Dose-Dependent Functions of FGF9

in Male Primordial Germ Cell Differentiation

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ABSTRACT

Primordial Germ Cells (PGCs) are the precursors of gametes. For PGC male differentiation, (i) inhibition of meiosis and (ii) male-inducing factor(s) are essential. It was recently revealed that Fibroblast Growth Factor 9 (FGF9) is one of male-inducing factors. Using isolated PGC culture system, we examined the function of FGF9 in PGC differentiation. We found low FGF9 treatment (0.2 ng/ml) induced PGC male differentiation. Conversely, high FGF9 treatment drastically stimulated PGC proliferation (40%) compared with the control and low FGF9 groups (5~10%). As a result, high FGF9 treatment (25 ng/ml) prevented PGCs to enter the male pathway. Also, we demonstrated that high FGF9 induced ERK1/2 signaling activation for stimulating PGC proliferation while low FGF9 enhanced phosphorylation of p38 signaling to push PGCs into the male pathway.

CHAPTER 1. INTRODUCTION

1.1 Primordial Germ Cell Development

Our bodies consist of two different cell types: somatic cells and germ (sperm or egg) cells. Somatic cells are essential for building up our bodies. On the other hand, germ cells generate next generation after fertilization. Germ cells have two different sexes (male, female), and they maintain both genetic integrity and diversity. All these features make germ cells uniquely valuable in our bodies.

Primordial germ cells (PGCs) are the embryonic precursors of germ cells. In mice, the extra-embryonic ectoderm produces signals, such as BMP4, BMP2, and BMP8b, at 6.0 day post coitum (dpc) (Western, 2009; Ewen and Koopman, 2010). These signals induce proximal epiblast cells to become the precursors of both PGCs (Western, 2009; Ewen and Koopman, 2010) and the extra-embryonic mesoderm. These cells migrate toward the posterior and drop out of the epiblast. By 7.25 dpc, these cells form a cluster and the cells located in the center acquire their PGC characteristics by expressing PGC-specific genes. Between 8.5 and 10.5 dpc, PGCs proliferate and migrate toward the gonad, future testis or ovary (Western, 2009; Ewen and Koopman, 2010). By 11.5 dpc, all of PGCs reach the gonad. Thereafter, XX and XY gonadal somatic cells undergo sex differentiation to form female and male gonads by 12.5 dpc (Brennan and Capel, 2004; Kim and Capel, 2006; Kocer et al., 2009). After the gonadal sex differentiation occurs, PGCs perform sex differentiation by 13.5 dpc. XX PGCs in the female gonad immediately enter meiosis to become female germ cells. In contrast to this, XY PGCs in

the male gonad enter mitotic arrest (G_0) and differentiate into male germ cells (Kocer et al., 2009; Western, 2009; Ewen and Koopman, 2010; Bowles and Koopman, 2013).

1.2 Sex Differentiation of Gonadal Somatic Cells

Gonadal environment determines the sex fate of PGCs. Molecular cues generated from gonadal supporting cells prompt PGCs to become male or female germ cells regardless of their sex chromosomes. Before sex differentiation, XX and XY gonads are morphologically equal. At 10.5 dpc, Y-specific *Sry* (Sex-determining Region of Y chromosome) expression occurs only in XY supporting cells to become pre-Sertoli cells (Brennan and Capel, 2004; Kim and Capel, 2006; Kocer et al., 2009). *Sry* promotes *Fgf9* and *Sox9* (*Sry-box 9*) expression to promote the pre-Sertoli cell differentiation. Then, pre-Sertoli cells enclose XY PGCs to form the male-specific testis cord by 12.5 dpc. Testis cord formation is essential for testis differentiation in mammals. XY PGCs inside the testis cord are isolated from interstitial space.

In the XX gonad, on the contrary, female-specific gene expression induce granulosa cell differentiation (Brennan and Capel, 2004; Kim and Capel, 2006; Kocer et al., 2009).

1.3 Sex Differentiation of PGCs

After gonadal supporting cells initiate sex differentiation, PGCs undergo their sex differentiation. By 13.5 dpc, XX PGCs in the female gonad immediately initiate meiosis to differentiate into female germ cells. On the other hand, XY PGCs in the male gonad

enter mitotic arrest (G_0) to differentiate into male germ cells (Kocer et al., 2009; Western, 2009; Ewen and Koopman, 2010; Bowles and Koopman, 2013).

In 2006, it was revealed that retinoic acid (RA) is a meiosis inducing factor (Koubova et al., 2006; Bowles et al., 2006). RA is produced in the adjacent mesonephros to be distributed into both the female and male gonads (Bowles and Koopman, 2007). In the female gonad, XX PGCs are easily exposed to RA, and resulting *Stra8* (Stimulated by Retinoic Acid 8) expression occurs in XX PGCs to initiate the meiotic process (Koubova et al., 2006; Bowles et al., 2006).

In the male gonad, XY PGCs are surrounded by Sertoli cells in the testis cord. Sertoli cells generate CYP26B1 (Cytochrome P450, family 26, subfamily B, polypeptide 1), a RA degenerating enzyme (Koubova et al., 2006). Therefore, XY PGCs are protected from any exposure to RA, and this results in entry into mitotic arrest to differentiate into male germ cells.

1.4 Male Factors for PGC Male Differentiation

The next question is whether inhibiting entry into meiosis is sufficient to induce PGC male differentiation or not. We have previously reported that inhibiting RA signaling is not sufficient for PGC male differentiation, but that some molecule(s) generated by stage-specific Sertoli cells are essential for this pathway (Ohta et. al., 2012). Guerquin et al. (2010) revealed that male gonads secrete diffusible factor(s) to induce PGC male pathway, as well as to inhibit entry into meiosis. They performed a co-culture experiment with an ovary and a liver or a fetal testis. As a result, XX PGCs in the ovary co-cultured with the testis enter mitotic arrest and express male specific genes. On the

other hand, XX PGCs in the ovary co-cultured with the liver enter meiosis and do not upregulate expression of the male specific genes. These findings strongly suggest that some molecules generated from Sertoli cells and/or gonadal somatic cells are necessary to induce male pathway.

1.5 Various Functions of FGF9 in the Male Gonad

Fibroblast growth factors (FGFs) are one of the growth factor families. To date 22 members of this family have been identified in human and mouse (Itoh and Ornitz, 2004). FGFs are mostly secreted proteins, except for FGF11-14 and FGF22 (Itoh and Ornitz, 2004). These FGF ligands bind to FGF receptors (FGFRs) to regulate diverse biological activities including patterning, morphogenesis, differentiation, cell proliferation, and migration (Thisse and Thisse, 2005). FGF9, one of the FGF family members, contributes to mammalian testis differentiation. In the fetal XY gonad, Y chromosome-specific *Sry* expression triggers *Sox9* upregulation to induce the male differentiation pathway. *Sox9* increases *Fgf9* mRNA transcription in the coelomic epithelial cells, then precursors of Sertoli cells proliferate to form the developing testis (Brennan and Capel, 2004).

FGF9 contributes exclusively to the proliferation of Sertoli precursor cells in the XY gonad. In FGF9-/- XY gonads, coelomic proliferation is markedly reduced, resulting male-to-female sex reversal is performed (Colvin et al., 2001). Interestingly, FGF9 also contributes to PGC development. It has been reported that FGF9 treatment directly increases the survival of 11.5 dpc XY PGCs (DiNapoli et al., 2006). Furthermore, Barrios et al. (2010) demonstrated that FGF9 treatment in the male and female gonads stimulates male-specific *Nanos2* expression in the PGCs to suppress meiotic process. They

suggested that FGF9 and RA regulate the expression of *Nanos2* in opposite ways, regarding entry into meiosis in PGCs (Barrios et al., 2010). Furthermore, Bowles et al. (2010) claim that FGF9 acts directly on PGCs to inhibit meiosis; in addition, FGF9 upregulates the PGC male-specific genes. They concluded that two independent and mutually antagonistic pathways involving RA and FGF9 act in concert to determine PGC male differentiation (Bowles et al., 2010).

1.6 MAPK Signaling Pathways

FGFs contributes to various important roles in mammalian development (Ornitz and Itoh, 2001). During embryonic development, FGFs have diverse roles in regulating cell proliferation, migration and differentiation (Ornitz and Itoh, 2001). FGFs function through the activation of their tyrosine kinase receptors, the FGF receptors (Thisse and Thisse, 2005). FGF receptors transmit extracellular signals to various cytoplasmic signal transduction pathways, such as mitogen-activated protein kinase (MAPK) and AKT. The best understood of these are the RAS-MAP kinase pathways which include ERK1/2, p38 and JNK kinases; the PI3K-AKT pathway, and the PLCγ pathway (Dailey et al., 2005).

In MAPK signal transduction pathways, it has been reported that ERK1/2 signaling stimulates cell proliferation, adhesion, migration, survival, and differentiation (Roskoski, 2012); p38 signaling regulates differentiation, inflammation, cell-cycle regulation, and cell death (Coulthard et al., 2009); JNK signaling plays roles in cell death and survival (Weston and Davis, 2007). Interestingly, MAPK signaling pathways affect germ cell proliferation and sex differentiation. ERK1/2 signaling contributes to avian PGCs proliferation (Choi et al., 2010) as well as mouse spermatogonial proliferation

(Dolci et al., 2001; He et al., 2008). It has been reported that p38 regulates *Sry* expression to produce differentiated Sertoli cells (Bogani et al., 2009; Gierl et al., 2012; Warr et al., 2012). Furthermore, p38 signaling is activated in the mouse germ cells after PGC male differentiation (Ewen et al., 2010).

In this project, we investigated effects of FGF9 at different concentrations on PGC differentiation. Using our germ cell culture system (Iwahashi et al, 2007), we revealed that low FGF9 (0.2 ng/ml) induces PGC male differentiation while high FGF9 (25 ng/ml) promotes PGC proliferation. We also demonstrated that high FGF9 suppresses PGC male differentiation. Moreover, we indicated that low FGF9 activates p38 signaling pathway to induce PGC male differentiation, whereas high FGF9 predominantly activates ERK1/2 signaling to stimulate PGC proliferation.

CHAPTER 2. MATERIALS AND METHODS

2.1 Animals

Transgenic mice expressing germ cell-specific green fluorescent protein (GFP) driven by the Pou5f1 (Oct4) gene promoter/enhancer (Tg OG2) were generated by microinjecting (CBA/Caj X C57BL/6J) F_2 zygotes (a generous gift from Dr. Jeff R. Mann, University of Melbourne, Melbourne, Victoria, Australia) (Szabó et al., 2002). Female CD1 (Jackson Laboratory) or Swiss (National Cancer Institute) mice were mated with Tg OG2 homozygous males to produce (CD1 X OG2) F_1 hybrid fetuses. Mating plug was checked the following day morning and the noon of the day was accepted 0.5 day post coitum (dpc). Pregnant mice were sacrificed by cervical dislocation and F_1 fetuses removed into cold Phosphate Saline Buffer (PBS; Invitrogen, Carlsbad, CA) and GFP-positive Primordial Germ Cells (PGCs) were obtained from these F_1 fetuses. All experiments involved mice were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Hawaii.

2.2 Media

Gonads were dissected in HEPES-Dulbecco Modified Eagle Medium (DMEM) (Invitrogen) with 15% Fetal Bovine Serum (FBS) (Hyclone Laboratories, Logan, UT). Isolated PGCs were cultured in high-glucose DMEM supplemented with 0.1 mM nonessential amino acids, 0.1 mM 2-merkaptoethanol, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 15% FBS. Depend on the purpose, various concentrations of Fibroblast Growth Factor 9 – 0, 0.2, 1, 5, 25, and 100

ng/ml – (FGF9) (Sigma-Aldrich, St. Louis, MO), 10 µM SB202190 – a p38 inhibitor – (Sigma-Aldrich), 10 µM BIRB796 – a p38 inhibitor – (Cayman Chemical Company, Ann Arbor, MI), or 10 µM U0126 – an ERK1/2 inhibitor – (Cayman Chemical Company, Ann Arbor, MI) were added to the culture medium.

2.3 Genotyping

Fetuses were sexed based on testis cord formation at 12.5 dpc. Sexing of 11.5 dpc fetuses was determined by PCR using Sex-Region of Y chromosome (*Sry*) specific primers (*5' – CTGTGTAGGATCTTCAATCTCT – 3' and 5' – GTGGTGAGAGGCACAAGTTGGC – 3'*) (Chuma and Nakatsuji, 2001). Heads of the fetuses were individually collected and boiled for 10 min and centrifuged with 13200 rpm at 4 ˚C for 5 min and supernatant was used for the sexing.

