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Signaling Roadmap Modulating Chicken Primordial Germ Cells Proliferation and Self-Renewal

Review Article

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ABSTRACT

In the life science technologies, the chicken Primordial Germ Cells (PGCs) are recognized as the most practical cells compared to the other chicken stem cells. For this purpose, the isolation and long-term culture of these cells are of great importance for the production of therapeutic proteins, such as monoclonal antibodies, vaccines, endangered species protection, and chimeric bird production. However, one of the major challenges of working with these cells is their lack of proliferation and self-renewal ability in the laboratory environment. Recent researches on chick PGCs have shown that the pluripotency-related signaling pathways active in these cells are highly effective in their *in vitro* proliferation and self-renewal. Therefore in this review, we tried to summarize and evaluate the mechanisms of the most important pluripotency-related signaling pathways in chicken PGCs, which may result in achieving the reproducible line for these cell types. Studies have shown that one method to induce pluripotency in PGCs is to manipulate signaling pathways, including TGF-β and Wnt/β-catenin, using growth factors and small molecules. Along with activation of signaling pathways involved in self-renewal, improving culture conditions can be an effective way to achieve chicken PGC cell line. It could be concluded that providing a defined culture condition and activating specific signaling pathways can lead to induction of proliferation in chick PGCs.

KEY WORDS pluripotency, primordial germ cell, self-renewal, signaling pathway, small molecule.

INTRODUCTION

Primordial germ cells (PGCs) are precursor germ cells and play a central role in the continuation of the species by the transmission of genetic information from one generation to the next. In comparison to the other chicken stem cells, PGCs have unique developmental characteristics (Han, 2009) and numerous signaling pathways play important roles in the development of their self-regulation (De Felici *et al*. 2009). Due to the lack of proper avian PGCs derivation methods during the early stages of embryonic development in the last 15 years, there is still much to investigate regarding PGCs. However, due to the complicated regulatory mechanism of PGCs, only insignificant progress was observed. It has also been shown that complex regulatory networks in PGCs are controlled by various external factors that lead to the activation of intracellular signaling pathways (De Felici *et al*. 2009). For instance, efficient production of cultured PGCs with increased cell survival and proliferation rate can be enriched by numerous factors and mechanisms. The most important of these factors and mechanisms include fibroblast growth factor (FGF), retinoic acid (RA), stem cell factor (SCF), leukemia inhibitory factor (LIF), and bone morphogenetic protein 4 (BMP4) (Matsui *et al*. 1992; Resnick *et al*. 1992; Tang *et al*. 2006). Although the signaling pathways involved in chicken PGCs

pluripotency are unclear, many recent studies have focused on TGF-β signaling pathways (Whyte *et al.* 2015), Wnt/Gsk3/β-catenin (Lee *et al.* 2016), BMP4 (Whyte *et al.* 2015), and basic FGF (bFGF) (Choi *et al.* 2010; Macdonald *et al.* 2010; Miyahara *et al.* 2014). The ones mentioned above are the best-studied signaling pathways in controling chicken PGCs' survival and proliferation. Recently, three different signaling pathways, including transforming growth factor beta (TGFβ), FGF, and insulin, have been reported to be required for PGC growth in chickens, suggesting the existence of a combined effect of multiple signaling pathways (Whyte *et al.* 2015). Moreover, many researches have proved the crosstalk between Wnt/β-catenin and FGF signaling (Lee *et al.* 2016). Therefore, it is necessary to study the regulatory mechanisms of PGCs growth. This can be achieved by investigating the role of endogenous and exogenous factors and finding out their roles in controlling the signaling pathways, which subsequently leads to the recognition of the key genes in the pathway. Despite numerous studies focusing on PGCs extraction and different techniques of long-term PGCs maintenance in the culture medium in the last decades, reproducibility, economic values, and long term maintenance of chicken PGCs are still remaining uncertain (Whyte *et al.* 2015).

