Midkine Promotes Proliferation of Primordial Germ Cells by Inhibiting the Expression of the Deleted in Azoospermia-Like Gene

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Primordial germ cell (PGC) development is an area of research that is hampered by low cell numbers as well as difficulty in isolation. They are, however, required for the production of gametes and as such represent an important area of understanding that has widespread implications for fertility and reproductive technologies. Here we investigated the role of the heparin-binding growth factor midkine (MK) on PGC development, first using our established model of porcine stem cell-derived PGC-like cells and then confirming our findings in PGC. Our results show that MK has a mitogenic effect on PGC, mediated through an increased cell proliferation as well as decreased apoptosis. Upon further investigation, we found these effects concomitant with the decreased expression of the germ cell-specific gene deleted in azoospermia-like (*DAZL*). This decrease in *DAZL* expression, and consequent decreases in the meiosis-related genes *SCP3* and *DMC1*, suggest a role for MK in preventing a shift in the PGC phenotype toward meiosis. MK instead increases activity of mitotic pathways in PGC, keeping them in a proliferative, less differentiated state. Lentiviral-mediated overexpression of *DAZL* further confirmed its role in promoting meiosis in and reducing proliferation of PGC. These effects were mitigated by the addition of MK, which was able to limit the effect of this *DAZL* overexpression. Furthermore, a loss-of-function study showed that a *DAZL* knockdown by small interfering RNA had the same effect as that induced by the addition of MK. Taken together, these data suggest that MK is able to maintain a proliferative PGC phenotype mediated by the suppression of *DAZL* in early germ cells. **(*Endocrinology* 153: 3482–3492, 2012)**

Differentiation of germ cells from stem cells may offer a valuable model for studying germ cell formation and oogenesis and provide insights into fertility. Extensive progress has been made in the field of *in vitro* differentiation technology over the past few years. Recent works have demonstrated that embryonic and somatic stem cells have the capacity to differentiate into early germ cells, oocyte-like cells (OLC), male gametes, and even offspring (1–8). Hübner *et al.* (3) first showed that mouse embryonic stem cells were capable of developing into oogonia. The developed oogonia were able to form oocytes, which underwent parthenogenic activation, leading to the formation of blastocysts. Subsequently, we reported that stem cells isolated from porcine fetal skin were able to give rise to primordial germ cell (PGC)-like cells (PLC) and OLC *in vitro*, substantiating the idea that somatic stem cells have an intrinsic ability to support the differentiation of germ cell-like cells (1, 9–11).

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Abbreviations: bFGF, Basic fibroblast growth factor; BrdU, 5-bromo-2-deoxyuridine; CCND3, cyclin D3; DAZL, germ cell-specific gene deleted in azoospermia-like; DNase, deoxyribonuclease; E13, embryonic d 13; E2, estradiol; GDNF, glial cell line-derived neurotrophic factor; GFP, green fluorescent protein; MK, midkine; OLC, oocyte-like cells; PBS-B, PBS with 2% BSA; PGC, primordial germ cell; PI3K, phosphatidylinositol 3-kinase; PLC, PGC-like cells; PTN, pleiotrophin; RNAi, RNA interference; SCF, stem cell factor; SCP, synaptonemal complex protein; siRNA, small interfering RNA.

PGC are the precursors of oocytes and spermatogonia. During migration, and for a short period of time after arriving at the genital ridge, PGC proliferate and increase in number. In the mouse, the number of germ cells reaches the highest at embryonic d 13 (E13) (12), the time of transition from mitosis to meiosis. After a number of mitotic divisions, PGC enter meiosis I, progressing through the leptotene, zygotene, and pachytene stages before arresting at the diplotene stage of prophase I (13). In the last decades, many studies have demonstrated that PGC depend on specific growth factors and other undetermined compounds for both their survival and proliferation (reviewed in Ref. 14). Several of these factors have been identified *in vivo*, including kit ligand [also known as stem cell factor (SCF)] (15), leukemia inhibitory factor (LIF) (16), basic fibroblast growth factor (bFGF or FGF-2) (17), and bone morphogenetic protein-4 (18). Applying this research to the *in vitro* culture of PGC, a combination of kit ligand, bFGF, leukemia inhibitory factor, and bone morphogenetic protein-4 has shown significant effects on the survival and proliferation of PGC cultured in the absence of somatic cell monolayer support (19). Despite these findings, additional factors that are involved in regulating PGC proliferation have yet to be identified. Moreover, factors and mechanisms controlling the PGC mitotic cycle and the shift from mitosis to meiosis are areas largely unexplored and are essential for gametogenesis.

We have previously reported the differentiation of PLC from stem cells isolated from the skin of porcine fetuses *in vitro*. These morphologically distinct PLC grow as either single cells or form loosely adherent clusters (becoming detached later on during differentiation) on a monolayer of fibroblast-like cells, are alkaline phosphatase positive, and express germ cell markers such as OCT_4, *FRAGILIS*, *STELLA*, *DAZL*, and *VASA*. Moreover, they undergo imprint erasure and are capable of differentiating into OLC (11). Our current study was intended to use stem cell-derived PLC as an alternative *in vitro* model to PGC to identify novel factors that regulate PGC mitosis and the mechanisms involved.

Materials and Methods

Isolation and culture of fetal porcine skin-derived stem cells

Stem cells were isolated and cultured from the skin of porcine fetuses collected at d 40–45 of gestation (E40–45) as previously described (1, 20). Briefly, skin from the back of individual fetuses was cut into 1- to 2-mm^2 pieces, which were washed three times in Hanks' balanced salt solution, digested with 0.25% trypsin for 40 min at 37 C, and treated with 0.1% deoxyribonuclease (DNase) for 1 min at room temperature. Tissue pieces were then washed twice with Hanks' balanced salt solution and twice with DMEM-F12 (1:1) and mechanically dissociated by vigorous pipetting. The resulting cell suspensions were passed through 40-μm cell strainers (BD Falcon, Franklin Lakes, NJ), centrifuged, and resuspended in stem cell medium [DMEM-F12 (1:1) containing penicillin/streptomycin supplemented with 1× B-27 (Life Technologies, Inc., Grand Island, NY), 40 ng/ml epidermal growth factor (Sigma Chemical Co., St. Louis, MO), and 40 ng/ml bFGF (Cell Signaling Technologies, Danvers, MA)]. Cells were cultured in 100-mm untreated culture dishes (Sarstedt, Montreal, Canada) at 38.5 C in 5% CO_2. Nonadherent clusters of individual cells, or skin spheres, formed within 48 h of culture. To remove contaminating cells, which attached to the bottom of the culture dishes, suspended skin spheres were subcultured twice during the 11-d culture before use. These skin spheres are undifferentiated skin stem cells.

Induced differentiation of skin-derived stem cells

For differentiation experiments, skin spheres were centrifuged, dissociated mechanically into single cells by pipetting, and plated at a final density of 8×10^4 cells per 60-mm tissue culture dish (Corning, Corning, NY) treated with 0.05 mg/μl poly-D-lysine (BD Biosciences, San Diego, CA) and 0.005 mg/ml laminin (BD Biosciences). Cell were maintained at 38.5 C in 5% CO_2 as an adherent monolayer through culture in 0.22-μm filtered differentiation medium [DMEM, penicillin/streptomycin, 5% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), 5% porcine follicular fluid, 0.23 mM sodium pyruvate, 0.1 mM nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), and 0.1 mM β-mercaptoethanol]. Half the differentiation medium was replaced every 4 d for up to 30 d of culture.