2.4 Germ Cell Culture

To isolate PGCs, male and female gonads were obtained from (CD1 X OG2) F_1 hybrid fetuses at 11.5 and 12.5 dpc. Gonads were dissected in HEPES- DMEM with 15% FBS and incubated in 0.2% Collagenase (Calbiochem, San Diego, CA) and Accumax (Innovative Cell Technologies, San Diego, CA) for 10 min at 37 ˚C. After enzymatic treatment, gonads were mechanically dissociated by using a 40 µm cell strainer (BD Falcon, Franklin Lakes, NJ) to prepare single cell suspension. GFP-positive male or female germ cells were sorted by using a FACSAria system (BD Bioscience, San Jose, CA). Purity of germ cells was more than 99.8%.

About 20,000 male or female germ cells were cultured on collagen-coated mesh inserts (Corning Life Science, Lowell, MA) (Iwahashi et al., 2007). The germ cells were cultured at 37 ˚C with 5% CO_2 in air for 1-3 days.

2.5 Germ Cell Collection after Culture

After 1-3 days of culture, 200-800 isolated germ cells were manually collected as one set with glass pipettes (diameter 40 μm).

2.6 Quantitative Gene Expression Analysis

cDNA was synthesized from 200-800 isolated germ cells by using SuperScript III Cells Direct cDNA synthesis kit (Invitrogen). Quantitative PCR analysis was performed by using MyIQ Single-Color Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA). 3-5 experiments were performed independently for each treatment group. The sequence of primers used were as follows (forward and revers, respectively): for *Dnmt3L*, 5' – GTGCGGGTACTGAGCCTTTTTAGA – 3' and 5' – CGACATTTGTGACATCTTCCACGTA – 3'; for *Stra8*, 5' – GTTTCCTGCGTGTTCCACAAG – 3' and 5' – CACCCGAGGCTCAAGCTTC – 3'; for *Actb*, 5' – CCTGTATGCCTCTGGTCGTA – 3' and 5' – CCATCTCCTGCTCGAAGTCT – 3'; and for *Nanos2*, Applied Biosystem Mm02525720_s1.

The relative expression of target mRNAs was calculated from target C_T values and Actb C_T values, using the standard curve method. Results were normalized to Actb gene expression (Ohta et al., 2012).

2.7 Cell Proliferation Analysis

Cell proliferation analysis was performed by using Click-IT EdU Alexa Flour HCS assay kit (Invitrogen). After two days of culture, male germ cells were incubated with EdU for 5 h at 37 ˚C. After incubation, approximately 200 germ cells were placed on glass slides and treated according to the manufacturer's instructions. EdU-positive cells were counted using an Axio Scope A1 microscope (Carl Zeiss Microimaging, Göttingen, Germany).

2.8 Protein Preparation and Western Blotting

For western blot analysis, germ cells were harvested and washed three times with ice-cold PBS. Cells were lysed with lysis buffer containing 50 mM Tris-HCl pH7.5 (Sigma-Aldrich), 1% TritonX-100 (Sigma-Aldrich), 150mM NaCl, and protease and phosphatase inhibitor cocktail (Cell Signaling, Danvers, MA). Lysates were centrifuged at 13,200 rpm for 10 min at 4 ˚C. The entire supernatants were mixed with 3.5X concentrated SDS sample buffer containing 62.5 mM Tris (Sigma-Aldrich), 2% SDS (Sigma-Aldrich), 10% glycerol (Sigma-Aldrich), 50 mM DTT (Promega, Madison, WI), 0.01% BPB (J. T. Baker Chemical Co., Phillipsburg, NJ) and denatured for 10 min at 95 ˚C. Polypeptides in the samples containing 10,000 cells were separated by SDS-PAGE in stacking gels – 5% (v/v) polyacrylamide – for 30 min at 80 V and separating gel – 10% (v/v) polyacrylamide – for 1 h at 200 V by using electrophoresis buffer containing 25 mM Tris (pH8.3), 192 mM glycine, and 0.1% SDS. After that, polypeptides was electrotransferred to nitrocellulose membranes (GE Healthcare, Amersham Hybond-P, Buckinghamshire, UK) by using blotting buffer containing 25 mM Tris (pH8.3), 192 mM

glycine, and 20% MeOH for 20 min at 20 V on Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell system. The membranes were blocked with blocking buffer containing Tris Based Saline (TBS), 0.1% (v/v) Tween-20 (Bio-Rad), and 5% (w/v) non-fat dry milk (Cell Signaling) – for 1 hour at room temperature. Then, membranes were probed with primary antibodies (1:1000 dilution) against β-Actin (Cell Signaling), total ERK1/2 (Cell Signaling), phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling), total p38 (Cell Signaling), phospho-p38 (Thr180/Tyr182) (Cell Signaling), total JNK (Cell Signaling), phospho-JNK (Thr183/Tyr185) (Cell Signaling), total AKT (Cell Signaling), and phospho-AKT (Ser473) (Cell Signaling) overnight with gentle shaking at 4 ˚C in TBS/T-5%(w/v) Bovine Serum Albumin (BSA). The following day, appropriate anti-rabbit IgG, HRP-linked secondary antibody (Cell Signaling) (1:1000 dilution) in blocking buffer was used for 1 hour at room temperature. The membrane was subjected on X-Ray film (GE Healthcare, Amersham Hyperfilm ECL, Buckinghamshire, UK) to visualize the protein bands.

CHAPTER 3. RESULTS

3.1 SA1: How External FGF9 Treatment Affects PGC Differentiation

Our previous study demonstrated that blocking of meiosis initiation is required for Primordial Germ Cell (PGC) male differentiation, but it is not sufficient (Ohta et al., 2012). Also, in the same study, our Sertoli + PGC aggregation culture system proved that in addition to meiosis inhibition, unknown male factor(s) from Sertoli cells is/are essential to push PGCs toward male pathway (Ohta et al., 2012). Recently, it has been shown that Fibroblast Growth Factor 9 (FGF9) directly induces PGC male differentiation (Bowles et al., 2010). This discovery makes FGF9 one of male-inducing factor candidate.

3.1.1 The effect of FGF9 on PGC male differentiation

First, in order to clarify the effect of FGF9 on PGC male differentiation, we examined expression of *DNA methyltransferase 3-like* (*Dnmt3l*) – a male specific gene – in 12.5 day post coitum (dpc) XY PGCs which cultured with various concentrations of FGF9 (0, 0.2, 1, 5, 25, and 100 ng/ml) for two days. *Dnmt3l* expression peaked at 1.5 times higher than the control under 0.2 ng/ml FGF9 treatment (Fig. 5). Then, expression levels of *Dnmt3l* were gradually decreased in the PGCs treated with higher concentrations of FGF9 (1-100 ng/ml) (Fig. 5). In a dose-dependent manner, *Dnmt3l* expression levels were the lowest in PGCs treated with 25 or 100 ng/ml FGF9 (Fig. 5). These data shows that low concentration of FGF9 (0.2 ng/ml) induces PGC male differentiation; in contrast, high concentration of FGF9 (25 or 100 ng/ml) inhibits PGCs entry towards the male pathway, although both not statistically significant ($p=0.75$, $p=0.95$, respectively). In the

following experiments, we examined effects of low (0.2 ng/ml) and high (25 ng/ml) FGF9 on PGC differentiation.

Next, 12.5 dpc XY PGCs were cultured for 3 days to confirm the influence of the low and high FGF9 in PGC male differentiation. The low FGF9 treatment increased *Dnmt3l* expression in the XY PGCs almost three times higher than the control (Fig. 6A). In contrast, high FGF9 treatment suppressed *Dnmt3l* expression in the cells, as shown previously (Fig. 6A). This findings are the same with our previous results (Fig. 5), and statistically significant ($p<0.05$).

To identify whether the PGCs exposed to the high FGF9 concentration enter the female pathway or not, we analyzed *Stimulated by Retinoic Acid gene 8* (*Stra8*) –a premeiotic marker– expression. We found that FGF9 treatment did not stimulate *Stra8* expression in XY PGCs regardless of the concentration (Fig. 6B), and not statistically significant ($p=0.20$, $p=0.13$). These results suggest that (1) low FGF9 treatment stimulates 12.5 dpc XY PGCs to enter the male pathway, and (2) high FGF9 treatment suppresses PGCs entry into either the male or female pathway.

3.1.2 The effect of FGF9 on undifferentiated PGCs

Since Sertoli cells have already been differentiated by 12.5 dpc, PGCs which are at this stage might have been exposed to some factor(s) from Sertoli cells. To avoid the influence of the unknown factor(s), we examined the effect of low and high FGF9 on undifferentiated XY and XX PGCs at 11.5 dpc. Under low FGF9 treatment, male-specific *Nanos2* expression became 7 times higher in XY PGCs relative to the control and the expression gradually decreased in a dose-dependent manner (25 and 100 ng/ml) (Fig. 7), and statistically significant ($p<0.05$). Conversely, in the XX PGCs, *Nanos2* expression

was not upregulated by the low or high FGF9 treatment (Fig. 7), and not statistically significant ($p=0.55$, $p=0.56$). These data shows that (1) low FGF9 treatment induces undifferentiated XY PGCs, to enter male-pathway, (2) high FGF9 treatment suppresses male differentiation in undifferentiated XY PGCs, and (3) FGF9 treatment does not influence undifferentiated XX PGCs to enter the male pathway.

3.1.3 Cell proliferation under FGF9 treatment

It is known that FGF9 stimulates cell proliferation in Sertoli cells (Brennan and Capel, 2004). Therefore, we examined the effect of FGF9 on 12.5 dpc XY PGC proliferation using EdU assay system. In the control group and PGCs treated with low FGF9, only 5 or 10 % of the cells became EdU positive, respectively (Fig. 8A, 8B). Under high FGF9 treatment, on the contrary, 40% of the PGCs became EdU positive and significantly increased their cell proliferation (Fig. 8A, 8B) ($p<0.05$). The results suggest that high FGF9 treatment drastically stimulates PGC proliferation, instead of inducing entry into male or female pathway.

3.2 SA2: What Signal Transduction Pathways Are Activated by FGF9 Treatment?

From our results, we concluded low FGF9 induces PGC male differentiation while high FGF9 stimulates PGC proliferation. Next, we hypothesized that these different functions of FGF9 might be caused by different signal transduction pathways activation. In order to verify this idea, we examined MAPK (ERK1/2, p38, JNK) and AKT signaling under low or high FGF9 treatments in 12.5 dpc XY PGCs. We chose these pathways

because it has been already known that growth factors stimulate these pathways and the cells are differentiated or proliferated at the end of the signaling cascades. Furthermore, it has been published that p38 signaling is activated in XY PGCs before sex differentiation (Ewen et al., 2010).

Western blot analysis showed that ERK1/2 phosphorylation was occurred under all conditions but it was almost four times higher in the PGCs treated with the high FGF9 than the control group (Fig. 10). Next, we analyzed p38 phosphorylation, and found that p38 was activated even without FGF9 treatment (Fig. 10). After treatment of low FGF9, p38 phosphorylation was enhanced in 12.5 dpc XY PGCs (Fig. 10). In contrast, high FGF9 treatment suppressed p38 phosphorylation in the cells (Fig. 10). JNK and AKT signaling pathways were not induced by either low or high FGF9 treatment compare with the control in the XY PGCs (Fig. 10). From these results, we conclude that the high FGF9 treatment activates ERK1/2 signaling pathway while the low FGF9 enhances p38 phosphorylation.

3.3 SA3: Effects of MAPK Signaling Pathways on PGC Proliferation and Male Differentiation

We performed inhibitor experiments to identify the relationship between cell proliferation and ERK1/2 signaling under the high FGF9 treatment, and PGC male differentiation and p38 signaling pathway under the low FGF9 treatment.