In this review, we discuss the signaling systems that control the regulation of long-term maintenance and self-renewal of chicken PGCs. The new findings from the signaling pathways and critical regulator genes of the chicken PGCs could provide insights into the biology of chicken pluripotent stem cell (PSC) lines and using them for the creation of recombinant proteins and vaccines.

Factors and mechanisms involved in chicken PGCs proliferation

Wnt/Gsk3/β-catenin signaling

It is shown that Wnt/Gsk3/β-catenin signaling can act as a key player in maintenance of PSC pluripotency and self-renewal. The Wnt is a member of the secretory protein family and signaling molecules that regulate numerous pathological and physiological processes during the development processes in all animal cells (Logan and Nusse, 2004). Also, it is shown that Wnt can act as a key player in promotion of proliferative capacity in both cancer and stem cells (Sato *et al.* 2004; Polakis, 2012). In the canonical Wnt pathway, Wnt ligands bind to frizzled (Fzd)-lipoprotein related protein (Lrp) 5/6 receptor complexes and activate Dishevelled (Dvl) proteins, leading to the phosphorylation and inactivation of GSK3β and accumulation of β-catenin in the cytoplasm (Figuer 1). Upon suppression of GSK3β, β-catenin enters the nucleus and creates heterodimers with T-cell factor (TCF) transcription factors and regulates various target genes (Fu *et al.* 2011; Valenta *et al.* 2012).

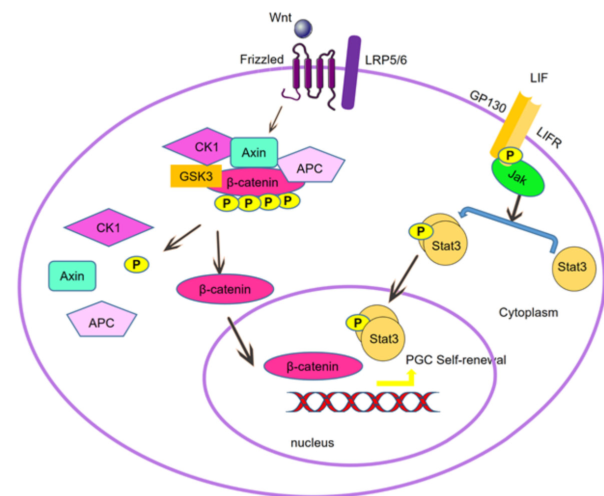

Figure 1 Wnt/β-catenin and LIF/STAT3 signaling pathways in chicken PGC self-renewal

Actually, studies accomplished by the activation of Wnt ligands showed improvement of self-renewal in mouse PGCs. Consistent with our results, recent studies on PGC development in mice have revealed the roles of several Wnt ligands including Wnt3 and Wnt3a in the specification (Ohinata *et al.* 2009; Bialecka *et al.* 2012; Aramaki *et al.* 2013; Tanaka *et al.* 2013), Wnt4 ligand in female sexual differentiation of these cells (Vainio *et al.* 1999; Chassot *et al.* 2012), and Wnt5a ligand and its receptor Ror2 in migration (Laird *et al.* 2011; Chawengsaksophak *et al.* 2012). Also, some experiments has demonstrated that self-renewal and proliferation of avian PGCs, similar to mammalian PGCs require Wnt/β-catenin signaling (Kimura *et al.* 2006; Ohinata *et al.* 2009; Chawengsaksophak *et al.* 2012) and Wnt/β-catenin signaling is considered to be an important pathway in chicken PGCs by governing their pluripotency. Compensatory pluripotency-related signaling is possible description of this observation, that begins after culturing PGCs under culture conditions (Hassani *et al.* 2014).