PGC isolation from fetal genital ridge

Porcine PGC were isolated using methods previously described (21, 22) with modifications. Briefly, genital ridges were collected from fetuses at E40–45. The tissue was incubated in 0.02% EDTA for 20 min at room temperature. The gonadal ridges were dissociated by gentle disruption using fine forceps. Supernatant containing dissociated cells was collected and centrifuged at $500 \times g$ for 5 min. The collected cells were cultured in a 24-well plate in our stem cell differentiation medium (described above) for 48 h. At the end of this initial plating period, the suspended and loosely attached cells with PGC morphology were collected by incubating in 0.02% EDTA for 5 min to separate them from the more firmly attached somatic cells. This separation culture method was previously shown to result in a nonadherent cell population composed of 63% PGC as determined by alkaline phosphatase staining (23).

Cell factor and hormone treatment

Morphologically distinct, nonadherent D25 PLC were collected from 60-mm dishes of differentiating cells (without disturbing the adherent monolayer) and counted. PGC isolated from E40–45 genital ridges served as controls to verify the findings in PLC. In each experiment, 1×10^5 cells were seeded into each well of a 24-well plate (Sarstedt) and cultured in differentiation medium in the absence or presence of 50 ng/ml human recombinant glial cell line-derived neurotrophic factor (GDNF) (Cell Science), 100 ng/ml human recombinant midkine (MK) (Cell Science), 25 ng/ml estradiol (E2) (Sigma, Canton, NY;

E2758), 40 ng/ml SCF (Sigma; S9915), 100 nM T_3 (Sigma; T6397), or 100 nM T_4 (Sigma; T1775), as well as a mixture of cell factors and hormones at half of the concentrations stated above, for 48 h.

5-Bromo-2-deoxyuridine (BrdU) incorporation assay

Nonadherent D25 PLC were collected from 60-mm dishes of differentiating cells and equal numbers of cells per treatment group were seeded into wells of a 24-well plate (Sarstedt) and cultured with 30 μg/ml BrdU in the presence and absence of 100 ng/ml MK for 48 h. The BrdU incorporation assay was performed as described previously (1). Individual fluorescent cells were counted based on propidium iodide nuclear staining and the BrdU signal. Three independent cultures were analyzed for BrdU uptake.

Caspase 3 assay

Cells were pelleted by centrifugation at 600 × *g* for 5 min at 4 C. Pellets were then washed once with 1 ml PBS and suspended in 1× lysis buffer (CASP3C; Sigma) at a concentration of 100 μl per 10^7 cells. After incubating the cells on ice for 20 min, the lysed cells were centrifuged at 20,000 × *g* for 10 min at 4 C. Analysis was performed after transferring the supernatants to new tubes. A 96-well plate microassay was then performed after a 90-min incubation at 37 C. Absorbance was read at 405 nm, and results were determined using a *p*-nitroaniline calibration curve.

RNA isolation and real-time RT-PCR

RNA was isolated using the Total RNA Kit (Norgen, Niagara-on-the-Lake, Canada) according to the manufacturer's protocol. Reverse transcription was performed as previously described (20). Briefly, samples were DNase treated with 1 U amplification-grade DNase (Invitrogen). Reverse transcription was then performed using random priming and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-time RT-PCR was performed using the SYBR Green PCR kit (TaKaRa, Otsu, Shiga, Japan) with 0.25 μM each forward and reverse primer. Reactions were run on an Mx3005P System (Stratagene, La Jolla, CA) for 38 total cycles. Primers and expected product sizes used for each set of reactions are shown in Table 1. Control reactions were run with water in place of cDNA to check for potential contamination. Product sizes were verified by agarose gel electrophoresis, and all products were sequenced to confirm identity. The RPII housekeeping gene was amplified for each sample to verify the presence of cDNA and as an internal control to calculate the relative level of target gene expression using the ΔΔCt method (24).

Lentiviral transduction of PGC-like cells

Cloning of the lentiviral gene transfer plasmid pL-SIN-EF1a-DAZL-GFP and production of recombinant lentiviral particles were as described (11). Transfected 293FT cells were cultured with differentiation medium to produce lentivirus. The lentiviral medium was collected, filtered with 0.45-μm filters, and frozen at −80 C. At d 3 of induced differentiation, the differentiated skin-derived stem cell medium was replaced with 3 ml lentiviral medium [germ cell-specific gene deleted in azoospermia-like (DAZL)-green fluorescent protein (GFP) or GFP] with polybrene at 8 μg/ml, followed by incubation for 24 h at 38.5 C, after which the medium was replaced with fresh differentiation medium. After 3–5 d, cells were evaluated for GFP expression.

Immunocytochemical analysis and OLC quantitation

Differentiating PLC were collected after 2 d of MK treatment using a 0.02% EDTA incubation for 10 min. All washes and incubations were carried out at room temperature unless otherwise stated. Cells were washed twice with PBS and fixed in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) in PBS for 20 min. Cells were then washed three times and incubated for 10 min in PBS with 0.1% Tween 20 (Fisher Scientific) and then incubated in 0.1% Triton X-100 (Fisher Scientific) in PBS for 20 min. Next, cells were blocked for 2 h in PBS with 2% BSA (PBS-B), and 0.05% Triton-X-100 (blocking solution), followed by an incubation with each primary antibody at 4 C overnight: 1:400 diluted rabbit polyclonal anti-DAZL (Abcam, Cambridge, MA), 1:400 diluted rabbit polyclonal anti-synaptonemal complex protein (SCP) 3 (Novus Biologicals, Littleton, CO), and 1:400 rabbit polyclonal anti-cyclin D3 (CCND3) (Novus). The next day, cells were washed in blocking solution and incubated for 2 h with 1:300 diluted goat antirabbit IgG-R-phycoerythrin (Sigma) for DAZL or 1:300 diluted goat antirabbit IgG-fluorescein isothiocyanate (Sigma) for SCP3 and CCND3. This was followed with

TABLE 1. List of primers used for real time RT-PCR

Target	Primers (5′–3′)	Accession no. (Ref.)	Product size (bp)
DAZL	cct cca acc atg atg aat cc ggg caa aat atc agc tcc tg	Gi:31542548	222
SYCP3	agc cgt ctg tgg aag atc ag aac tcc aac tcc ttc cag ca	NM_153694	197
DMC1	ggg ata caa atg aca aca ag cga aat tct cca aaa gct tc	D64107, CV876801	239
REC8	att cga cac ctt tta gag gct g aag tct cct cga ctg atc tct g	NM_020002	203
BAX	aag cgc att gga gat gaa ct cga tct cga agg aag tcc ag	AJ606301 (51)	251
BCL2	gaa acc cct agt gcc atc aa ggg acg tca ggt cac tga at	NM214285 (52)	196
CCND3	tca cag gca ctg agt gga c gat ggc tgt gac atc tgt gg	(53)	176

a wash with PBS-B and incubation with Hoechst333258 (Sigma-Aldrich, St. Louis, MO) for 5 min, followed by three washes with PBS-B. Cells were mounted using fluorescent mount medium (DakoCytomation, Carpinteria, CA) and viewed using an Olympus BX-UCB (Center Valley, PA) microscope and MetaMorph analysis software (Universal Imaging Corp., Downingtown, PA). For semiquantitative immunocytochemistry analysis, positive cells were counted, and a ratio of positive cells to total cells was calculated for both control and MK groups, using the same exposure conditions to detect fluorescence.

Differentiating PLC were collected at d 25 and treated with MK for 2 d, before having the medium replaced with MK-free medium, at which point they were further cultured for another 18 d (d 45 of differentiation). At d 45 of differentiation, emerging OLC were counted in both control and MK groups, and the rate of OLC development was determined.

RNA interference (RNAi)

Morphologically distinct, nonadherent d 25–30 PLC were collected from 60-mm dishes of differentiating cells and counted. Cells were seeded into wells of a 24-well plate (Sarstedt) at 2×10^5 cells per well and cultured in 500 μl differentiation medium without antibiotics overnight. These cells were transfected with 20 pmol DAZL or control small interfering RNA (siRNA) using Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations. After 24 h at 38.5 C, the medium was changed. After another 48 h, these cells were collected and counted. The RNA of these cells was isolated to determine the expression of *DAZL* and *SCP3*. Sequences for DAZL siRNA are as follows: DAZL target sequence, 5′-cac aaa taa att tcc atg gta-3′; siRNA sense strand, 5′-caa aua aau uuc cau ggu att-3′; and siRNA antisense strand, 5′-uac cau gga aau uua uuu gtg-3′ (QIAGEN, Valencia, CA).