3.3.1 Effect of ERK1/2 signaling pathway on PGC proliferation

We investigated whether ERK1/2 signaling pathway activated by high FGF9 treatment regulates PGC proliferation. For this purpose, we used an ERK1/2 inhibitor, U0126. This inhibitor binds to MEK1/2, the MAPKK for ERK1/2 activation, to suppress both phosphorylation and the catalytic activity of ERK1/2. First, we performed western blot analysis to confirm whether U0126 successfully suppresses the phosphorylation of ERK1/2 activated by high FGF9 treatment in 12.5 dpc XY PGCs (Fig. 11A). High FGF9 treatment clearly phosphorylated ERK1/2 in XY PGCs like our previous result (Fig. 10), but an additional treatment of 10 μM U0126 completely suppressed phosphorylation of ERK1/2 in the cells (Fig. 11A). Optical density analysis presented evidences that the phosphorylation level of ERK1/2 was eight times lower in the presence of the inhibitor even high FGF9 presented in the PGCs than the only high FGF9 treated group (Fig. 11A). These results clearly indicates that the inhibitor successfully inhibits the activation of ERK1/2.

We examined PGC proliferation in the presence of U0126. XY PGCs at 12.5 dpc were cultured with the inhibitor for 2 days after that we performed EdU assay. Under the high FGF9 treatment without the inhibitor, 40% of the cells became EdU positive like we previously showed (Fig. 8A) ($p<0.01$), an increase 10 times higher than in the control (Fig. 11B). In PGCs treated with both high FGF9 and the ERK1/2 inhibitor at 1 or 10 μM, cell proliferation was dose-dependently blocked and 20 or 10% of the cells were EdU positive, respectively (Fig. 11B) ($p<0.01$, $p<0.05$).

Next, we examined the effect of ERK1/2 inhibitor on sex-specific genes expressions in 12.5 dpc XY PGCs (Fig. 11C). The cells were cultured with or without the

high concentration of FGF9 and/or ERK1/2 inhibitor for one day. In the absence of FGF9, ERK1/2 inhibition did not affect male-specific *Nanos2* expression and its level was the same as the control (Fig. 11C). In the presence of the high FGF9, *Nanos2* expression was decreased in XY PGCs by 25% compared to the control (Fig. 11C), and not statistically significant (p=0.27). When the cells were treated with both FGF9 and the inhibitor, we observed a 2-fold increase in *Nanos2* expression in the XY PGCs compared with the cells treated with the high FGF9 only (Fig. 11C), and not statistically significant (p=0.93). In addition to *Nanos2*, we also analyzed the female-specific *Stra8* expression (Fig. 11C). Results showed *Stra8* expression was decresed in all the three groups (FGF9 only, the inhibitor only, or both) (Fig. 11C). Our data demonstrates that ERK1/2 signaling pathway stimulated by high FGF9 treatment induces PGC proliferation, resulting in suppression of PGC male or female differentiation.

3.3.2 Effect of p38 signaling pathway on PGC male differentiation

We, next, determined whether the p38 signaling pathway stimulated by low FGF9 treatment induces PGC male differentiation or not. For this purpose, we used two different p38 inhibitors, BIRB796 and SB202190. These two inhibitors are distinct from each other according to their inhibition strategy and target isoforms of p38. It is known that p38 consists of 4 isoforms (*α, β, γ, δ*). BIRB796 binds to the conserved DFG motif of all p38 isoforms (*α, β, γ, δ*) and inhibits both phosphorylation and catalytic activities of all four isoforms. On the other hand, SB202190 binds to the ATP-binding site of *α* and *β* isoforms of p38 and inhibits the catalytic activity of both of them. However, this inhibitor does not block the phosphorylation of *α and β* isoforms.

3.3.2.1 Inhibition of p38 phosphorylation

First, we performed western blotting in 12.5 dpc XY PGCs treated with low FGF9 and/or BIRB796. In 12.5 dpc XY PGCs treated with 10 μM BIRB796, phosphorylation of all p38 isoforms was successfully blocked compare with the control (Fig. 12A). Low FGF9 treatment enhanced p38 phosphorylation, and optical density (O.D) analysis showed that the increase in the phosphorylation level of p38 δ and γ isoforms were higher than the increase of p38 α and β isoforms phosphorylation in the cells (Fig. 12A). When both the inhibitor and low FGF9 treatment were used together, the phosphorylation of p38 was significantly inhibited in the PGCs (Fig. 12A). Again, optical density analysis results showed that the phosphorylation of p38 α, β isoforms was decreased almost six times compared to the only low FGF9 treated group, and the phosphorylation of p38 γ, δ isoforms was diminished half of the only low FGF9 treated group (Fig. 12A). These results show that the inhibitor successfully suppresses all p38 isoforms.

3.3.2.2 Effect of inhibition of p38 α and β isoforms on PGC male differentiation

To find out which isoform(s) are responsible for PGC male differentiation, we used an isoform specific inhibitor, SB202190, for p38 α and β.We analyzed the male-specific *Nanos2* expression in 11.5 dpc XY PGCs treated with or without low FGF9 and/or SB202190 for 1 day (Fig. 12B). The inhibitor treatment without low FGF9 did not affect *Nanos2* expression in the cells and showed the same expression level as the control (Fig. 12B). When PGCs were treated with low FGF9, *Nanos2* expression was drastically upregulated and was nearly 6 times higher than that of the control (Fig. 12B), and statistically significant ($p<0.05$). In contrast, when both low FGF9 and the inhibitor were

administered together, *Nanos2* expression was completely suppressed in PGCs (Fig. 12B) ($p<0.01$). These results indicate that p38 α and β isoforms contribute to PGC male differentiation.

Next, we performed the same experiment in 12.5 dpc XY PGCs. In PGCs treated with the inhibitor only, *Nanos2* expression was the same level as the control (Fig. 12C). Interestingly, only low FGF9 treatment did not induce *Nanos2* expression (Fig. 12C). In the presence of both low FGF9 and the inhibitor, *Nanos2* expression in the PGCs was decreased to less than half of that in the only low FGF9 treated PGCs (Fig. 12C), and not statistically significant ($p=0.07$). These results show that, again p38 α, β isoforms regulate PGC male differentiation.

Additionally, we measured *Stra8* expression which was similar to the control with the inhibitor treatment only (Fig. 12C). In the presence of FGF9, *Stra8* expression decreased independently from the p38 inhibition (Fig. 12C). Together, data indicates that the p38 signal transduction pathway promotes PGC male differentiation and does not contribute to meiotic process (Fig. 12C).

CHAPTER 4. DISCUSSION

We have previously proven that a PGC male differentiation is induced both by inhibition of retinoic acid (RA) signaling, and by factor(s) production by age-specific fetal Sertoli cells (Ohta et al., 2012). FGF9 has been reported as one of the male-inducing factors (Barrios et al., 2010; Bowles et. al., 2010). Therefore, we tested the effect of FGF9 on PGC male differentiation at the same concentration (25 ng/ml) as has been previously reported. Surprisingly, the FGF9 treatment suppressed PGC male differentiation in our preliminary experiments. From this result, we suspected that FGF9 might function differently in the PGCs under different culture conditions. In this project, isolated PGCs were cultured under different concentrations of FGF9 (0 ~ 100 ng/ml) to determine if FGF9 has different functions in the PGCs in a dose-dependent manner. We revealed (1) that FGF9 in the low concentration (0.2 ng/ml) induces PGC male differentiation by stimulating a p38 signal transduction pathway, and (2) that the high FGF9 (25 ng/ml) treatment induces PGC proliferation by activating ERK1/2 signaling pathway. Our data demonstrates that FGF9 has distinct functions in XY PGCs in a dose-dependent manner.

4.1 Low FGF9 Induces PGC Male Differentiation

Using a germ cell culture system (Iwahashi et al., 2007), we revealed different dose-dependent functions of FGF9 on PGC differentiation. The low (0.2 ng/ml) FGF9 treatment induces PGC male differentiation, whereas the high (25 ng/ml) FGF9 induces PGC proliferation to suppress the male pathway in the XY PGCs. Our results indicate

that the FGF9 functions as a male-inducing factor, but also regulates cell proliferation in a dose-dependent manner.

Retinoic Acid (RA) is a meiosis-inducing factor, as well as a key molecule to help determine the sex fate of PGCs (Koubova et al., 2006; Bowles et al., 2006). XX PGCs exposed to RA immediately enter meiosis to become female germ cells, but XY PGCs enter mitotic arrest (G_0) to then be differentiated into male germ cells. In the male gonad, Sertoli cells produce CYP26B1, a RA-degrading enzyme, to protect XY PGCs from any exposure to RA, and with a result that XY PGCs do not enter meiosis. Is inhibiting the RA signaling sufficient for PGCs to enter the male pathway? Recent studies have revealed that inhibiting RA is not sufficient, and some molecule(s) in the male gonads are essential for PGC male differentiation (Barrios et al., 2010; Bowles et al., 2010; Guerquin et al., 2010; Ohta et al., 2012). FGF9 has been reported as a male factor for PGC male differentiation (Bowles et al., 2010; Barrios et al., 2010). FGF9 is expressed in both XX and XY gonadal supporting cells at 11.5 dpc, but its expression becomes male specific by 12.5 dpc (Scmahl et al., 2004). It has been reported that an *Fgf9*$^{-/-}$ XY gonad has performed male-to-female sex reversal (Colvin et al., 2001). Furthermore, in this *Fgf9*$^{-/-}$ gonad, most of the XY PGCs are predominantly lost, while XX PGCs are not affected at all (DiNapoli et al., 2006). These findings attract researchers to identify the direct effect of FGF9 on PGC differentiation (Barrios et al., 2010; Bowles et al., 2010).

Barrios et al. (2010) cultured 12.5 dpc XX and XY gonads with FGF9 and detected an up-regulation of male-specific *Nanos2* expression in both XX and XY PGCs (Barrios et al., 2010). They proposed that RA and FGF9 in the male gonad regulate *Nanos2* expression and meiosis initiation in the PGCs in opposite ways. In the *Nanos2*-

null XY gonads, the PGCs ectopically expressed *Stra8*, a pre-meiotic marker, and they eventually entered meiosis, suggesting that NANOS2 represses the *Stra8* expression in order to prevent meiotic initiation (Suzuki and Saga, 2008).

Bowles et al. (2010) first revealed that FGF9 directly suppresses meiosis. They demonstrated that the PGCs in the *Cyp26b1* KO XY gonad express a pre-meiotic marker *Stra8* less than any wild-type XX PGCs, suggesting that FGF9 in the XY gonad reduces the sensitivity of the PGCs. Furthermore, Bowles et al. (2010) also demonstrated that the FGF9 treatment increases the male-specific gene expression in isolated XY PGCs at 12.5 dpc, suggesting that the FGF9 directly induces PGC male differentiation.

Our results that were obtained from low (0.2 ng/ml) FGF9 treatment strongly support previous reports that FGF9 directly induces PGC male differentiation. However, the main difference lies in the FGF9 concentration that is treated. The other groups treated the PGCs with 25 ng/ml FGF9 to induce male differentiation (Bowles et al., 2010; Barrios et al., 2010; Guerquin et al., 2010). In our experiments on the other hand, 25ng/ml (high) FGF9 treatment strongly suppressed PGC male differentiation. We expect this difference was caused by different culture conditions, especially by the concentration of fetal bovine serum (FBS) in the media. Our culture medium contained 20% FBS. On the contrary, they did not add FBS in the media or reduce the FBS concentration up to 10% (Bowles et al. 2010; Barrios et al., 2010). Because FBS should contain some amount of growth factors including FGF9, it may affect the amount of the exogenous FGF9 that is essential for PGC male differentiation in vitro. However, FBS supports germ cell survival in vitro; therefore, it is essential to add FBS in the culture medium.

4.2 High FGF9 Induces PGC Proliferation

In our data, the high (25 ng/ml) FGF9 treatment in the 12.5 dpc XY PGCs stimulated PGC proliferation, and these PGCs failed to enter male differentiation (Fig. 5 and Fig. 6A). In general, XY PGCs enter mitotic arrest at around 13.5 dpc, and reach the G_0 cell cycle stage to commit to the male pathway. Therefore, it is strongly suggested that the high Fgf9 treatment has disturbed a male-specific cell-cycle clock in XY PGCs. It has been shown that some members of the FGF family proteins, for examples FGF2, FGF4, and FGF8 induce PGC proliferation (Resnick et al., 1998; Kawase et al., 2004; Choi et al., 2010). Resnick et al. (1998) reported that bFGF directly binds to PGCs and stimulates PGC proliferation at 11.5 dpc. In another study the researchers indicate that FGF4 and FGF8 independently promote PGC proliferation in culture (Kawase et al., 2004). According to the expression patterns of FGF4 and FGF8 in the mouse embryos, they proposed that stage-specific mechanisms regulate PGC proliferation. First, during PGC migration, neighboring somatic cells produce FGF4 and FGF8 and regulate PGC proliferation via paracrine stimulation. Second, after PGCs reach the gonad, they express FGF4 and FGF8 by themselves, and PGCs continuously proliferate by their own autocrine stimulation. Lastly, PGCs stop proliferation at around 13.5 dpc, although they still express FGF4 and FGF8 by themselves. At the stage of PGC sex differentiation, gonadal somatic cells produce proliferation inhibitors, like SCF and TGF-β1, to suppress PGC proliferation. Eventually, PGCs commit to the male or female pathway.