To examine the importance of the Wnt/β-catenin pathway in chicken PGCs, in 2016, researchers applied JW74 and BIO, inhibitors of β-catenin and GSK3-β, respectively (Lee *et al.* 2016). The results of their study suggested that inhibition of Wnt/β-catenin signaling causes cell cycle arrest through downregulation of CDK6 and CCND1 (Sun *et al.* 2008), therefore, it showed that Wnt/β-catenin signaling is needed for the maintenance of PGC proliferation. Since BIO and JW74 could activate and inactivate β-catenin, respectively, in cultured chicken PGCs trhough modulating its active form level. Lee *et al.* (2016) used Wnt ligands or BIO to activate the Wnt signaling pathway in chicken PGCs. The outcomes of their proliferation assay proposed that the Wnt/β-catenin signaling activation caused *in vitro* proliferation of chicken PGCs, whereas JW74 inhibitrd this.

These outcomes suggested that Wnt/β-catenin signaling is needed for *in vitro* proliferation of chicken PGCs (Lee *et al.* 2016).

In the other hands, studies showed that β-catenin is a target gene of FGF signaling using RAS/ ERK or PI3K/AKT cascades (Israsena *et al.* 2004; Ding *et al.* 2010). Notably, if bFGF is not exist, β-catenin will be only located in the membrane with E-cadherin, where it accumulates and in the presence of bFGF is translocated to the nucleus to activate downstream genes (Ding *et al.* 2010; Valenta *et al.* 2011). Also, some experiments have demonstrated that bFGF increases β-catenin signaling in cells via increasing the level of β-catenin mRNA, nuclear translocation of β-catenin, and phosphorylation of GSK-3-β (Otero *et al.* 2004). Thus, β-catenin through the overexpression of bFGF lead to *in vitro* expansion of stem cells, while its absence enhances differentiation (Israsena *et al.* 2004). Also, due to the β-catenin role in the chicken PGCs proliferation that was previously reported (Ge *et al.* 2012), Lee *et al.* (2016) suggested that bFGF signaling develop the chicken PGCs proliferation through the β-catenin activation and the downstream signaling. Overall, activition of Wnt/β-catenin signaling was found to be necessary for proliferation of chicken PGCs.

FGF and MAPK/Erk signaling

The FGFs are a family of cell-signaling proteins that are classified into six different FGF sub-families (Itoh, 2007). FGF2, which is also known as bFGF encodes FGF-β which is a signaling protein in the FGF family (Dionne *et al.* 1990; Kim, 1998). Like other members of FGF family, bFGF possesses cell survival characteristics and broad mitogenic and is involved in various biological processes such as cell proliferation regulation, embryonic development, migration, differentiation, and cell growth (Feldman *et al.* 1995; Delaune *et al.* 2005). Also, bFGF is a potent mitogen in mammalian PGCs medium culture (Resnick *et al.* 1998), and FGF signaling is vital to PGCs migration and controls the number of germ cells in mice (Takeuchi *et al.* 2005). In chickens, bFGF is among the factors supporting preblastodermal cells (Park *et al.* 2006), embryonic germ cells (EGCs) (Park and Han, 2000), and PGCs proliferation (van de Lavoir *et al.* 2006). Choi *et al.* (2010) and Macdonald *et al.* (2010) identified bFGF as an fundemental factor for *in vitro* proliferation of chicken PGCs. In PGCs of chicken, FGF2 activates the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), that has anti-apoptosic features and promotes the cell cycle. In the FGF/MAPK/Erk pathway, the FGF ligand that binds to FGFR, a family of receptor tyrosine kinases (RTKs), causes receptor dimerization and then leading to phosphorylation RTKs.

Phosphorylated RTKs activate important signal pathways, such as the MAPK/Erk and the PI3K/Akt signaling pathways (Figure 2).