Statistical analyses

For each set of data, independent experiments were repeated at least three times, with data representing the mean ± SEM of all repeats. Data were analyzed by one-way ANOVA followed by the Tukey test for multiple comparisons to determined statistical differences between groups using GraphPad Prism analysis software. Results were considered significant at $P < 0.05$.

Results

GDNF and MK stimulate the proliferation of both PLC and PGC

After stem cell isolation from E40–45 porcine fetuses and 24 h culture, undifferentiated skin stem cells within nonadherent spheres (Fig. 1A) were formed. These spheres of skin-derived stem cells were dissociated into single cells and induced to attach and differentiate in differentiation medium. Figure 1, B–D, shows images of PLC at d 15, 20, and 25 of differentiation, respectively. Consistent with what was reported previously (1, 11), these shiny, round PLC appeared either as single cells or formed loosely adherent clusters resting on top of the existing fibroblast-like monolayer cells (Fig. 1, B–D). A subpopulation of the

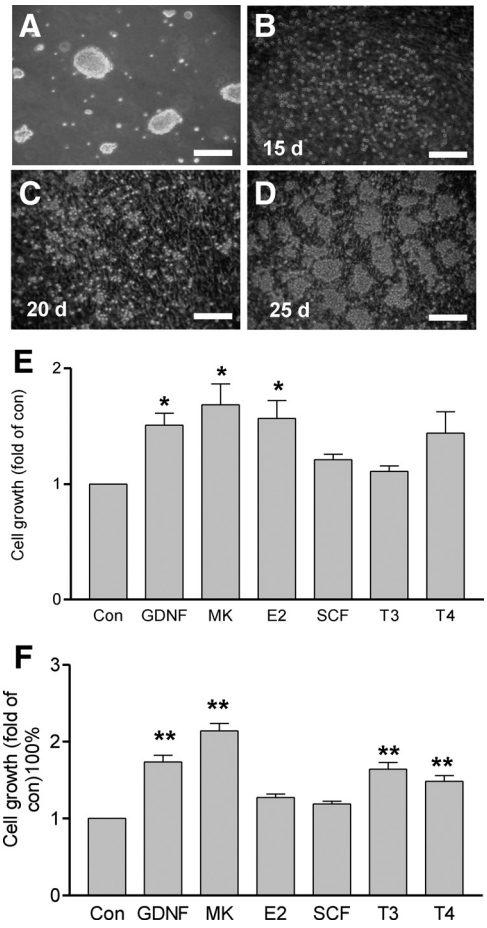

FIG. 1. Derivation and proliferation of PLC in the presence of selected mitogens. A, Spheres of skin-derived stem cells at passage 2; B–D, PLC at d 15, 20, and 25 of differentiation; E and F, proliferation response of PLC derived from skin stem cells (E) and PGC derived from fetal genital ridges (F) cultured in the absence and presence of GDNF (100 ng/ml), MK (100 ng/ml), E2 (100 nM), SCF (40 ng/ml), T_3 (100 nM), or T_4 (100 nM). Data represent the mean ± SE of four (E) and six (F) experiments, respectively. Significantly different from control: *, $P < 0.05$; **, $P < 0.01$.

round cells gradually detached and grew as suspended single or clustered cells at later differentiation time points (Fig. 1, C and D). We next screened a panel of potential growth factors and hormones for their potential role in PLC proliferation. The d-25 detached PLC were collected and cultured in the absence and presence of GDNF, MK, E2, SCF, and thyroid hormones T_4 and T_3 for 48 h. It was found that the number of PLC was increased in the presence of GDNF (1.51 ± 0.23-fold), MK (1.68 ± 0.41-fold), and E2 (1.57 ± 0.34-fold) compared with cells cultured in its absence ($P < 0.05$; Fig. 1E). To confirm the physiological relevance of this finding, the mitogenic effects of the same panel of factors on genital ridge-derived PGC were tested. As shown in Fig. 1F, the number of PGC was also increased in response to stimulation by GDNF (1.74 ± 0.15-fold) and MK (2.14 ± 0.17-fold). Interestingly, T_3 (1.65 ± 0.15-fold) and T_4 (1.48 ± 0.13-fold) also in-

creased PGC cell number by 1.65 ± 0.15-fold and 1.48 ± 0.13-fold, respectively, whereas PLC showed only trends of cell number increase to these hormones.

MK-stimulated proliferation is accompanied by decreased apoptosis

MK is a heparin-binding growth factor widely expressed in somatic tissues that is strongly induced during oncogenesis and tissue repair, although its role on PGC proliferation was unknown. Previous work on transcriptional profiling in mouse PGC revealed that the expression of MK is 30-fold higher in 10.5 d postcoitum (actively proliferating) than in 12.5 d postcoitum (transitioning from mitosis to meiosis) PGC (25), suggesting a possible role in PGC proliferation. Because MK significantly increased both PLC and PGC numbers in our screening study, we next sought to focus our study to further investigate the mechanism of MK action on PLC. The effect of different concentrations of MK, from 0–200 ng/ml, on PLC proliferation *in vitro* was assessed. As shown in Fig. 2A, the number of PLC was increased in the presence of 50 ng/ml (1.5 ± 0.22-fold) and 100 ng/ml (1.7 ± 0.20-fold) MK compared with cells cultured in its absence and other concentrations. To determine whether the increase in cell number was a consequence of cell proliferation, decreased apoptotic cell death, or both, the incorporation of the thymidine homolog BrdU was examined to assess DNA synthesis. A 1.47-fold increase in the percentage of cells incorporating BrdU was achieved when MK was added to the culture medium (36.41 ± 6.27%) compared with PLC cultured without MK (24.77 ± 3.41%, $P < 0.05$; Fig. 2B), suggesting MK stimulates PLC DNA synthesis and thus proliferation. To test whether the increase in cell number was also mediated by antiapoptosis pathways, the expression of apoptosis-related genes was analyzed using a caspase 3 activity assay and real-time RT-PCR. As shown in Fig. 2, C–E, the proliferation seen in MK-treated cells was significantly associated with a decreased level of caspase 3 activity (0.77 ± 0.08). In addition, the expression of *BAX* (0.70 ± 0.13), another apoptotic marker, was also suppressed by MK, although the expression of *BCL2* was unaffected compared with control ($P < 0.05$). This finding suggests that MK also suppress apoptosis of PLC.

MK alters expression of meiosis- and mitosis-specific genes

To test whether the observed effect of MK on PLC proliferation was mediated via an inhibition of meiosis, the expression of meiosis-specific genes was analyzed using real-time RT-PCR in PLC cultured with or without 100 ng/ml MK. Figure 3A shows that MK-stimulated proliferation was significantly associated with an inhibition of meiotic gene expression. MK decreased the expression of *DAZL* (0.132 ± 0.06), *SCP3* (0.625 ± 0.14), *DMC1* (0.27 ± 0.12), and *REC8* (0.526 ± 0.16) compared with that of cells cultured in its absence. By contrast, the expression of the mitosis-specific gene *CCND3* in PLC cultured with the same concentration of MK was increased significantly compared with the control (1.85 ± 0.28-fold). This antimeiotic effect of MK was also verified by its ability to decrease the expression of meiosis-specific genes in PGC; MK decreased the expression of meiosis-specific genes *DAZL* and *SCP3* and increased the expression of the mitosis-specific gene *CCND3* (1.655-fold) compared with that of cells cultured in its absence (Fig. 3B). Semiquantitative immunocytochemistry analysis also confirmed MK's regulation of these meiotic and mitotic genes at the protein level (Fig. 4). To investigate whether MK-stimulated PLC proliferation alters the germ cell differentiation potential, PLC were treated with MK for 2 d and further cultured in the absence of