Recently, Choi et al. (2010) proved that bFGF, and not other factors such as stem cell factor (SCF) and leukemia inhibitory factor (LIF), enhances avian PGC proliferation in vitro. They suggested that bFGF is essential for PGC proliferation because they could

prove that culturing the isolated PGCs with bFGF causes increased cell number (Choi et al., 2010).

The XY-specific FGF9 expression contributes to both Sertoli cell proliferation and PGC male differentiation. Our question is whether a high FGF9 also regulates PGC proliferation in vivo. At 11.5 dpc, FGF9 is highly expressed in the XY gonad, but not in the XX gonad. It is not likely that XX and XY PGCs use a different machinery for their proliferation before sex differentiation. We speculate that PGC proliferation is regulated by other factor(s), such as FGF4 and/or FGF8 in vivo.

4.3 XX and XY PGCs at 11.5 dpc are Not Equivalent

In this study we revealed that sexually undifferentiated XX and XY PGCs at 11.5 dpc have different responses to an FGF9 treatment. In the presence of the low FGF9, only XY PGCs, but not XX PGCs at the same stage, entered the male pathway. In general, XX and XY PGCs at 11.5 dpc have been recognized as sexually bipotent, and they can commit to either a male or female fate regardless of their sex chromosomes (Hilscher et al., 1974; Adams and McLaren, 2002; Chuma and Nakatsuji, 2001). Our results strongly suggest that XX and XY PGCs at 11.5 dpc are not equivalent, and they already initiate their sex-specific differentiation in the morphologically undifferentiated gonads. We expect that XX and XY gonads are already distinctive at 11.5 dpc. At 11.5 dpc, XX and XY gonads are morphologically equivalent; however, the sex-specific gene expression is already triggered in the somatic cells. In the XY gonad, a Y-chromosome-specific *Sry* expression is initiated as early as 10.5 dpc (Koopman et al., 1990; Gubbay et al., 1990); therefore, different gonadal environments impose different characteristics on XX and XY

PGCs once they reach the XX or XY gonads by 11.5 dpc. The different response to FGF9 between XX and XY PGCs is one of these characteristics.

DiNapoli et al. (2006) found that the FGF9 treatment supports cell survival only in XY PGCs, but not in XX PGCs at 11.5 dpc. They also confirmed that the PGC number is predominantly decreased in the XY *Fgf9*$^{-/-}$ gonad, but not in the XX mutant gonad (DiNapoli et al., 2006). From these results, they suggested that the masculinizing factor(s) secreted in the male gonadal environment make XY PGCs dependent on FGF9 (DiNapoli et al., 2006).

There are two possibilities why XY PGCs predominantly react against FGF9 even before their sex differentiation occurs. First, XX and XY PGCs may produce distinct FGF receptors (FGFRs) (DiNapoli et al., 2006). In mice, there are four FGF receptors (FGFR 1-4) and three splicing variants (FGFR1IIIb-c, FGFR2IIIb-c, and FGFR3IIIb-c). However, it was reported that 11.5 dpc XX and XY PGCs express the same FGF receptors, FGFR1, FGFR3, and FGFR4 (Scmahl et al., 2004). Second, XY and XX PGCs may synthesize sex-specific co-factors such as heparin sulfate proteoglycans (DiNapoli et al., 2006). It is known that heparin molecules bind to both FGFRs and to FGF ligands on the cell surface, and they contribute to signal and stabilize the FGFR-FGF ligand complex (reviewed by Lemmon and Schlessinger, 2010; Turner and Grose, 2010). Scmahl (2004) and DiNapoli (2006) suggest that XY gonad at 11.5 dpc synthesizes sex-specific heparin sulfate proteoglycan molecules, which support FGF9-FGFRs; therefore, only a synthesis can induce a sex-specific response to FGF9 in XY PGCs.

To the contrary, other reports have claimed that XX PGCs can commit to the male pathway under the exogenous FGF9 treatment (Bowles et al., 2010; Barrios et al., 2010).

In these reports, however, the researchers used the gonadal organ culture system. The XX gonads at 11.5 dpc were cultured in the presence of FGF9 (25 ng/ml), then XX PGCs in the gonads upregulated male specific *Nanos2* and *Dnmt3l*. We speculate that an exogenous FGF9 directly affects XX gonadal supporting cells to differentiate them into the male supporting cells. In the cultured XX gonads, FGF9 stimulate XX gonadal somatic cells to be differentiated into male somatic cells. As a result, a sex-reversed XX gonadal environment may induce XX PGCs to be responsive to FGF9, as male PGCs.

4.4 Different Functions of FGF9 in XY PGCs in a Dose-Dependent Manner

In this study we present evidence that FGF9 has dose-dependent and different functions in XY PGCs: the low FGF9 (0.2 ng/ml) induces PGC male differentiation, whereas the high FGF9 (25 ng/ml) stimulates PGC proliferation. It is already shown that certain FGF family proteins generate different effects on the target cells in a dose-dependent manner (Qian et al., 1997; Lovicu and McAvoy, 2001; Kelly et al., 2003; Iyengar et al., 2007).

When multipotent cortical stem cells were cultured with FGF2 at different concentrations, low FGF2 (0.1 ng/ml) induced neuronal differentiation, whereas high FGF2 (10 ng/ml) treatment promoted both neuronal and glial cell differentiation in vitro (Qian et al., 1997). Because multipotent cortical stem cells synthesize multiple FGF receptors (Qian et al., 1997), it was proposed that different concentrations of FGF2 activate distinct FGF receptors (FGFRs) to activate different signal transduction pathways (Wang et al., 1994; Shaoul et al., 1995). Another study has proven that bFGF

regulates multiple functions in lens epithelial cells in a dose-dependent manner (McAvoy and Chamberlain, 1989). Three different concentrations (0.15, 3, and 40 ng/ml) of bFGF induce three different responses: proliferation, migration, and fibre differentiation, in the lens cells. It was also reported that embryonic neural precursors (ENPs) perform either differentiation or proliferation depending on the concentration of FGF2 in vitro (Kelly et al., 2003). Neuronal differentiation was stimulated under the low FGF2 (1 ng/ml) treatment, while ENP proliferation occurred under the high FGF2 (10 and 20 ng/ml) treatments (Kelly et al., 2003).

There are several possible reasons to explain why different concentrations of FGFs cause different functions in the same target cells. We suspect at least three mechanisms as follows: (1) Different frequency of receptor-ligand binding: Different concentrations of the FGF ligands cause a different association-dissociation rate, and as a result generate distinct signal frequencies to induce different cellular response (Li et al, 2009). (2) Different receptor-ligand binding affinity: It is known that extracellular domains of the FGF receptors are morphologically different from each other (Mohammadi et al., 2005); therefore, their receptor-ligand binding affinities are diverse - For example, a high-affinity receptor could be triggered by the low concentration, but a low-affinity receptor is required for the high concentration to be activated (Dailey et al., 2005). (3) Different magnitude of activity of the receptors: FGF receptors also have different intracellular kinase domains, like their extracellular domains; thus, the FGF receptors have different magnitudes of activities (Raffioni et al., 1999). As a consequence, different concentrations of the FGF ligands may generate different magnitudes of activities in the target cells.

4.5 Mitogen Activated Protein Kinase (MAPK) Signal Transduction Pathways

It has been proven that the FGF family proteins stimulate MAPK and AKT signaling cascades in various cell types via FGF receptors (FGFRs) (reviewed by Dailey et al., 2005; Turner and Grose, 2010; Dorey and Amaya, 2010). There are several reports that a single FGF ligand, depending on its concentrations, activates different cellular signaling to induce different functions. For example, an FGF2 treatment induces three different functions in NIH3T3 cells in a dose-dependent manner (0-1000 ng/ml) (Garcia-Maya et al., 2006). A low FGF2 (<10 ng/ml) treatment activates p38 signaling to induce cell differentiation and survival (Garcia-Maya et al., 2006), whereas, FGF2 at 10 ng/ml stimulates ERK1/2 phosphorylation to induce cell proliferation (Garcia-Maya et al., 2006). Lastly, it has been shown that a high concentration of FGF2 (100 and 1000 ng/ml) predominantly stimulates p38 phosphorylation, but not ERK1/2 phosphorylation (Garcia-Maya et al., 2006) to induce cell survival. Furthermore, it is indicated that α-isoform, one of two FGFR2 splicing variants, is activated by FGF2 at or above 10 ng/ml. However, the β-isoform can be activated by lower FGF2 concentrations. From these results, it is suggested that these isoforms of FGFR2 have different receptor-ligand binding affinities. The low FGF2 treatment is enough to activate the high-affinity receptor (β-isoform) engaged with p38 signaling, but the low-affinity receptor (α-isoform) requires the high FGF2 treatment for activation, and it is associated with ERK1/2 signaling pathway.

Interestingly, a certain FGF ligand activates one signaling pathway, but induces different functions in a dose-dependent manner. Lovicu and McAvoy (2001) show that both low (5 ng/ml) and high (100 ng/ml) concentrations of FGF2 activate ERK1/2

signaling in the lens epithelial cells, but activated ERK1/2 promotes either cell proliferation or differentiation, respectively. They found that the high FGF2 treatment induced a phosphorylation of ERK1/2 greater than what resulted from the low FGF2 treatment. From these indications, it is suggested that the different intensity of active ERK1/2 stimulates different functions (Marshall, 1995). Iyengar et al. (2007) present evidence that a low FGF2 (1 ng/ml) treatment induces ERK1/2 phosphorylation for a short time (6h), whereas a high FGF2 (100-150 ng/ml) treatment continuously stimulates ERK1/2 phosphorylation for a long time (18h) in the lens epithelial cells, inducing cell proliferation or cell differentiation, respectively. In conclusion, it is suggested from this research that different concentrations of FGF ligands induce a different intensity and/or duration of the MAPK signaling pathways, stimulating different functions in the target cells (Iyengar et al., 2007).

4.6 ERK1/2 Signaling Stimulated by High FGF9 Induces PGC Proliferation

Our data demonstrate that the high FGF9 treatment predominantly enhances ERK1/2 signal pathway in XY PGCs to induce PGC proliferation. Under the PGC proliferation status, XY PGCs cannot enter mitotic arrest (G_0), a typical event within PGC male differentiation; therefore, these PGCs cannot differentiate into male germ cells.

Other studies have also shown that ERK1/2 signaling pathway induces germ cell proliferation (Choi et al., 2010; Dolci et al., 2001; He et al., 2008). Choi et al. (2010) reported that bFGF treatment stimulates ERK1/2 signaling in cultured chicken PGCs to

induce PGC proliferation. Their microarray and q-PCR analyses have demonstrated that phosphorylated ERK1/2 upregulates the expression levels of cell proliferation- or cell cycle-related genes (Choi et al., 2010). Another study also show that there is a relationship between ERK1/2 activation and cell proliferation. It was shown that glial cell line-derived neurotropic factor (GDNF) treatment stimulates proliferation of a mouse spermatogonial stem cell line via ERK1/2 signaling pathway (He et al., 2008). They also obtained the same results by using type A spermatogonia freshly isolated from six-day-old mice (He et al., 2008). They reveal that ERK1/2 signaling activates several transcription factors, such as CREB-1, ATF-1, CREM-1, and c-fos. Eventually, c-fos upregulate Cyclin A transcription. Cyclin A is a cell cycle protein, and it regulates G_1/S phase transition, resulting in the promotion of cell proliferation. Stem cell factor (SCF) treatment stimulates co-activation of ERK1/2 and PI3K-dependent AKT signaling pathways, and this induce A_1-A_4 spermatogonial cell proliferation in vitro (Dolci et al., 2001). Inhibiting either ERK1/2 or AKT signaling pathway results in disrupting the spermatogonial cell proliferation (Dolci et al., 2001). Further, these researchers found that the SCF treatment induces nuclear translocation of Cyclin D3, a regulator protein of G1/S transition in the cells, to induce cell proliferation (Dolci et al., 2001). In conclusion, it is suggested that ERK1/2 pathway stimulated by various growth factors upregulates both cell cycle- and cell proliferation- related genes to regulate cell proliferation in the target cells.