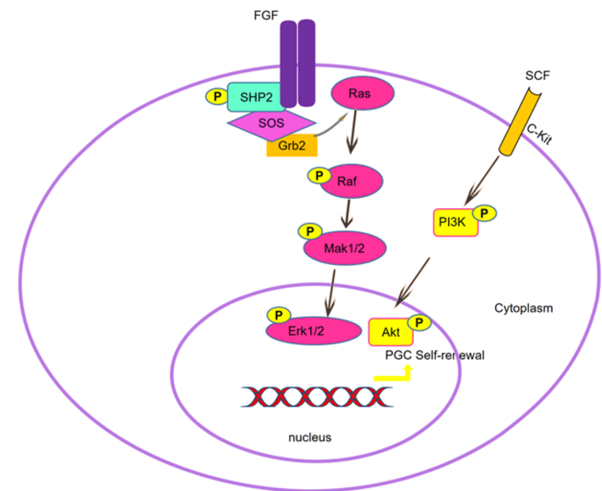

Figure 2 FGF and GFs signaling pathway in chicken PGC self-renewal

When MEK and FGF2 receptors are prohibited by PD0325901 and PD173074, respectively, ERK activation does not happen. It should be noted that when MEK/ ERK signaling is not activated, PGCs started degeneration and failed to proliferate (Choi *et al.* 2010; Macdonald *et al.* 2010). It can be concluded that bFGF via MEK/ERK signaling plays a crucial role in the long-term proliferation and survival of chicken PGCs (Choi *et al.* 2010). The data demonstrated that MEK/ERK is a downstream target of bFGF, which activates a wide range of second messengers and supports cell proliferation in PGCs of chicken (Choi *et al.* 2010).

In the other hands, FGF9 and FGF8 are other FGFs family members, which involved in a variety of biological processes including differentiation, and cell growth. Among FGFs, Ulu *et al.* (2017) showed that FGF9 regulates PGCs in a dose-dependent manner. FGF9 promotes differentiation into SSCs at low concentration (0.2 ng/mL), while it prohibits differentiation and facilitates PGCs self-renewal at high concentration (25 ng/mL) (Ulu *et al.* 2017). It has been showed that the FGF family such as FGF8 can cause *in vitro* proliferation of mouse PGCs (Kawase *et al.* 2004) and stimulate PGCs self-renewal through regulating the MAPK/ERK signal (Dolci *et al.* 2001; Choi *et al.* 2010). It was found that adding FGF8 to the mouse embryos promotes PGCs proliferation (Kawase *et al.* 2004). Another study showed that this was obtained through activating ERK1/2 in MAPK/ERK signaling pathway (Choi *et al.* 2010).

Also, FGF8 was differentially expressed in the chicken SSCs, PGCs, and ESCs. It has been demonstrated that it participated in cell differentiation regulation and embryogenesis. Wang *et al.* (2018), found that FGF8 knockdown promotes formation of SSCs whereas its overexpression can promote chicken PGCs self-renewal and prohibit SSCs formation. In conformation with the earlier studies, researchers found the positive correlation between MAPK/ERK signaling pathway and the FGF8 expression level (Astick, 2012). The FGF8 knockdown can prohibit the MAPK/ERK signaling pathway, whereas FGF8 overexpression can enhance it.

There are pieces of literature that report the differentiation of other types of germ cells that are controlled by FGFs. Kurimoto *et al.* (2014) revealed that ERK signaling negative regulators such as Spry2 and Dusp6, are explicitly up-regulated in PGC precursors and nascent PGCs during PGCs specification. Kimura *et al.* (2014) showed that FGF-ERK signaling inhibition causes induction of PGC-like cells from mouse ESCs. Significantly, overexpression of FGF8 can promote proliferation of PGCs and also prevent its differentiation into SSCs (Kimura *et al.* 2014). In general, the FGFs family members by activation the FGF/MAPK/Erk signaling pathway plays an important role in PGCs proliferation and consequently, it is necessary that the addition of bFGF to the cell culture medium.