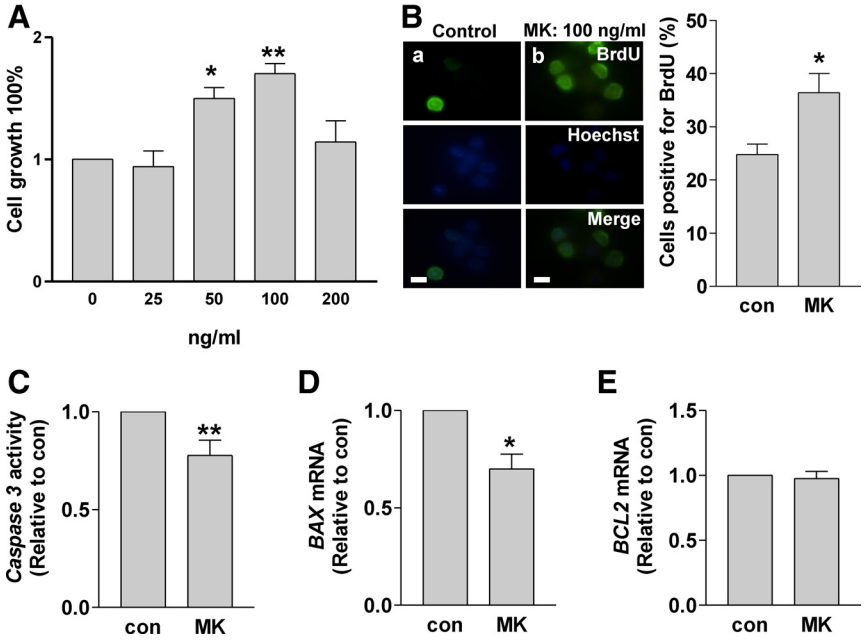

FIG. 2. Regulation by MK of PLC cell number, DNA synthesis, and apoptosis. A, Dose-dependent increase of PLC in response to MK; B, MK up-regulated DNA synthesis measured by BrdU uptake analysis; C–E, caspase 3 activity (C) and mRNA expression of *BAX* (D) and *BCL2* (E) in the absence [control (con)] and presence of MK (100 ng/ml); Real-time RT-PCR data were analyzed with the ΔΔCt method using RPII for normalization and is expressed as the fold change relative to the control. Data represent the mean ± SEM of six independent experiments. Significantly different from control: *, $P < 0.05$; **, $P < 0.01$.

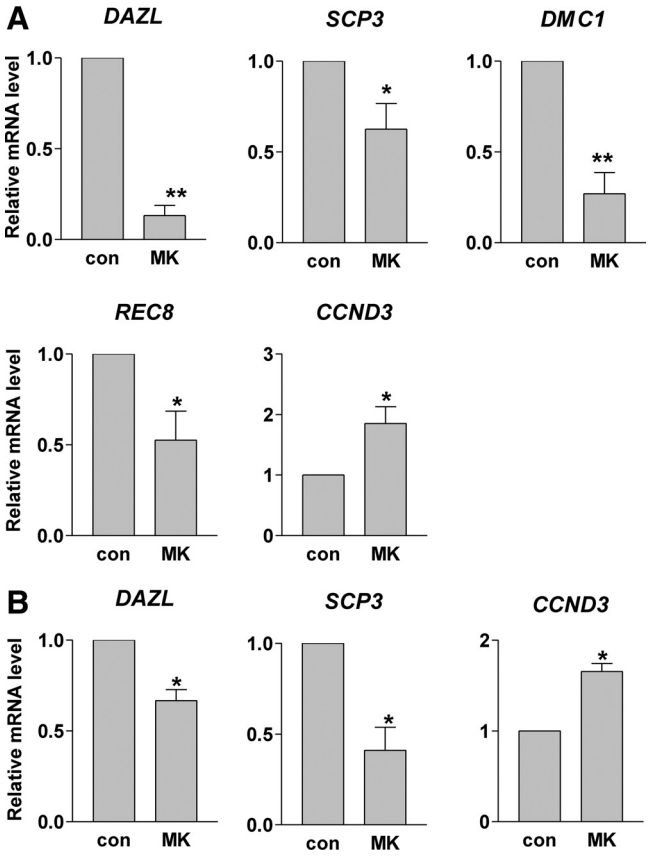

FIG. 3. MK suppresses meiosis- and increases mitosis-specific gene expression in both PLC (A) and PGC (B). Real-time RT-PCR data were analyzed with the ΔΔCt method using RPII for normalization and is expressed as the fold change relative to the control (con). Data represent the mean ± SEM of three to four independent experiments. Significantly different from control: *, $P < 0.05$; **, $P < 0.01$.

MK for another 4 and 8 d. As shown in Fig. 5A, the expression levels of *DAZL*, *SCP3*, and *DMC1* in the MK-treated group reached that of the control at d 6 (4 d after MK removal) and began showing a trend of higher expression at d 10 (8 d after MK removal). Longer culture of both groups resulted in OLC generation (Fig. 5B), with more OLC generated from those cells initially treated with MK (Fig. 5C).

Overexpression of *DAZL* by lentiviral transduction reverses MK-induced proliferation

We next used a gain-of-function approach to analyze whether the effect of MK on PLC proliferation was mediated through the inhibition of *DAZL*. We produced an EF1α-DAZL-GFP lentivirus, in which the expression of *DAZL* is driven by the EF1α promoter. The *GFP* coding sequence was linked to *DAZL* by an intraribosomal entering site so it was expressed simultaneously for monitoring the transduction efficiency. An EF1α-GFP construct was used as a control virus. Figure 6, A and B, shows images of PLC from the control (GFP) and *DAZL* overexpression groups, respectively. Figure 6, C and D, shows representative images showing the decreased PLC (shiny cell) density in the MK-stimulated *DAZL* overexpression group (D) compared with the MK control (GFP) group (C). As shown in Fig. 6E, *DAZL* overexpression not only completely reversed MK-induced cell growth but also decreased it to the level that is lower than the non-MK control (GFP). The number of DAZL-transduced PLC was significantly higher in the presence of MK compared with its control (no MK) ($P < 0.05$; Fig. 6B). Figure 6E shows confirmation of the overexpression of *DAZL* in the EF1α-DAZL-GFP lentivirus-transduced group. It is worth noting that *DAZL* mRNA levels were suppressed by MK when compared with their respective controls. Interestingly, a similar trend in expression was observed for *SPC3*, in which the mRNA level of *SCP3* in DAZL-transduced PLC was increased compared with that of the GFP-transduced cells in the control group, whereas the *SCP3* transcript level was decreased in DAZL-transduced PLC in the presence of MK compared with DAZL-transduced cells cultured in its absence (Fig. 6G).

DAZL knockdown by siRNA increases PLC proliferation

We next used a loss-of-function approach to investigate the role of DAZL on MK-induced PLC proliferation. Cells transduced with EF1α-DAZL-GFP lentivirus were cultured in the absence and presence of MK, and *DAZL* expression in PLC was knocked down using siRNA. Similar to treatment with MK, PLC transfected with DAZL-RNAi showed a 1.27-fold increase in cell number over control, although no further increase in cell proliferation was evident with a combination of MK and DAZL-RNAi ($P < 0.05$; Fig. 7A). Figure 7B shows the corresponding decrease in *DAZL* mRNA levels by DAZL-RNAi. Consistent with the cell growth response in Fig. 6A, no further decrease in the *DAZL* mRNA level was observed in the DAZL-RNAi plus MK group when compared with that of the MK group.