We believe our findings have added unique data to the literature: ERK1/2 pathway stimulated by the high FGF9 treatment induces XY PGC proliferation in vitro. We expect that the activated ERK1/2 signaling in XY PGCs will promote transcription of

cell cycle-related genes, such as Cyclins. The XY PGCs treated with the high FGF9 continuously perform their DNA replication, and cannot exit from the cell cycle to enter G_0 stage. Because of this, PGC proliferation that has been stimulated by ERK1/2 signaling will inhibit PGC male differentiation.

4.7 p38 Stimulated by Low FGF9 Induces PGC Male Differentiation

We have revealed that the low FGF9 treatment enhances p38 signaling pathway in XY PGCs to induce PGC male differentiation. Ewen et al. (2010) reported that p38 signaling is activated in the mouse PGCs in a sex-dependent manner. In the mouse fetal gonads, only XY PGCs, but not XX PGCs, express phosphorylated p38 after sex differentiation. To determine the role of p38 signaling in XY PGCs, Ewen et al. (2010) cultured 11.5 dpc XY gonads with two p38 inhibitors (SB202190 and SB203580) for three days. Interestingly, the XY PGCs in the treated gonads performed male-to-female sex reversal, and initiated meiosis. From these results they concluded that p38 signaling pathway is critical for PGC male differentiation to occur (Ewen et al., 2010). However, p38 signal transduction pathway is also activated in Sertoli cells within the male gonad. It was reported that p38 signaling trigger *Sry* expression in the undifferentiated XY gonadal supporting cells to induce pre-Sertoli cell differentiation (Bogani et al., 2009). The researchers cultured 11.5 dpc XY gonads in the presence of a p38 inhibitor (SB202190) and found that XY somatic cells performed male-to-female sex reversal (Bogani et al., 2009). This latter data strongly suggests that p38 signaling is essential for male-specific gonadal differentiation to occur (Bogani et al., 2009). In the mouse gonads, first gonadal supporting cells differentiate into either pre-Sertoli cells or granulosa cells. Thereafter

these environments regulate PGC sex differentiation. Because Ewen et al. (2010) used the gonadal organ culture system, it is difficult to conclude that the p38 inhibitors directly affected XY PGC sex differentiation. It is more likely that the p38 inhibitors directly influenced XY supporting cells to perform male-to-female sex reversal. Then these female-like somatic cells eventually induced XY PGC sex reversal. The option they can use is Cre-LoxP conditional knock-out system. Using this system, it may be possible to confirm the direct effect of p38 on PGC differentiation. In this study, we have used isolated PGCs to help analyze the effects of p38 on PGC sex differentiation. As a result, we believe we clearly demonstrate the direct effects of p38 signaling on PGC male differentiation.

p38 consists of four different isoforms (α, β, γ, and δ). Using q-PCR analysis, Ewen et al. (2010) suggested that p38 β and δ isoforms are predominantly expressed in XY PGCs. In this study, by using western blot, we reveal that the FGF9 treatment upregulates the phosphorylation levels of both isoform groups (α & β, γ & δ) in XY PGCs. Furthermore, we demonstrate that XY PGCs cultured with the p38 inhibitor specifically for p38 α and β isoforms do suppress male differentiation. Our data strongly suggest that p38 α and β isoforms stimulated by the low FGF9 regulate PGC male differentiation. Additionally, it has been reported that p38γ- and p38δ-deficient mice are viable and fertile, suggesting that these isoforms may not have critical roles to play in the sex differentiation in somatic cells and/or PGCs (Sabio et al., 2005). As we already mention above, p38 signal pathway is also essential for Sertoli cell differentiation. Warr et al. (2012) showed that lack of p38 α and β isoforms in vivo causes XY gonadal sex reversal. Other researchers generated p38α- and p38β-deficient mice failed to express

Sry, and performed male-to-female gonadal sex reversal (Warr et al., 2012). All in all, ours and previous findings strongly suggest that p38α and p38β isoforms are vital for male differentiation in both PGC and Sertoli cells.

In conclusion, we revealed that different FGF9 concentrations induce different functions in the XY PGCs, both entry into PGC male differentiation, and PGC proliferation. These functions are regulated by distinct MAPK signaling pathways, p38 and ERK1/2, respectively. We expect that different concentrations of FGF9 activate p38 or ERK1/2 signaling pathway with the participation of three mechanisms: (1) the frequency of the ligand-receptor binding, (2) the affinity of the ligand-receptor binding, and (3) the magnitude of activity of the receptors. Future work will focus on which downstream molecules of p38 signaling are activated. The appearance of these molecules would help us to understand more about sex differentiation of PGC.

FIGURES

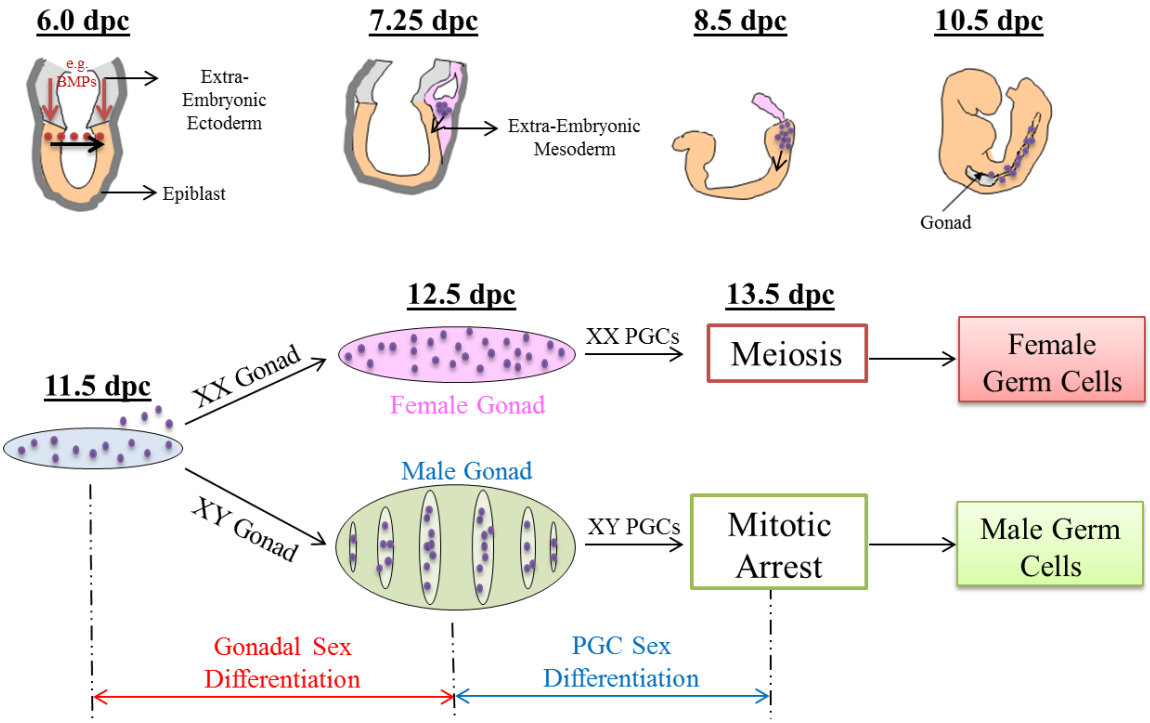

Figure 1. Development of Primordial Germ Cells (PGCs)

At 6.0 dpc, extra-embryonic ectoderm secretes factors, such as BMPs, to induce the neighboring epiblast cells to make PGC precursors. These cells migrate toward the posterior of fetus and drop out of the epiblast. At 7.25 dpc, PGCs acquire distinct characteristics and go back into the epiblast. Between 8.5 and 10.5 dpc, PGCs proliferate and migrate toward the undifferentiated gonad (future testis or ovary). By 11.5, all PGCs reach the gonad. By 12.5 dpc, gonadal sex differentiation occurs and XX gonad differentiates into the female gonad whereas XY gonad differentiates into the male gonad. After gonadal sex differentiation, PGCs undergo sex differentiation by 13.5 dpc. XX PGCs in the female gonad immediately enter meiosis to become female germ cells. On the contrary, XY PGCs in the male gonad enter mitotic arrest (G0) to become male germ cells.

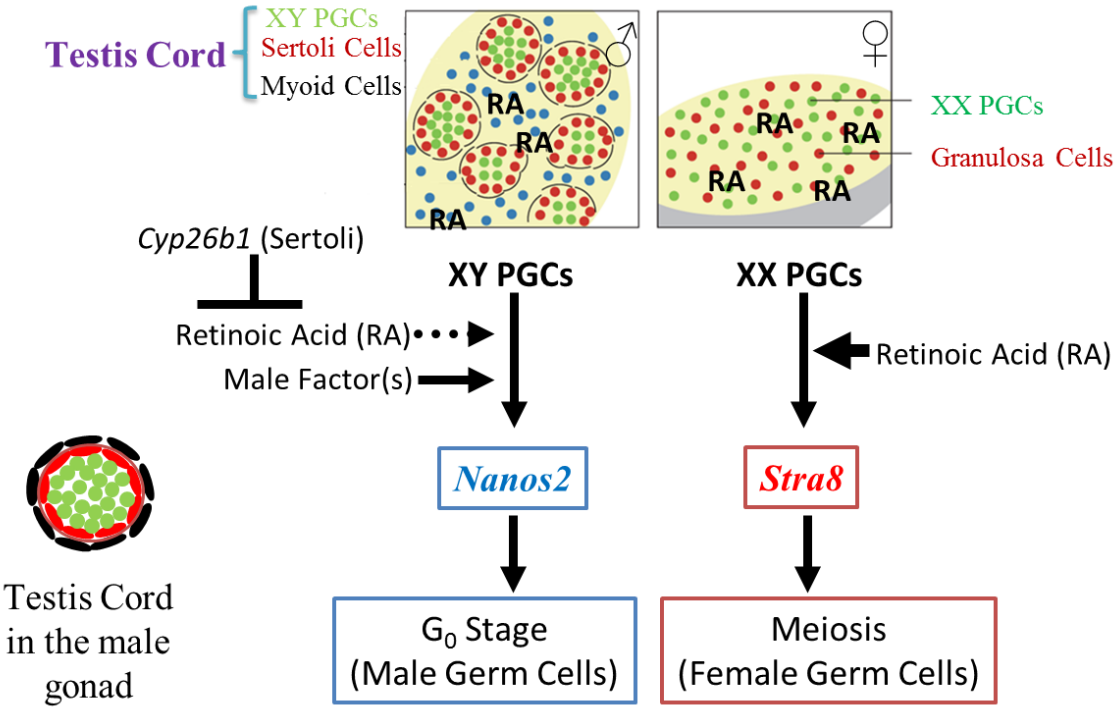

Figure 2. Gonadal environment decides sex fate of PGCs

After sex differentiation, male specific sertoli cells form the testis cord and XY PGCs are surrounded by the cord in the male gonad. On the other hand, female specific granulosa cells do not form a special structure in the female gonad. Retinoic Acid (RA) is a meiosis inducing factor. In the female gonad, XX PGCs are exposed to RA and immediately enter meiosis. In the male gonad, XY PGCs surrounded within the testis cord do not initiate meiosis, but enter mitotic arrest (G0). Sertoli cells produce RA degrading enzyme, CYP26B1, in male gonads and XY PGCs are protected from the exposure to RA.