PI3K/Akt signaling

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway is another factor and mechanism involved in proliferation and survival in PSCs (Brazil *et al.* 2002). Indeed, the PI3K signaling, has belonged to the family of lipid kinases, exerts its biological effects through the activation of various downstream molecules such as AKT (Brazil *et al.* 2002; Cantley, 2002). The PI3K/AKT signaling pathway activated by growth factors such as LIF, FGF, SCF and RA through phosphorylation of the 3-hydroxyl group of the plasma membrane cell. Interestingly, activation of the PI3K/Akt signaling is performed by the interaction between Receptor tyrosine kinase (c-Kit) and its ligand (KL) (Figuer 2). In other cell types, KL binding induces autosphorylation of the c-Kit receptor, and the activated receptor, in turn, phosphorylates various substrates, thus activating cascades or distinct signaling pathways such as the Janus kinase (JAK)_signal transducer and activator of transcription (STAT), the PI3K_AKT_mTOR_p70S6K, Ras_mitogen-activated protein kinase kinase (MEK)_mitogen-activated protein kinase (MAPK), and the Src signaling pathways (Blume-Jensen *et al.* 1993; Rameh and Cantley, 1999; Ueda *et al.* 2002).

So, the Akt serine/threonine kinase (also known as protein kinase B) activation is necessary for the proliferation and survival of mammalian endogenous PGCs and the *in vitro* establishment of EG cells (Kimura *et al.* 2008; Matsui *et al.* 1992). A number of studies suggest that PI3K/Akt signaling promotes the dedifferentiation of mice PGCs (Kimura *et al.* 2006). However, in chicken PGCs, the inhibition of PI3K/Akt signaling arrests *in vitro* proliferation and promotes apoptosis (Macdonald *et al.* 2010; Whyte *et al.* 2015).

On the other hand, growth factors such as SCF, as the c-Kit ligand, activate PI3K/Akt signaling. Thus, c-Kit and its ligand are the key regulators of PGCs growth and survival (Besmer, 1993), and interaction between c-KIT (In mice, c-KIT is expressed on the surface of PGCs) and SCF is needed for migration, inhibition of apoptosis, and germ cell proliferation (Dolci *et al.* 1991; De Felici *et al.* 1999; Gu *et al.* 2009). These data propose that SCF has been apploed as a support factor for *in vitro* proliferation and survival of mouse PGCs (Dolci *et al.* 1991; Matsui *et al.* 1992; De Felici *et al.* 1999). Mouse SCF1 ability for the survival of PGCs culture is limited, and it is not involved in its proliferation (Dolci *et al.* 1991). Meanwhile, PGCs proliferation *in vitro* is enhanced by SCF2 and can be used for the long-term cultivation of murine PGCs (Dolci *et al.* 1991; De Felici *et al.* 1999). In various studies, SCF has also been used for the culture of PGCs in chickens (van de Lavoir *et al.* 2006; Choi *et al.* 2010; Macdonald *et al.* 2010; Naito *et al.* 2012). Some studies have suggested that administration of chicken SCF (SCF) may positively affect *in vitro* proliferation of chicken PGCs (Karagenç and Petitte, 2000; Glover and McGrew, 2012). However, chicken SCF2 can significantly improve the proliferation of chicken PGCs *in vitro* by activating the PI3K/Akt signaling pathway (Miyahara *et al.* 2016).

RA is another important factor in the activation of the PI3K/Akt signaling pathway (Koshimizu *et al.* 1995; Yu *et al.* 2011). RA treatment can activate PI3K in cultured PGCs and subsequently induce Akt phosphorylation. These outcomes peopose that RA stimulates NF-kB activation as a downstream target modulator of PI3K/Akt signaling (Yu *et al.* 2011). Activated Akt induces NF-kB nuclear import and thus gives access to target gene promoters (Paling *et al.* 2004; Chen and Khillan, 2010; Han *et al.* 2010). Some of the signals that act on PGCs to initiate proliferation are produced by targeted cell cycle gene products (Ge *et al.* 2007; Yu *et al.* 2011). Inhibition of PI3K/Akt and NF-kB signaling causes the down-regulation of cell cycle regulatory genes and suppresses cell proliferation in RA-treated PGCs.

