GDNF stage-dependently enhanced the developmental potential of SM and LG follicle-derived oocytes in vitro, an effect that was blocked by GFRα-1 blocking antibody. Oocytes from SM and LG follicles were in vitro matured in the absence (−) or presence (+) of 50 ng/ml GDNF for 40–44 h. Parthenogenetic embryos were generated by electroactivation and cultured for 7 d. A, Cleavage rate (number of embryos at the two-cell stage/number of activated MII oocytes) 24 h after activation; B, percentage of embryos reaching the eight-cell stage (number of embryos at the eight-cell stage/number of two-cell-

**Fig. 5.** Stage-dependent influence of GDNF on cyclin B1 protein expression in SM and LG follicle-derived oocytes collected at MII. A, A representative Western blot of SM and LG oocyte lysates collected after 40–44 h of IVM in the absence (−) or presence (+) of 50 ng/ml GDNF, depicting the migration of porcine cyclin B1 protein at 62 kDa and GAPDH protein at 36 kDa; B, results of the densitometric analysis depicting relative expression levels of cyclin B1 normalized by GAPDH as the fold increase of the SM−GDNF group. Data represent the mean ± SEM of three independent experiments. Different letters denote statistical differences between groups (P < 0.05).
though GDNF had no effect on LG follicle-derived oocyte nuclear maturation during IVM, it significantly increased the percentage of oocytes from SM follicles that reached the MII stage to a rate comparable to untreated LG oocytes. This effect was found to be specific, because a GFRα-1 blocking antibody completely neutralized the contribution of exogenously added GDNF. Interestingly, the blocking antibody reduced the percentage of SM follicle-derived oocytes extruding a first polar body to levels below controls cultured in the absence of GDNF without antibody, suggesting that the contribution of endogenous sources of GDNF were also affected. Unlike the majority of LG follicle-derived oocytes, which have completed their final growth phase at the time of collection, most oocytes from SM follicles are meiotically incompetent and cytoplasmically deficient in terms of sustaining early embryonic development (32). Their competence to resume meiosis is dependent on adequate stimulation by intraovarian factors, and our data suggest that GDNF is one of these autocrine/paracrine factors.

Similar to the effect observed for nuclear maturation, GDNF significantly increased the percentage of SM IVM oocyte-derived parthenogenetically activated embryos that reached the eight-cell and blastocyst stages, again at rates comparable to the untreated LG embryo group. The percentage of embryos reaching the blastocyst stage was also increased in the group derived from LG IVM oocytes treated with GDNF, suggesting that this factor may further enhance the developmental competence of LG oocytes in a manner independent from nuclear maturation. Again, the effect of GDNF on preimplantation embryo development was found to be specific, because a GFRα-1 blocking antibody significantly impeded the progression of LG follicle-derived embryos to the blastocyst stage. Similar to the neutralizing effect observed in oocytes from SM follicles at MII, the antibody induced a further decrease in the percentage of LG embryos...
reaching the blastocyst stage to levels below controls cultured in the absence of GDNF and antibody.

In further support of the differential effect on the nuclear and cytoplasmic maturation of LG follicle-derived oocytes, cyclin B1 protein levels were significantly higher in the presence of GDNF compared with untreated controls. Cyclin B1 protein expression was also increased in the SM oocyte GDNF treatment group to levels comparable to LG controls. It is possible that the effect of GDNF on the cytoplasmic maturation of both SM and LG follicle-derived oocytes is elicited through mechanisms similar to those engaged in GFRα-1/RET-positive spermatogonial stem cells, in which cyclin B1 gene expression has been shown to be highly up-regulated in response to GDNF stimulation (33).

Recent studies have demonstrated that brain-derived neurotrophic factor (BDNF) enhances the rate at which parthenogenetically activated bovine and neurotrophic factor (BDNF) enhances the rate at which cyclin B1 gene expression has been shown to be highly up-regulated by androgens linked to the FSH receptor signaling cascade (13). Thus, GDNF could potentially signal through one or more of these pathways to regulate the functional effects described here in COCs at different stages of folliculogenesis.

Maturation of cumulus-oocyte complexes derived from early antral follicles could potentially provide a substantial source of competent oocytes for *in vitro* embryo production. However, oocytes derived from these SM follicles are less developmentally competent than those isolated at later stages of folliculogenesis (43). Nevertheless, culture systems have been developed that support the growth and maturation of oocytes from rodent primordial follicles, resulting in the birth of live pups (44, 45). More recently, live offspring were produced from murine premeiotic fetal germ cells by first transplanting ovarian grafts under kidney capsules followed by IVM (46). Although significant progress has been made in enhancing the meiotic resumption of IVM porcine oocytes derived from early antral follicles, the majority of these SM oocytes are unable to support preimplantation embryo development (24, 32, 47), which may be due to shortfalls in the culture environment (29, 48). In addition, there is room to improve the percentage of LG follicle-derived oocytes that are able to sustain the formation of blastocysts. Our finding that GDNF significantly enhances the developmental potential of SM and LG follicle-derived oocytes during IVM suggests that inclusion of this factor in oocyte culture medium may increase the pool of competent oocytes for transgenesis, human-assisted reproduction, and therapeutic cloning applications.

Acknowledgments

We thank Queenie Cheung and the staff at Conestoga Farm Fresh Pork for their assistance in collecting porcine ovaries.

Received January 9, 2007. Accepted May 21, 2007.

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This work was supported by the Premier’s Research Excellence Award, Natural Sciences and Engineering Research Council of Canada, and Canadian Institutes for Health Research.

Disclosure Summary: The authors have nothing to disclose.

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