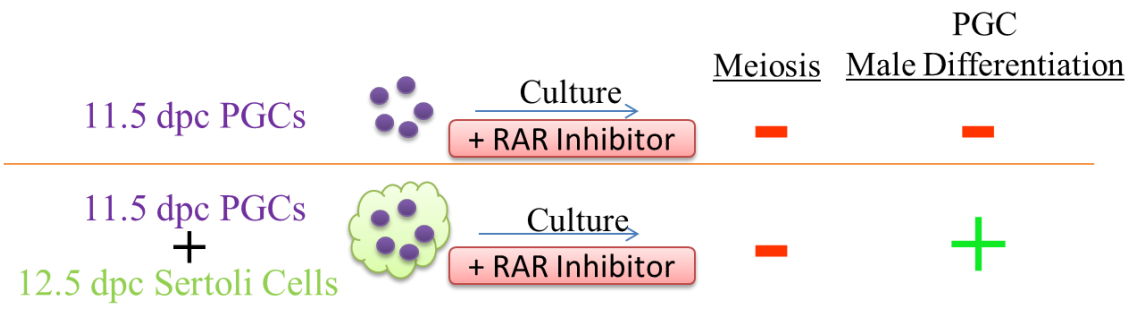

Figure 3. Sertoli cells produce male factor(s) to induce PGC male differentiation

Is inhibiting RA signaling sufficient to induce PGC male differentiation? To answer this question, we cultured 11.5 dpc XX and XY PGCs with RA receptor inhibitor (RARI). These PGCs did not enter meiosis, but failed to differentiate into male germ cells. Next, 11.5 dpc XX and XY PGCs were aggregated with Sertoli cells at different stages (12.5, 15.5, and 18.5 dpc) and cultured with RARI. The PGCs aggregated with 12.5 dpc Sertoli cells entered male pathway regardless of their sex chromosomes. From these results, we concluded that (*i*) inhibiting meiosis is essential but not sufficient for PGC male differentiation, and *(ii)* 12.5 dpc Sertoli cells produce some male factor(s) to induce PGC male differentiation (Ohta et al., BOR 2012). Recently, it was suggested that Fibroblast Growth Factor 9 (FGF9) is a male factor.

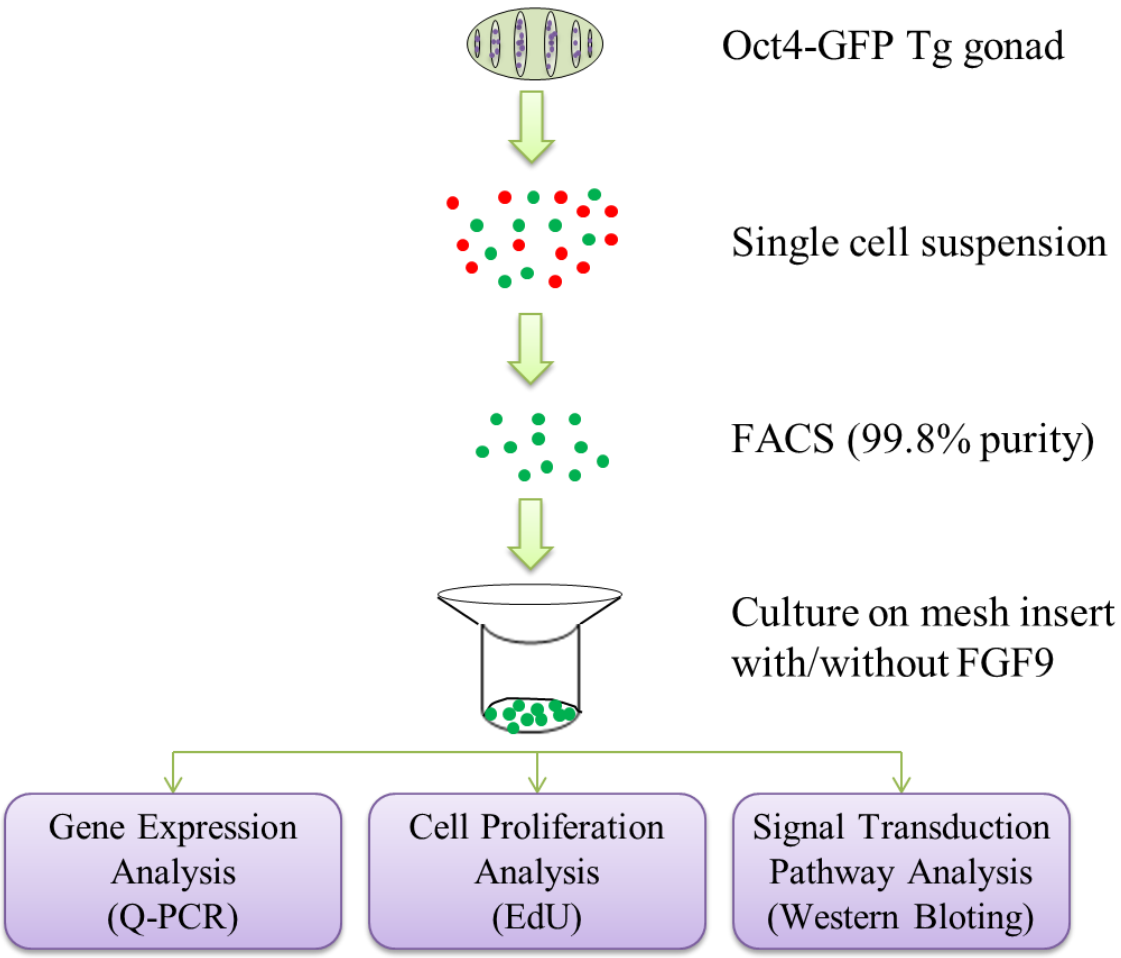

Figure 4. Experimental procedures

1) XX and XY gonads were dissected from Oct4-GFP Tg fetuses at 11.5 or 12.5 dpc.
2) Gonads were dissociated into single cell suspension.
3) GFP positive PGCs were isolated by using FACS (99% purity).
4) PGCs were cultured on mesh inserts with/without FGF9 at different concentrations.
5) After culture, PGCs were manually collected under microscope and subject to gene expression, cell proliferation, and signal transduction pathway analyses.

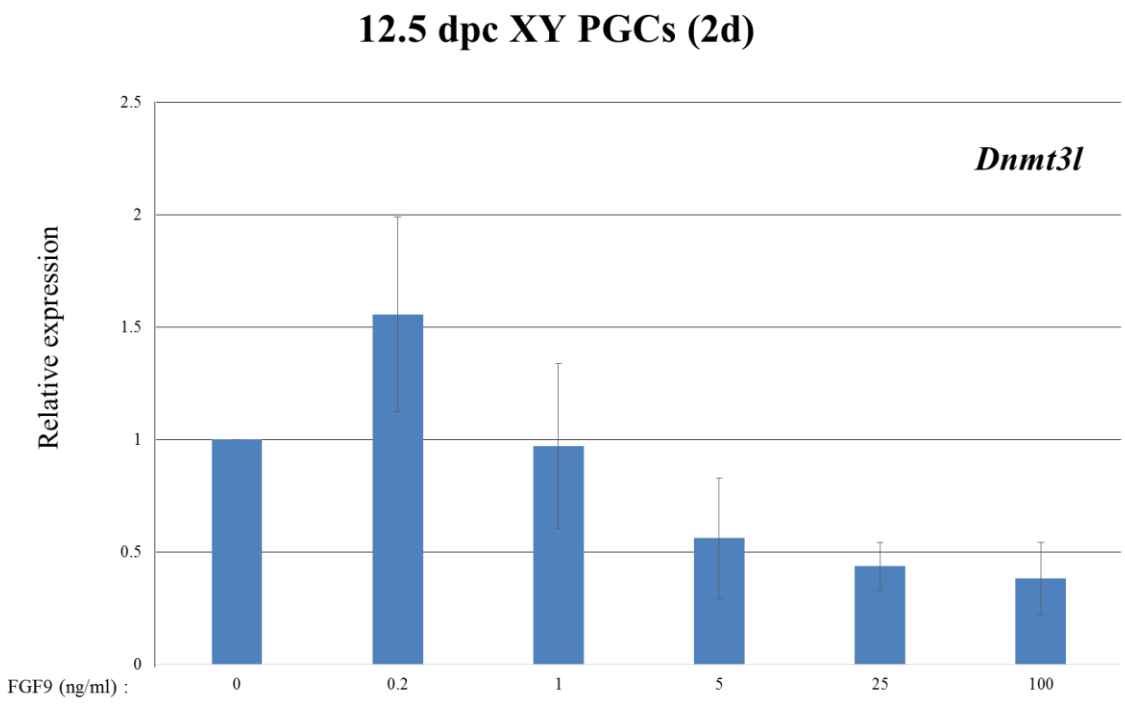

Figure 5. *Dnmt3l* expression under of FGF9 treatment at various concentration

To examine the effect(s) of FGF9 on PGC male differentiation, 12.5 dpc XY PGCs were cultured with different concentrations of FGF9. After two days of culture, male specific *Dnmt3l* expression peaked under 0.2 ng/ml FGF9 treatment, then their expression levels were gradually decreased in a dose-dependent manner. Under the high concentrations (25 and 100 ng/ml), *Dnmt3l* expression was suppressed in the cells. From these results, we set 0.2 ng/ ml as low FGF9 and 25 ng/ml as high FGF9 treatment for the following experiments.

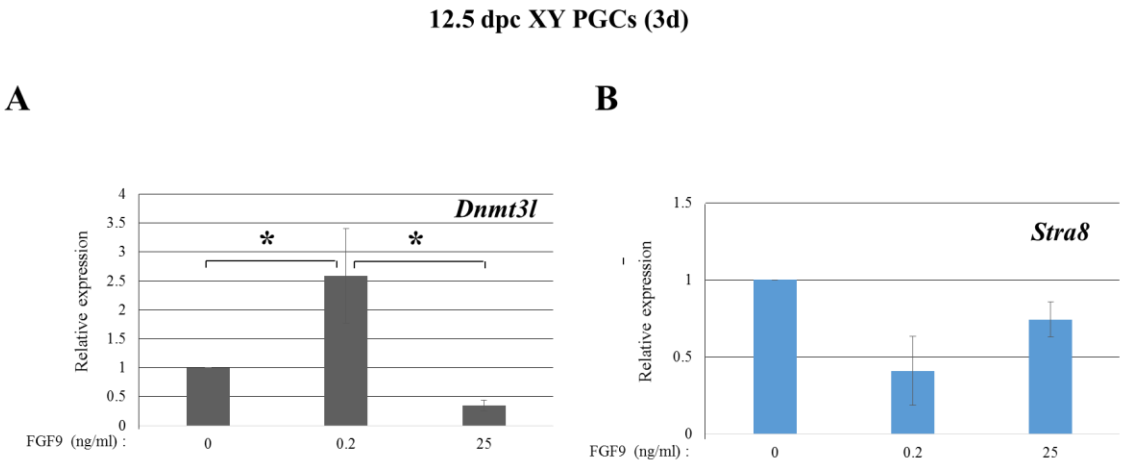

Figure 6. Low FGF9 induces PGC male differentiation

We confirmed the influence of the low and high FGF9 treatments in PGC male differentiation. **(A)** XY PGCs at 12.5 dpc cultured with low FGF9 increased *Dnmt3l* expression at three times higher than in the control. High FGF9 treatment suppressed *Dnmt3l* expression in the cells. **(B)** Low or high FGF9 did not increase female specific *Stra8* in XY PGCs. *P<0.05.

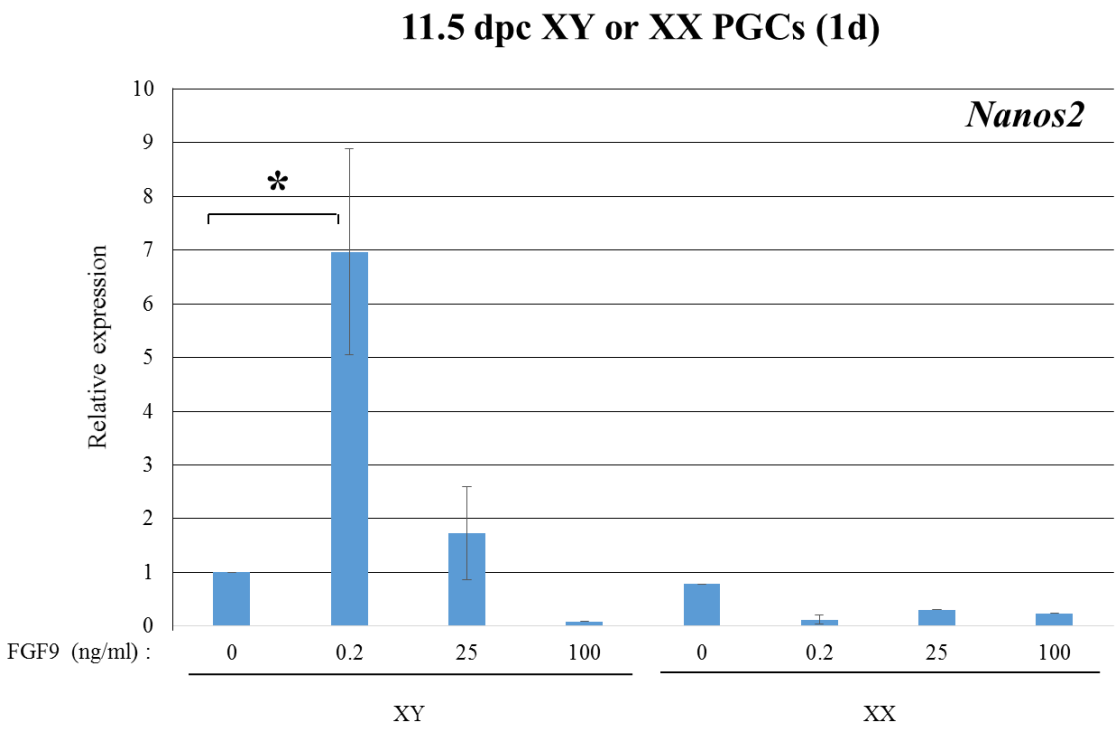

Figure 7. Only XY PGCs respond to FGF9 treatment

In XY PGCs at 11.5 dpc under the low FGF9 treatment, male specific *Nanos2* expression elevated 7 times relative to the control. The *Nanos2* expression gradually decreased in a dose-dependent manner (25 and 100 ng/ml). XX PGCs at 11.5 dpc did not response to FGF9 regardless of their concentrations.