RA remarkably increases the number of migratory phase PGCs and attenuates the depletion of gonadal PGCs *in vitro* (Matsui *et al.* 1992; Koshimizu *et al.* 1995). Yu *et al.* (2012) revealed that RA significantly promotes colony for-

mation in PGCs and enhances their survival and mitotic activity. Accordingly, the results showed that the addition of RA stimulates the survival and proliferation of chicken PGCs through activation of the PI3K/Akt pathway, which includes the NF-kB signaling cascade. This suggests that activation of PI3K/Akt signaling is necessary for the proliferation and survival of chicken PGCs.

LIF/Stat3 signaling

Leukemia inhibitory factor (LIF) is a member of the cytokine family which contains cardiotrophin, oncostatin M, IL-11, and IL-6. LIF acts on various types of cell, such as neurons, adipocytes, hepatocytes,osteoblasts, and embryonic stem (ES) cells (Nicola and Hilton, 1997; Taga and Kishimoto, 1997). Initially, LIF was detected as an inducer of M1 leukemia cell differentiation (Tomida *et al.* 1984) and rediscovered as an inhibitor of mouse ES cell differentiation (Smith *et al.* 1988). Activation of the LIF/Stat3 signaling pathway begins when LIF binds to the cell surface via two transmembrane proteins, the LIF receptor beta (LIFRβ) and gp130. LIF uses the membrane protein gp130 to form the heterodimer receptors (Figuer1). This heterodimer then phosphorylates and activates a gp130-associated cytoplasmic tyrosine kinase - Janus kinase (Jak) - which then activates three intracellular signaling pathways: the Jak/signal transducer and activator of transcription 3 (Stat3) pathway, the PI3K/protein kinase Akt pathway, and the MAPK/ Erk pathway (Hassani *et al.* 2014).

LIF/Jak/Stat3 are the first pluripotency-related signal transductions in mouse ES cells. Activated Jak can phosphorylate tyrosine residues on gp130 and provide a docking site for the proteins that include src homology 2 (SH2) domains such as Stat3. Furthermore, Jak directly phosphorylates Stat3 on tyrosine 705 (Stat3pTyr705), thereby leading to its homodimerization. Dimerized Stat3 enters the nucleus and regulates the expression of target genes (Hassani *et al.* 2014). Some research suggests a role for the JAK-STAT signaling pathway in the growth of male germ cells in Drosophila and mice (Sheng *et al.* 2009; Oatley *et al.* 2010). In mammalian PGCs, short-term PGC culture experiments and genetic knockout models have involved in the growth factor LIF in proliferationand early survival (Dolci *et al.* 1991; Matsui *et al.* 1992; Dolci *et al.* 1993; Farini *et al.* 2005). Besides, Horiuchi *et al.* (2004) showed that in the undifferentiated state, chicken LIF could maintain chicken ES cell cultures (Horiuhci *et al.* 2004). Moreover, Zhang *et al.* (2017) showed that the JAK-STAT pathway is involved in the proliferation, differentiation, and signal transduction of chicken PGCs. Furthermore, Zhang *et al.* (2017) proposed that JAK-STAT effectively promoted the PGCs formation in the genital ridge during early embryogenesis *in vivo* and played a positive role in the regulation of ESC differentiation into SSC *in vitro*, with STAT3 and JAK2 function as pivotal factors for intracellular signal transduction (Zhang *et al.* 2017). Thus, mammalian cytokines such as human and mouse LIF (Petitte *et al.* 2004) as well as avian cytokines such as LIF are able to maintain self-renewal state in chicken stem cells (Nakano *et al.* 2011). In general, LIF activation of the JAK/STAT pathway can lead to long-term culture of chicken stem cells (Petitte *et al.* 2004).