Discussion

The current study used our previously established stem cell-to-germ cell differentiation model (11) to screen for factors that may regulate the proliferation of PLC and the mechanism involved. It was found that PLC and PGC derived from fetal genital ridge had a proliferative response to a panel of growth factors and hormones in a similar manner. Of the factors tested, GDNF and MK were shown to significantly increase numbers of PLC and PGC. Estrogen significantly increased PLC numbers, and although

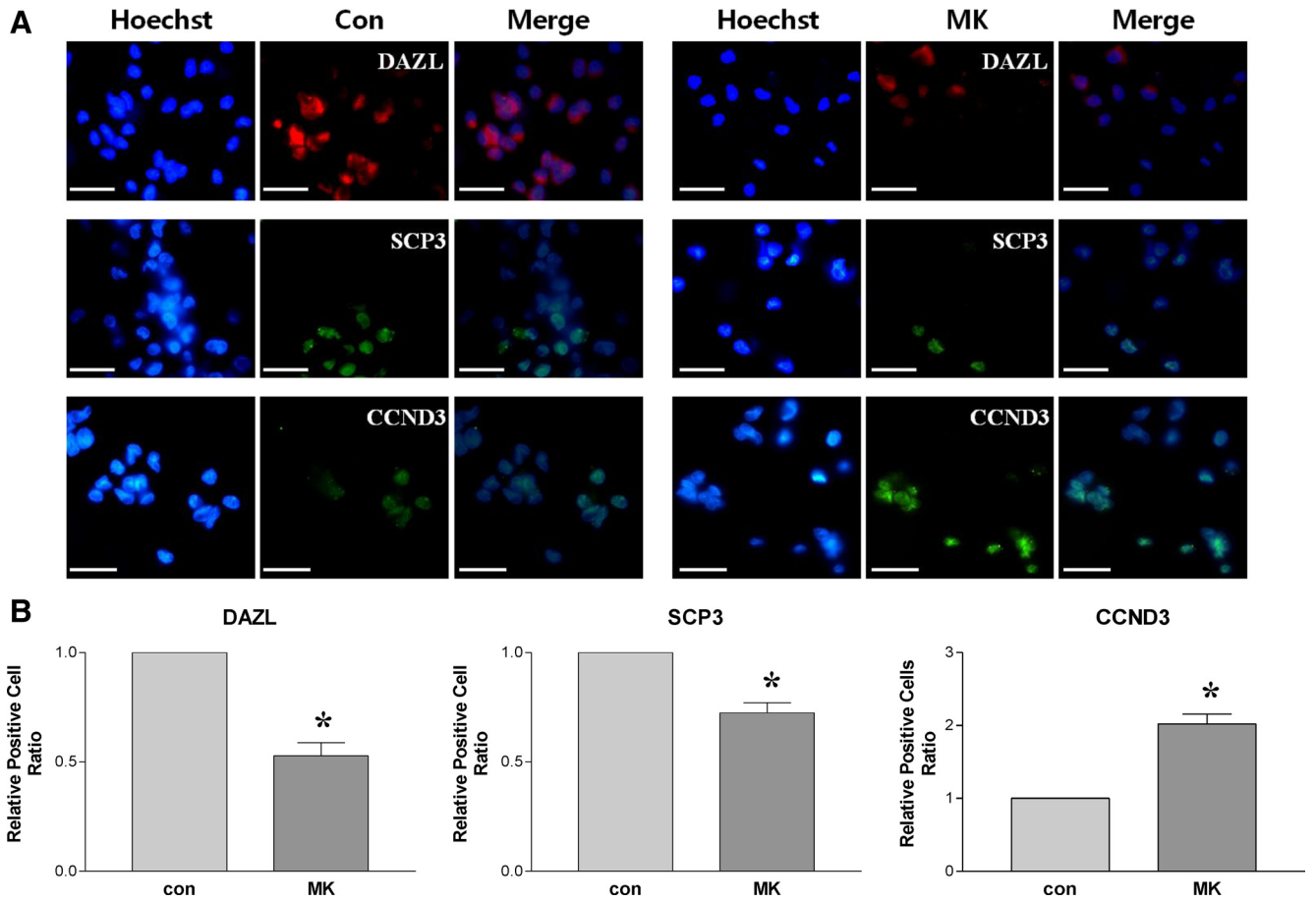

FIG. 4. MK suppresses meiosis-, and increases mitosis-specific protein expression in PLC. Immunocytochemistry and semiquantitative analysis for DAZL, SCP3, and CCND3 proteins in the MK-treated (MK) and untreated [control (con)] PLC. A, The cytoplasmic expression of DAZL and the nuclear expression of SCP3 and CCND3 were detected in the control and MK-treated PLC; B, numbers of DAZL- and SCP3-positive PLC were decreased, whereas CCND3-positive cells were increased, in the MK-treated group. Data represent the mean ± SEM of four independent experiments. *, Significantly different from control, $P < 0.05$. *Scale bar*, 50 μm.

not significant, the same trend of increased cell numbers was seen in PGC. Likewise, a similar pattern was observed for T_3 and T_4, where PGC numbers were increased significantly, whereas a trend of increased cell numbers was observed in PLC (Fig. 1, E and F). The proliferation and gene expression pattern changes in response to factors (*e.g.* MK and GDNF) between PLC and PGC in our study further suggest that stem cell-derived PLC may be an alternative *in vitro* model for PGC, which is far less hampered by limited cell numbers and difficult isolation to study early germ cell development.

MK is the product of a retinoic acid-responsive gene belonging to the family of heparin-binding growth and differentiation factors (26, 27). The role of MK in promoting cell proliferation, migration, survival, and other activities in many cell types has been well documented (for review, see Ref. 28). MK has been identified as a strong inducer of somatic tissue repair as well as oncogenesis; aberrant expression of MK is found in numerous cancers (28). Pleiotrophin (PTN), the only other known member of the PTN/MK family (29), shares 50% of its amino acid sequence with MK, and both have identical conservation of 10 cysteine residues that align in five disulfide bonds (30). Although MK- or PTN-knockout mice are born without major defects in themselves, mice deficient in both have impaired fertility (small litter size), their offspring born were small in size, and about 50% died before the age of 4 wk (28, 31), suggesting the critical and compensatory role of MK and PTN in fertility. The presence of the MK protein has been confirmed in follicular fluid, and the addition of MK to *in vitro* oocyte maturation media increased developmental competence to the blastocyst stage after *in vitro* fertilization. It was suggested that the enhancement of oocyte competence to embryo development was likely mediated by cumulus cells because the effect of MK was abolished in the absence cumulus cells (32). The same group also demonstrated that MK suppresses the apoptosis that occurs in cumulus cells during *in vitro* oocyte maturation of bovine oocyte-cumulus complexes (33). MK was also detected in granulosa cells, theca cells,