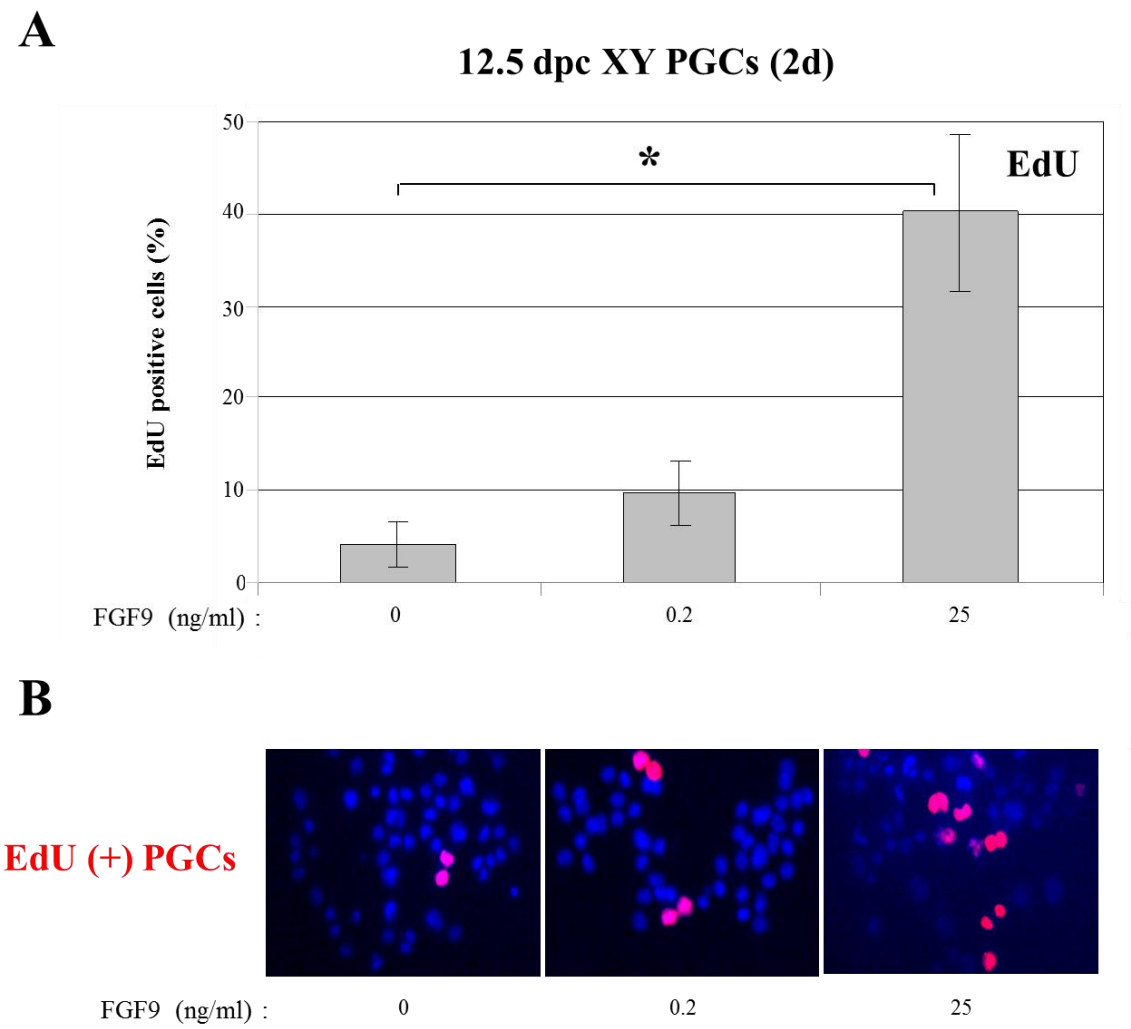

Figure 8. High FGF9 induces XY PGC proliferation

To examine the effect of FGF9 on PGC proliferation, we performed EdU assay. Isolated 12.5 dpc XY PGCs were cultured for 2 days and EdU was treated for the last 5 hours. **(A)** In the control and the low FGF9 treated groups, 5 and 10% of the cells proliferated, respectively. High FGF9 treatment significantly increased PGC proliferation (40%). **(B)** Red: EdU (+) cells, blue: Hoechst staining. *P<0.05. Scale Bar = 50 μm.

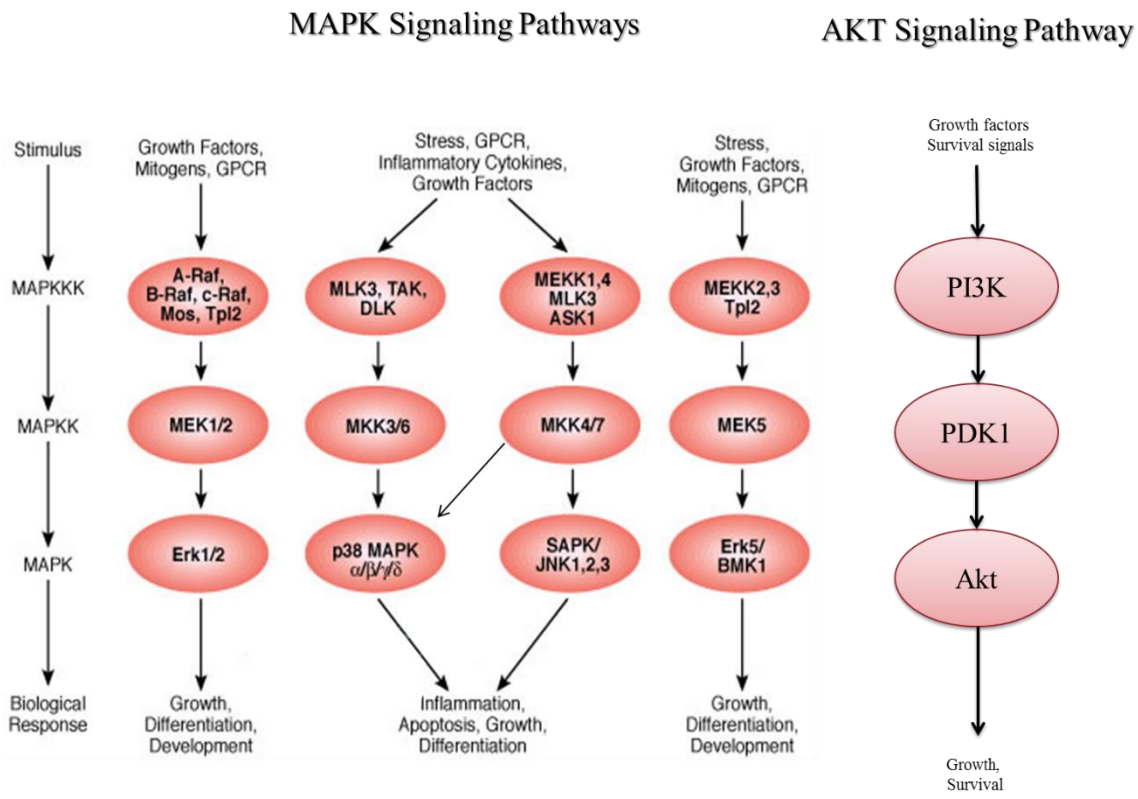

Figure from Cell Signaling Technology

Figure 9. MAPK and AKT signal transduction pathway activated by various stimulus regulate different cellular functions

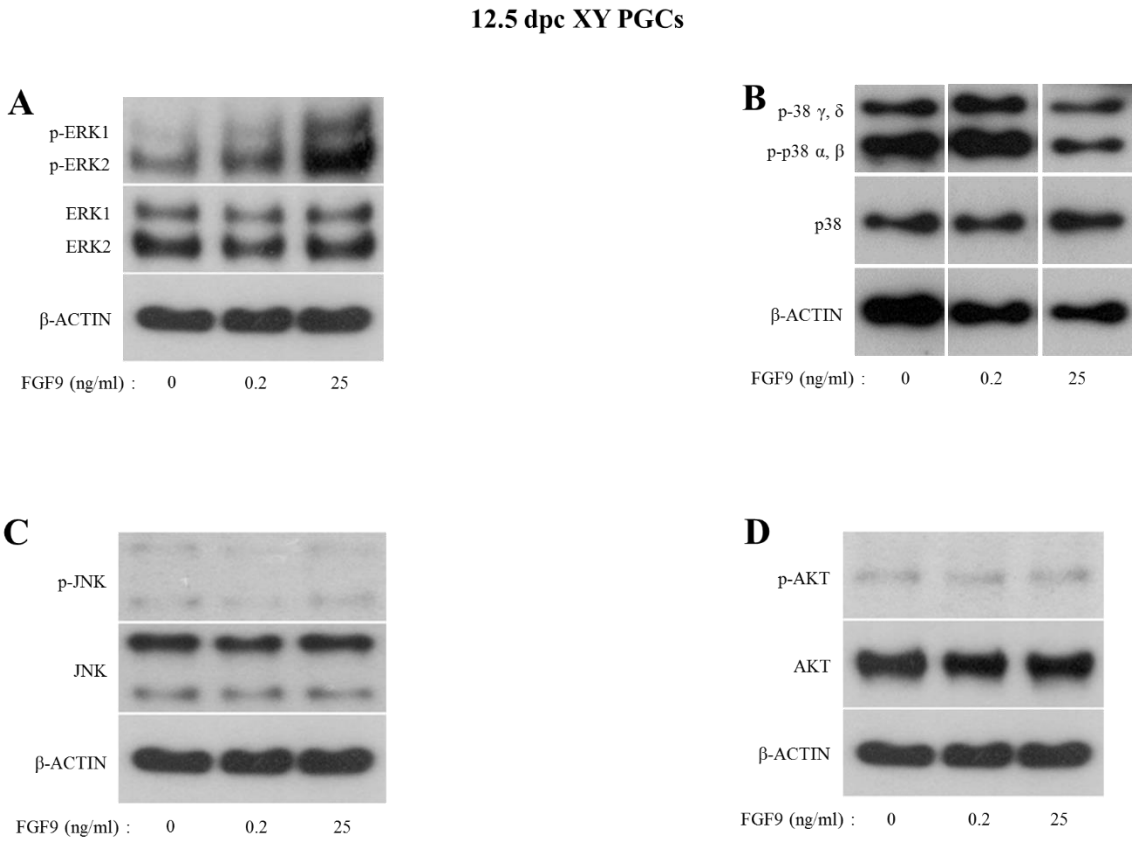

Figure 10. High FGF9 activates ERK1/2 whereas low FGF9 enhances p38 phosphorylation

We examined which signal transduction pathways (ERK, p38, JNK, AKT) are activated by low and high FGF9 treatments in the PGCs. Isolated 12.5 dpc XY PGCs were treated with FGF9 for 30 min and subject to western blots. **(A)** High FGF9 treatment significantly activated ERK1/2 signaling pathway in the PGCs. **(B)** p38 was activated in the XY PGCs without FGF9 treatment. Low FGF9 treatment enhanced p38 phosphorylation. In contrast, the high FGF9 treatment suppressed p38 phosphorylation in these cells. **(C, D)** JNK and AKT signaling pathways were not induced by either low or high FGF9 treatment in the XY PGCs.