TGFβ, Activin and Nodal/Smad2/3 signaling

Recent studies have shown that the signal transduction through TGF-β/Activin/SMAD2/3 has been shown to be essential for the proliferation and self-renewal of PSCs. It is suggested that TGF/β signaling inhibiting mouse PGCs proliferation in culture (Godin and Wylie, 1991). While some experiments has demonstrated that self-renewal of avian PGCs (Whyte *et al.* 2015), similar to mammalian epiblast stem cells (epiSCs), human embryonic stem cells (hESC) and human iPSC require a TGF/β signaling for self-renewal.

TGF/β ligands such as Activin A and Nodal lead to phosphorylate regulatory Smad (R-Smad) proteins through heterodimers of TGF-βR 1 and 2. R-Smads bind to co-Smad (Smad4) following phosphorylation and translocate into the nucleus and eventually lead to hundreds of genes up or down regulated within 1-2 hours (Figuer 3).

This phosphorylation was ablated by the addition of one of the type II receptor chemical inhibitors SB431542, which selectively suppresses ALK1/5/7 type I receptors. Furthermore, the addition of SB431542 resulted in abrupt exit from the pluripotent state and self-renewal, and as well the activation of lineage specific genes (Yakhkeshi *et al.* 2018). Thus, the TGF-β/Activin and Nodal/Smad2, 3 signaling are known to perform pivotal functions in the differentiation, proliferation, migration, and apoptosis of reproductive cells. Furthermore, TGF/β has an inhibitory effect on the proliferation of at least two stem cell populations: hemopoietic stem cells and intestinal crypt cells in the mice. Many studies have suggested that TGF/β inhibiting mouse PGC proliferation in culture may be a general mechanism in the complicated process of stem cell proliferation (Godin and Wylie, 1991). Although several studies have identified TGFβ as an active signaling pathway in PGCs, the role of TGFβ in chicken PGCs remains unclear. Recently, it is represented that TGF-β/Activin/Nodal signaling pathways are active in chicken PGCs both *in vivo* and *in vitro*. It can support the self-renewal of germ-line competent PGCs in combination with insulin and FGF2 (Whyte *et al.* 2015). Moreover, Yakhkeshi *et al.* (2018) showed that through the activation of TGF-β signaling pathways by SMAD2/3 phosphorylation, Activin A leads to *in vitro* expansion of quail gonadal-derived PGCs.

Therefore, their findings suggested that Smad2/3 signaling is a critical pathway in quail PGCs proliferation (Yakhkeshi *et al.* 2018). Furthermore, it has been showed that signal transduction by TGF-β/Activin/Nodal is crucial for chicken PGC proliferation (Macdonald *et al.* 2010; Whyte *et al.* 2015; Lee *et al.* 2016).

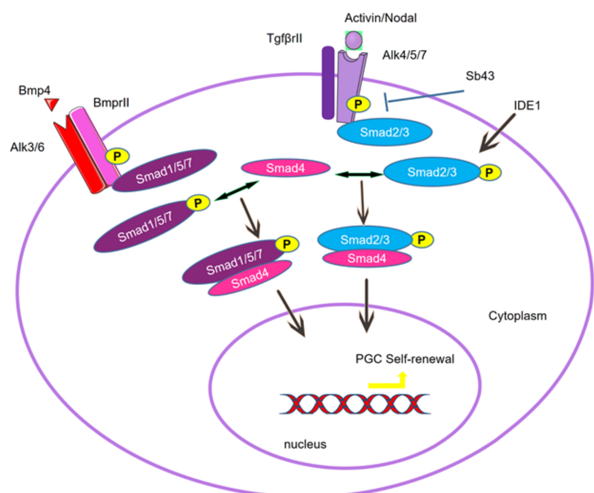

Figure 3 BMP/SMAD and Activin/Nodal Signaling Pathway in Chicken PGC Self-renewal

BMP4/Smad1/5/8 signaling

Bmp4 is a multifunctional cytokine that belongs to the TGFβ superfamily of intercellular signaling proteins. As other members of the TGFβ superfamily, BMPs transduce their signals by binding and inducing the dimerization of different type I and type II serine/threonine kinase receptors, and cause phosphorylation of the type I receptors (BMP receptor 1A (BMPR1A) or BMP receptor1B (BMPR1B)). The activation of BMP receptors causes phosphorylation of SMAD1/5/8 (R-Smads) proteins (Figuer 3). Afterwards, R-Smads bind to co-Smad (Smad4) and translocate into the nucleus, where they act as transcription factors and regulate the expression of target genes (Kawabata *et al.* 1998).