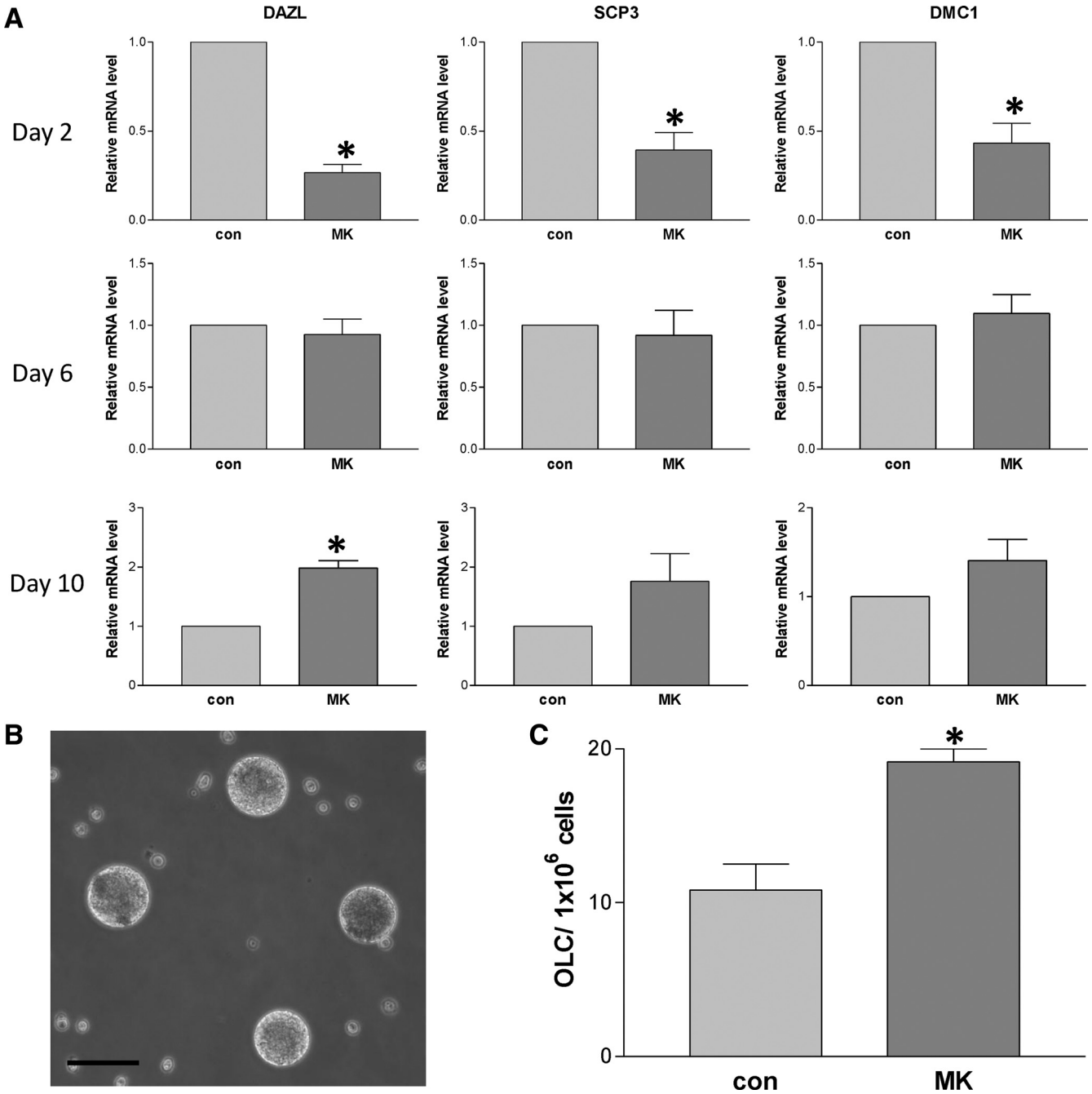

FIG. 5. Influence of MK treatment on the differentiation of PLC. The d-25 PLC were collected and cultured in the absence [control (con)] and presence (MK) of MK for 2 d. At the end of d 2, medium was replaced without MK, and PLC were cultured for 20 additional days. A, Expression levels of meiosis-related genes (*DAZL*, *SCP3*, and *DMC1*) at the end of MK treatment (d 2) as well as 4 and 8 d after MK removal (d 6 and 10, respectively) as determined by real-time RT-PCR; B, upon further differentiation (15–20 d after MK treatment), OLC were detected in both treatment groups; C, the number of OLC per 10^6 PLC was determined for MK-treated and untreated groups. Data represent the mean ± SEM of three independent experiments. *, Significantly different from control, $P < 0.05$. *Scale bar*, 200 μm.

and follicular fluid from human follicles. In addition to increased BrdU uptake by granulosa cells, the level of MK was found to be positively correlated with estradiol level in the follicular fluid (34). These findings demonstrated the role of MK in a late stage of follicular development and oocyte maturation. However, whether MK also plays a role in early germ cell development was unknown. Here we report that MK induces a shift similar to that seen in embryonic stem cells (35) away from differentiation to a more proliferative phenotype in early germ cells.

PGC enter meiosis during differentiation after arriving at the gonadal ridge. In prophase I of meiosis, paired homologous chromosomes undergo recombination. The synaptonemal complex, located along the paired meiotic

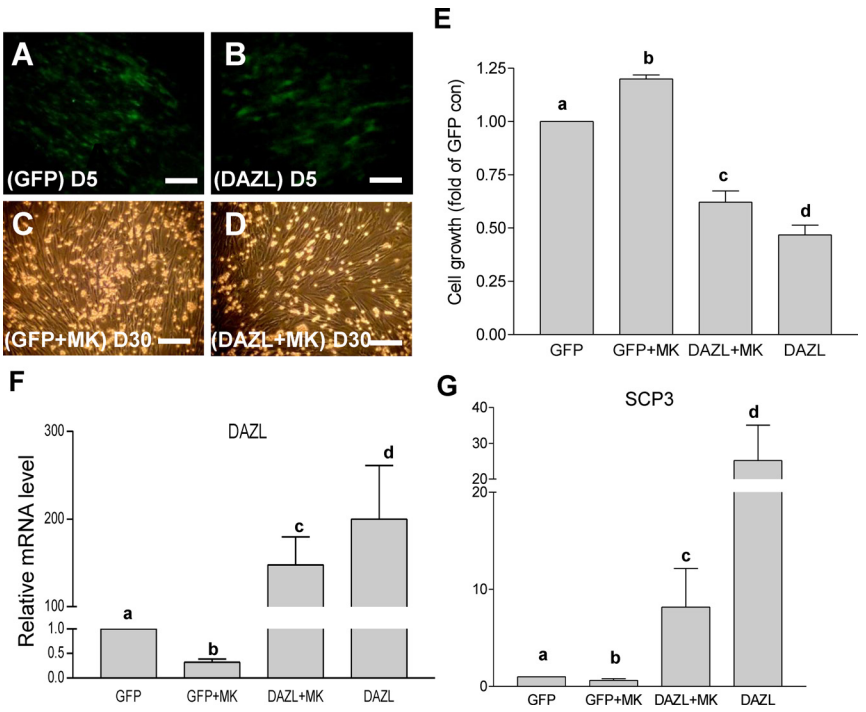

FIG. 6. Overexpression of *DAZL* suppresses MK-induced proliferation and *SCP3* expression. A and B, Representative images of EF1α-GFP (A; GFP) and EF1α-GFP-DAZL (B; DAZL) lentivirus-transduced cells at d 5 of transduction; C and D, representative images of GFP plus MK (C) and DAZL plus MK (D) showing that DAZL decreased MK-induced PLC (top layer of shiny cells) density at d 30 of transduction; E, proliferation in response to MK in DAZL-transduced and GFP-transduced (control) PLC at d 30 of differentiation; F, relative mRNA levels of *DAZL* and *SCP3* in transduced (±*DAZL*) PLC with and without MK (100 ng/ml). Data represent the mean ± SEM of four independent experiments. *Different letters* denote statistical differences between groups ($P < 0.05$).

chromosomes, is required for this process, and the *SCP3* gene is essential for synaptonemal complex assembly. SCP3 is thus regarded as a marker for identifying the meiotic transition because it is present from this initial step (36). DMC1, a mammalian homolog of RecA, is thought to play a role during chromosome synapsis and homologous recombination (37–39). In addition, the meiosis-specific cohesin subunit REC8 is reported to be involved in homologous chromosome sister chromatid separation in mice (40). *DAZL* is up-regulated in migrating PGC and remains expressed throughout postmigratory stages (41). It was recently shown in mice that Dazl interacts with *Mvh* and *Scp3* mRNA, stimulating their expression in germ cells by binding to a site within their 3′ untranslated regions via its RNA recognition motif (42, 43). In *Dazl*-knockout mice, germ cell-specific genes like *Oct4*, *Stella*, and *Mvh*, and germ cell nuclear antigen show reduced expression in the embryos (44). Dazl is an intrinsic meiotic competence factor and may have an obligatory function upstream of meiotic initiation because its expression has been shown to be necessary for the expression of *Stra8* and meiotic initiation in embryonic ovaries (45). In a comprehensive study using *Dazl*-null mice, Haston *et al.* (46) suggest that Dazl is required for the differentiation of PGC, a multifaceted cellular event that involves the preparation and entry of the cell into meiosis as well as erasure of the genomic imprint. Our finding that overexpression of *DAZL* increased expression of the meiosis marker *SCP3* by more than 20-fold, and significantly reduced numbers of PLC at the same time support the role of DAZL in suppressing mitosis and enhancing the differentiation of PGC with entry into meiosis. In contrast, addition of MK to the culture stimulated the proliferation of PLC. This finding is supported by the fact that MK increased cell number, it stimulated DNA synthesis, and it increased the expression of mitotic gene such as *CCND3*. Increased cell numbers was also the result of MK's suppression of PLC apoptosis because the activity of caspase 3 and the expression of *BAX*, important members of the apoptosis pathway (47), were decreased in the presence of MK.