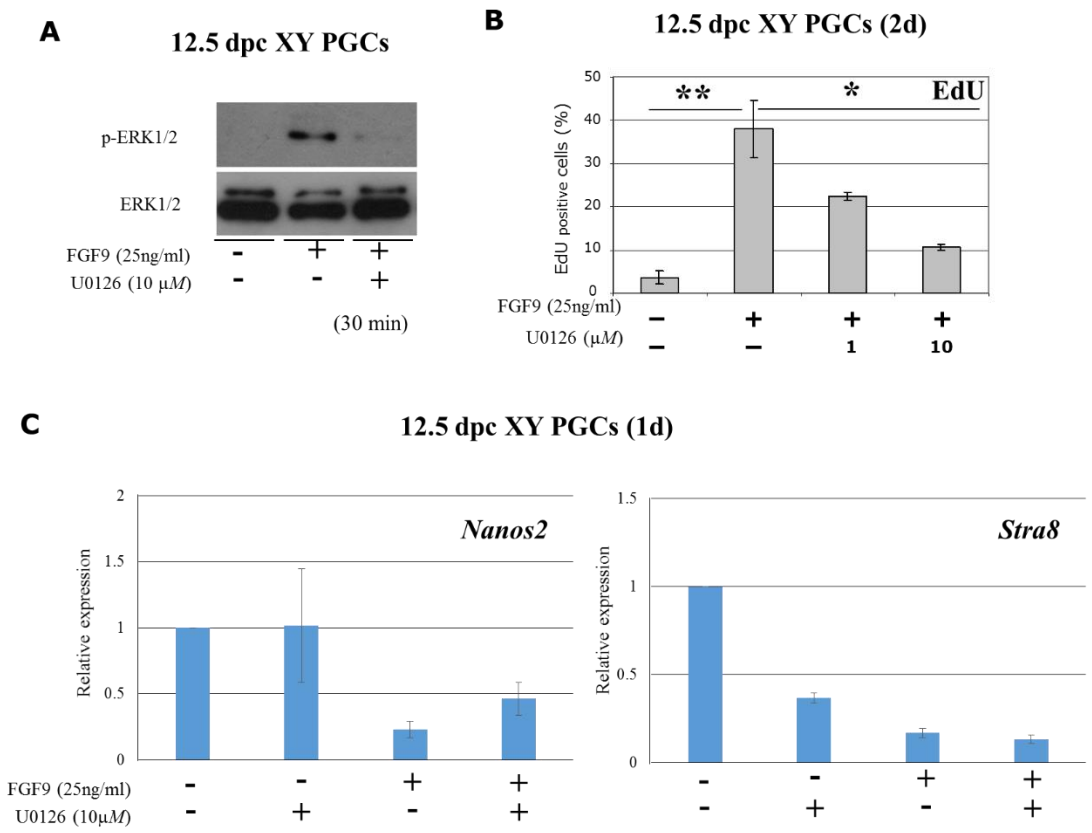

Figure 11. ERK1/2 signaling pathway induces PGC proliferation

To examine whether ERK1/2 signaling pathway activated by high FGF9 treatment regulates PGC proliferation, 12.5 dpc XY PGCs were cultured with high FGF9 and/or ERK1/2 inhibitor (U0126).
(A) High FGF9 treatment clearly activated ERK1/2 pathway in XY PGCs, but 10 μM U0126 treatment completely suppressed phosphorylation of ERK1/2 in the cells. **(B)** EdU assay shows that under the high FGF9 treatment, 40% of the PGCs underwent cell proliferation. In the PGCs treated with both FGF9 and the inhibitor at 1 or 10 μM, cell proliferation rates were suppressed at 20 or 10%, respectively. *P<0.05, **P<0.01. **(C)** Without FGF9 treatment, the ERK inhibitor did not affect male-specific *Nanos2* expression. In the presence of high FGF9, *Nanos2* expression was decreased in XY PGCs by 25% of the control. When the cells were treated with both FGF9 and the inhibitor, *Nanos2* expression increased 2-fold in the XY PGCs compared with the cells treated with high FGF9 only. Female specific Stra8 expression was very low in all 4 groups.

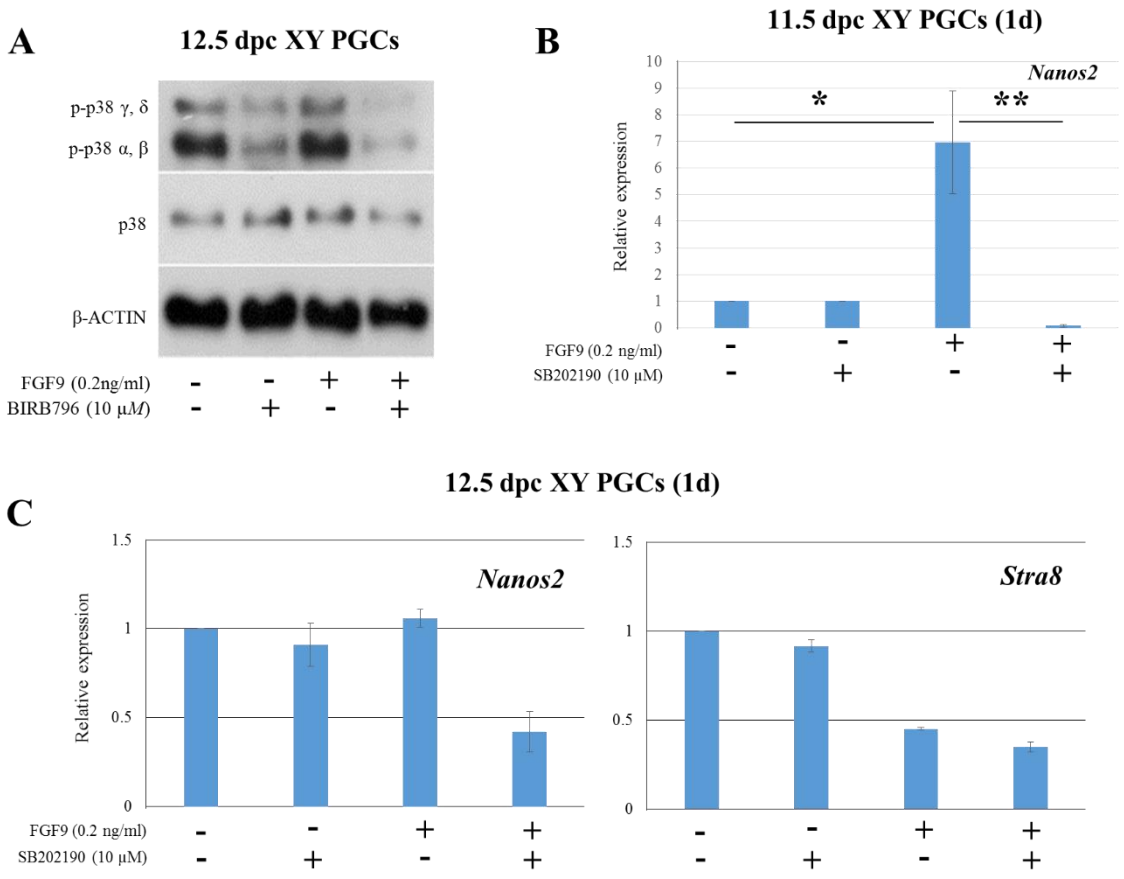

Figure 12. p38 signaling pathway induces PGC male differentiation

To determine the whether p38 signaling pathway, activated by low FGF9, regulates PGC male differentiation, 12.5 or 11.5 dpc XY PGCs were cultured with low FGF9 and/or p38 inhibitors (BIRB796 and SB202190). p38 has 4 isoforms: *α, β, γ*, and *δ*. BIRB796 inhibits all p38 isoforms and SB202190 inhibits only *α* and *β* isoforms. **(A)** In 12.5 dpc XY PGCs treated with 10 μM BIRB796, p38 phosphorylation was successfully blocked. Low FGF9 treatment enhanced p38 phosphorylation in the cells, in particular, *α* and *β* isoforms were predominantly phosphorylated. When both low FGF9 and BIRB796 were treated together, the phosphorylation of p38, especially *α* and *β* isoforms were suppressed in the 12.5 dpc XY PGCs. **(B)** We examined effects of *α* and *β* isoforms on PGC male differentiation. XY PGCs at 11.5 dpc were treated with low FGF9 and/or SB202190 for 1 day and the male-specific *Nanos2* expression was analyzed. The inhibitor treatment without low FGF9 did not affect *Nanos2* expression in the cells. When PGCs were treated with low FGF9, *Nanos2* expression was significantly upregulated at nearly 6 times higher than that of the control. In contrast, when both low FGF9 and the inhibitor were administered together, *Nanos2* expression was completely suppressed in the PGCs. **(C)** In 12.5 dpc XY PGCs, the control, only FGF9, and only SB202190 treated groups had similar *Nanos2* expression levels after 1 day of culture. When SB202190 treated with FGF9, *Nanos2* expression was downregulated. *Stra8* expression was not affected in the presence of SB202190.

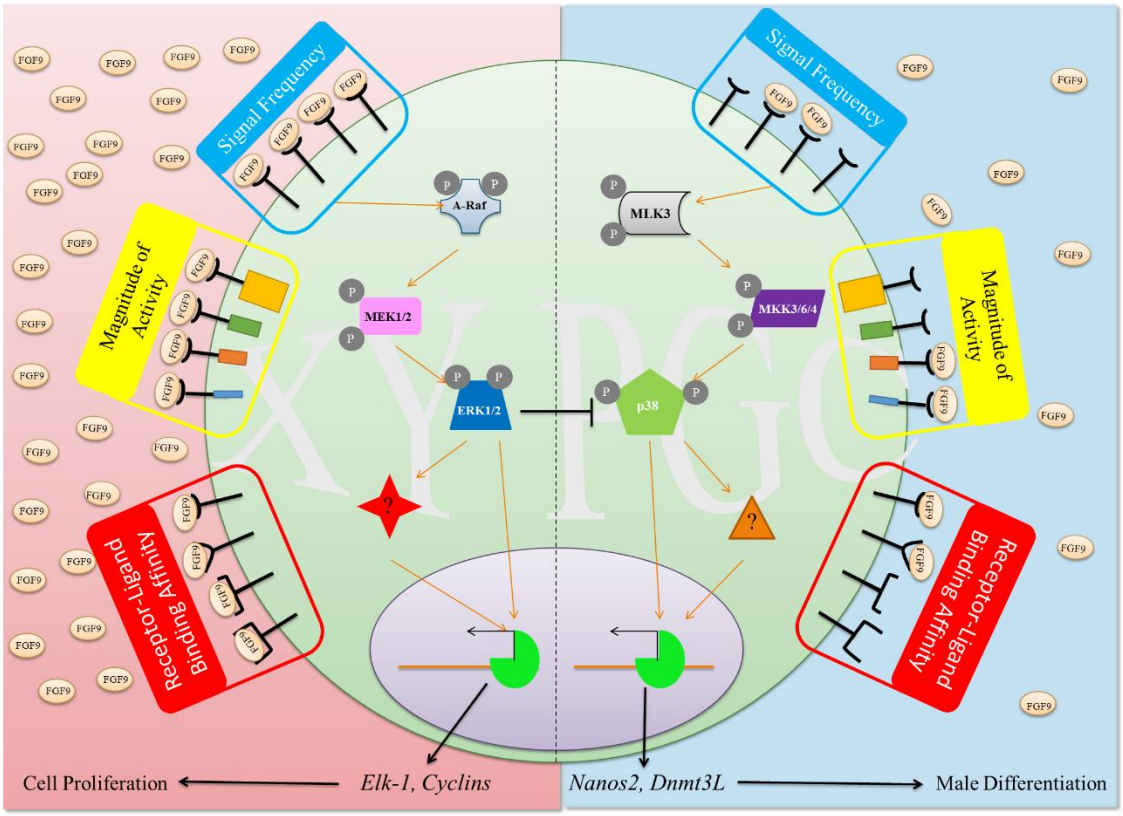

Figure 13. Dose-dependent functions of FGF9 in PGC differentiation

Different concentrations of FGF9 can cause different signal frequency which may lead activation of p38 or ERK1/2 (Dailey et al., 2005). Low or high FGF9 treatments generate low or high signal rate in the cell and these different rates activate distinct MAPKKKs (MAP3K). Therefore, p38 or ERK1/2 is activated at the end of the cascades. Then, p38 directly or indirectly induces transcription of male specific genes, such as *Nanos2* and *Dnmt3l*, or ERK1/2 directly or indirectly induces transcription of cell cycle-related genes, such as *Cyclins* and *Elk-1* (Dailey et al., 2005).

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