The functional significance of BMP4 and its signal transduction pathway components in PGCs differentiation during embryonic gonad development has been documented through gene knockout mouse models. For instance, Bmp4-null mutant embryos are deficient in the development of PGCs (Lawson *et al.* 1999), and other studies have shown that BMPR1 (de Sousa Lopes *et al.* 2004) and its signal transducers Smad1 (Tremblay *et al.* 2001; Hayashi *et al.* 2002) and Smad5 (Chang and and Matzuk, 2001) are also essential for PGCs formation. Researches in the mouse (Lawson *et al.* 1999; Ying and Zhao, 2000; Ying and Zhao, 2001) and the axolotl (Johnson *et al.* 2003; Chatfield *et al.* 2014) showed that BMP signal transduction is essential for specifying PGCs through induction and for the initial formation of the germ cell lineage and PGC survival (Lawson *et al.* 1999; Ying and Zhao, 2001; Farini *et al.* 2005). While few studies have concentrated on the specific role of BMP signaling in the PGC formation in chickens, some researches show that this pathway promotes PGCs proliferation in the chicken, and their migration in embryos may be regulated by this pathway (Whyte *et al.* 2015).

Recently Zuo *et al.* (2019) showed that BMP4 and RA signaling play a different role in PGC formations (Zuo *et al.* 2019). RA is the most usual reagent applied during *in vitro* germ cell differentiation of pluripotent cells (Imamura *et al.* 2014). Among genes influenced by RA treatment, there are members of the BMPs family including BMP2, BMP4, and BMP8b which activate Smad1/5 in the PGCs. Moreover, the BMP-Smad1/5 signaling pathway is necessary for PGCs proliferation and specification (Tremblay *et al.* 2001; Chang *et al.* 2012; Whyte *et al.* 2015). As a result, the BMP4 and RA signaling pathways play opposite roles in the formation of germ cell driven by epigenetic processes including histone acetylation, phosphorylation, and ubiquitination. Also, Smurf and Smad5 are crucial to the interaction between the two pathways (Zuo *et al.* 2019).

CONCLUSION

Maintaining a well-balanced network for transmitting signals has been shown to play an important role in self-renewal and cell line production in stem cell cultures. Wnt/Gsk3/β-catenin, TGF-β/Activin/Nodal and FGF/MAPK/Erk signal pathways are considered to be the most vital pathways for self-renewal and long-term culture of PCGs. Interestingly, the signaling pathways that support PGC pluripotency and proliferation exhibit diverse functions in different species. For example, the TGF-β/Activin pathways in mouse PGCs lead to inhibition of self- renewal, which are thought to be prominent signaling pathways regarding maintenance of pluripotency and long-term culture of chicken PGCs. TGF-β signaling pathway appears to be the only signaling pathway which is essential in maintaining pluripotency in chicken PGCs. However, a closer look reveals that the connections of all signaling pathways lead to the production of cell lines. Overall, *in vivo* and *in vitro* evidence suggest that chicken PGCs are quite different from mammalian PGCs due to their unique development in terms of functional behavior and signal transduction mechanisms. Although some recent studies have shown the production of PGCs, the demonstration of all pluripotent properties in long-term culture has not yet been achieved. It appears the pluripotency to be a unique phenomenon, and the production of PGC lines requires further efforts to identify interactions between underlying molecular mechanisms

and signaling pathways. To use PGCs for biotechnology purposes, it is essential to obtain evidence of how to stabilize pluripotency in culture.

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