Our study also showed that MK-stimulated PLC proliferation is accompanied by its suppressed expression of meiosis-associated genes such as *DAZL*, *SCP3*, *DMC1*, and *REC8*, and its enhancement of the expression of mitosis-related genes such as *CCND3*. Although the mechanism by which MK increases PGC proliferation is currently unclear, our findings suggest that it is at least in part via its suppression of *DAZL*. This notion is supported by the data that overexpression of *DAZL* completely reversed the effect of MK on cell proliferation as well as its suppression of meiotic marker expression (Fig. 6). In addition, when DAZL expression was down-regulated by siRNA, a proliferative PLC phenotype, similar to that occurring with MK stimulation, was observed.

It was recently demonstrated that MK can improve cardiac function by activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (48). The same signaling pathway has also been shown to play a role in embryonic stem cells, where MK promoted self-renewal and proliferation by inhibiting apoptosis (35). Interestingly, the activation of this pathway increased telomerase activity and thus mitosis in PGC. In fact, it was shown that CCND3 is one of the targets for the PI3K-Akt pathway that up-regulates cardiac fibroblast proliferation (49). In addition, antiapoptosis effects via the PI3K pathway were also re-

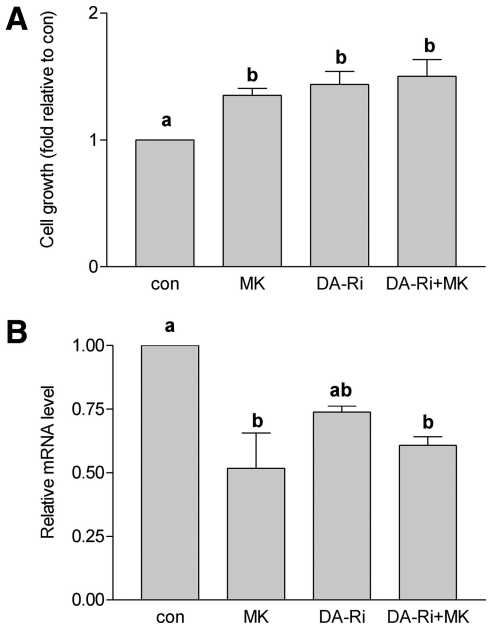

FIG. 7. Proliferation of DAZL-transduced PLC in response to DAZL RNAi knockdown. A, Effect of DAZL RNAi on the proliferation of PLC cultured with and without MK. Relative mRNA levels of *DAZL* in DAZL RNAi-treated PLC with and without MK. Real-time RT-PCR data were analyzed with the ΔΔCt method using RPII for normalization and is expressed as the fold change relative to the control (con). Data represent the mean ± SEM of three independent experiments. *Different letters* denote statistical differences between groups ($P < 0.05$). DA-Ri, DAZL-specific siRNA.

ported in germ cells within the primordial follicle (50). Whether MK exerts its mitotic-enhancing and antiapoptotic effects on PGC through this pathway is yet to be investigated.

In summary, our current study demonstrated that MK plays an important role in enhancing the proliferation of PGC. The mitotic action of MK may be mediated by its down-regulation of the expression of *DAZL* and subsequent suppression of PGC meiotic entry. Our finding uncovers the role of MK in PGC and may shed light on understanding the mechanism underlining the mitosis to meiosis switch in the early stages of germ cell development.

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References

1. **Dyce PW, Wen L, Li J** 2006 In vitro germline potential of stem cells derived from fetal porcine skin. Nat Cell Biol 8:384–390
2. **Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ** 2004 Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature 427:148–154
3. **Hübner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, Wood J, Strauss 3rd JF, Boiani M, Schöler HR** 2003 Derivation of oocytes from mouse embryonic stem cells. Science 300:1251–1256
4. **Kehler J, Tolkunova E, Koschorz B, Pesce M, Gentile L, Boiani M, Lomelí H, Nagy A, McLaughlin KJ, Schöler HR, Tomilin A** 2004 Oct4 is required for primordial germ cell survival. EMBO Rep 5:1078–1083
5. **Nayernia K, Nolte J, Michelmann HW, Lee JH, Rathsack K, Drusenheimer N, Dev A, Wulf G, Ehrmann IE, Elliott DJ, Okpanyi V, Zechner U, Haaf T, Meinhardt A, Engel W** 2006 In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. Dev Cell 11:125–132
6. **Toyooka Y, Tsunekawa N, Akasu R, Noce T** 2003 Embryonic stem cells can form germ cells in vitro. Proc Natl Acad Sci USA 100: 11457–11462
7. **West JA, Park IH, Daley GQ, Geijsen N** 2006 In vitro generation of germ cells from murine embryonic stem cells. Nat Protoc 1:2026–2036
8. **Zwaka TP, Thomson JA** 2005 A germ cell origin of embryonic stem cells? Development 132:227–233
9. **Dyce PW, Li J** 2006 From skin cells to ovarian follicles? Cell Cycle 5:1371–1375
10. **Dyce PW, Shen W, Huynh E, Shao H, Villagómez DA, Kidder GM, King WA, Li J** 2011 Analysis of oocyte-like cells differentiated from porcine fetal skin-derived stem cells. Stem Cells Dev 20:809–819
11. **Linher K, Dyce P, Li J** 2009 Primordial germ cell-like cells differentiated in vitro from skin-derived stem cells. PLoS One 4:e8263
12. **Tam PP, Snow MH** 1981 Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. J Embryol Exp Morphol 64:133–147
13. **Monk M, McLaren A** 1981 X-chromosome activity in foetal germ cells of the mouse. J Embryol Exp Morphol 63:75–84
14. **De Felici M, Scaldaferri ML, Lobascio M, Iona S, Nazzicone V, Klinger FG, Farini D** 2004 Experimental approaches to the study of primordial germ cell lineage and proliferation. Hum Reprod Update 10:197–206
15. **Dolci S, Williams DE, Ernst MK, Resnick JL, Brannan CI, Lock LF, Lyman SD, Boswell HS, Donovan PJ** 1991 Requirement for mast cell growth factor for primordial germ cell survival in culture. Nature 352:809–811
16. **De Felici M, Dolci S** 1991 Leukemia inhibitory factor sustains the survival of mouse primordial germ cells cultured on TM4 feeder layers. Dev Biol 147:281–284
17. **Resnick JL, Bixler LS, Cheng L, Donovan PJ** 1992 Long-term proliferation of mouse primordial germ cells in culture. Nature 359: 550–551
18. **Pesce M, Gioia Klinger F, De Felici M** 2002 Derivation in culture of primordial germ cells from cells of the mouse epiblast: phenotypic induction and growth control by Bmp4 signalling. Mech Dev 112: 15–24
19. **Farini D, Scaldaferri ML, Iona S, La Sala G, De Felici M** 2005 Growth factors sustain primordial germ cell survival, proliferation and entering into meiosis in the absence of somatic cells. Dev Biol 285:49–56
20. **Dyce PW, Zhu H, Craig J, Li J** 2004 Stem cells with multilineage potential derived from porcine skin. Biochem Biophys Res Commun 316:651–658
21. **Linher K, Cheung Q, Baker P, Bedecarrats G, Shiota K, Li J** 2009 An epigenetic mechanism regulates germ cell-specific expression of the

porcine deleted in azoospermia-like (DAZL) gene. Differentiation 77:335–349
22. **Shim H, Anderson GB** 1998 In vitro survival and proliferation of porcine primordial germ cells. Theriogenology 49:521–528
23. **Takabayashi S, Sasaoka Y, Yamashita M, Tokumoto T, Ishikawa K, Noguchi M** 2001 Novel growth factor supporting survival of murine primordial germ cells: evidence from conditioned medium of ter fetal gonadal somatic cells. Mol Reprod Dev 60:384–396
24. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2^{--C}T method. Methods 25:402–408
25. **Molyneaux KA, Wang Y, Schaible K, Wylie C** 2004 Transcriptional profiling identifies genes differentially expressed during and after migration in murine primordial germ cells. Gene Expr Patterns 4:167–181
26. **Matsubara S, Take M, Pedraza C, Muramatsu T** 1994 Mapping and characterization of a retinoic acid-responsive enhancer of midkine, a novel heparin-binding growth/differentiation factor with neurotrophic activity. J Biochem 115:1088–1096
27. **Tsutsui J, Kadomatsu K, Matsubara S, Nakagawara A, Hamanoue M, Takao S, Shimazu H, Ohi Y, Muramatsu T** 1993 A new family of heparin-binding growth/differentiation factors: increased midkine expression in Wilms' tumor and other human carcinomas. Cancer research 53:1281–1285
28. **Muramatsu T** 2010 Midkine, a heparin-binding cytokine with multiple roles in development, repair and diseases. Proc Jpn Acad Ser B Phys Biol Sci 86:410–425
29. **Deuel TF, Zhang N, Yeh HJ, Silos-Santiago I, Wang ZY** 2002 Pleiotrophin: a cytokine with diverse functions and a novel signaling pathway. Arch Biochem Biophys 397:162–171
30. **Li YS, Milner PG, Chauhan AK, Watson MA, Hoffman RM, Kodner CM, Milbrandt J, Deuel TF** 1990 Cloning and expression of a developmentally regulated protein that induces mitogenic and neurite outgrowth activity. Science 250:1690–1694
31. **Zou P, Muramatsu H, Sone M, Hayashi H, Nakashima T, Muramatsu T** 2006 Mice doubly deficient in the midkine and pleiotrophin genes exhibit deficits in the expression of beta-tectorin gene and in auditory response. Lab Invest 86:645–653
32. **Ikeda S, Ichihara-Tanaka K, Azuma T, Muramatsu T, Yamada M** 2000 Effects of midkine during in vitro maturation of bovine oocytes on subsequent developmental competence. Biol Reprod 63:1067–1074
33. **Ikeda S, Saeki K, Imai H, Yamada M** 2006 Abilities of cumulus and granulosa cells to enhance the developmental competence of bovine oocytes during in vitro maturation period are promoted by midkine; a possible implication of its apoptosis suppressing effects. Reproduction 132:549–557
34. **Hirota Y, Osuga Y, Nose E, Koga K, Yoshino O, Hirata T, Yano T, Tsutsumi O, Sakuma S, Muramatsu T, Taketani Y** 2007 The presence of midkine and its possible implication in human ovarian follicles. Am J Reprod Immunol 58:367–373
35. **Yao X, Tan Z, Gu B, Wu RR, Liu YK, Dai LC, Zhang M** 2010 Promotion of self-renewal of embryonic stem cells by midkine. Acta Pharmacol Sin 31:629–637
36. **Yuan L, Liu JG, Zhao J, Brundell E, Daneholt B, Höög C** 2000 The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility. Mol Cell 5:73–83
37. **Pittman DL, Cobb J, Schimenti KJ, Wilson LA, Cooper DM, Brignull E, Handel MA, Schimenti JC** 1998 Meiotic prophase arrest with failure of chromosome synapsis in mice deficient for Dmc1, a germline-specific RecA homolog. Mol Cell 1:697–705
38. **Sato S, Hotta Y, Tabata S** 1995 Structural analysis of a recA-like gene in the genome of *Arabidopsis thaliana*. DNA Res 2:89–93
39. **Yoshida K, Kondoh G, Matsuda Y, Habu T, Nishimune Y, Morita T** 1998 The mouse RecA-like gene Dmc1 is required for homologous chromosome synapsis during meiosis. Mol Cell 1:707–718
40. **Lee J, Okada K, Ogushi S, Miyano T, Miyake M, Yamashita M** 2006 Loss of Rec8 from chromosome arm and centromere region is required for homologous chromosome separation and sister chromatid separation, respectively, in mammalian meiosis. Cell Cycle 5:1448–1455
41. **Yen PH** 2004 Putative biological functions of the DAZ family. Int J Androl 27:125–129
42. **Reynolds N, Collier B, Bingham V, Gray NK, Cooke HJ** 2007 Translation of the synaptonemal complex component Sycp3 is enhanced in vivo by the germ cell specific regulator Dazl. RNA 13: 974–981
43. **Reynolds N, Collier B, Maratou K, Bingham V, Speed RM, Taggart M, Semple CA, Gray NK, Cooke HJ** 2005 Dazl binds in vivo to specific transcripts and can regulate the pre-meiotic translation of Mvh in germ cells. Hum Mol Genet 14:3899–3909
44. **Lin Y Page DC** 2005 Dazl deficiency leads to embryonic arrest of germ cell development in XY C57BL/6 mice. Dev Biol 288:309–316
45. **Lin Y, Gill ME, Koubova J, Page DC** 2008 Germ cell-intrinsic and -extrinsic factors govern meiotic initiation in mouse embryos. Science 322:1685–1687
46. **Haston KM, Tung JY, Reijo Pera RA** 2009 Dazl functions in maintenance of pluripotency and genetic and epigenetic programs of differentiation in mouse primordial germ cells in vivo and in vitro. PLoS One 4:e5654
47. **Rucker EB 3rd, Dierisseau P, Wagner KU, Garrett L, Wynshaw-Boris A, Flaws JA, Hennighausen L** 2000 Bcl-x and Bax regulate mouse primordial germ cell survival and apoptosis during embryogenesis. Mol Endocrinol 14:1038–1052
48. **Takenaka H, Horiba M, Ishiguro H, Sumida A, Hojo M, Usui A, Akita T, Sakuma S, Ueda Y, Kodama I, Kadomatsu K** 2009 Midkine prevents ventricular remodeling and improves long-term survival after myocardial infarction. Am J Physiol 296:H462–H469
49. **Harding P, LaPointe MC** 2011 Prostaglandin E2 increases cardiac fibroblast proliferation and increases cyclin D expression via EP1 receptor. Prostaglandins Leukot Essent Fatty Acids 84:147–152
50. **Jin X, Han CS, Yu FQ, Wei P, Hu ZY, Liu YX** 2005 Anti-apoptotic action of stem cell factor on oocytes in primordial follicles and its signal transduction. Mol Reprod Dev 70:82–90
51. **Lee K, Wang C, Chaille JM, Machaty Z** 2010 Effect of resveratrol on the development of porcine embryons produced in vitro. J Reprod Dev 56:330–335
52. **Kumar BM, Jin HF, Kim JG, Ock SA, Hong Y, Balasubramanian S, Choe SY, Rho GJ** 2007 Differential gene expression patterns in porcine nuclear transfer embryos reconstructed with fetal fibroblasts and mesenchymal stem cells. Dev Dyn 236:435–446
53. **Liu GY** 2009 cDNA cloning, sequence identification and tissue expression distribution of three novel porcine genes: UCHL3, RIT1 and CCND3. Mol Biol Rep 36:521–528