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# Basic fibroblast growth factor is critical to reprogramming buffalo (*Bubalus bubalis*) primordial germ cells into embryonic germ stem cell-like cells

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ABSTRACT

Primordial germ cells (PGCs) are destined to form gametes *in vivo*, and they can be reprogrammed into pluripotent embryonic germ (EG) cells *in vitro*. Buffalo PGC have been reported to be reprogrammed into EG-like cells, but the identities of the major signaling pathways and culture media involved in this derivation remain unclear. Here, the effects of basic fibroblast growth factor (bFGF) and downstream signaling pathways on the reprogramming of buffalo PGCs into EG-like cells were investigated. Results showed bFGF to be critical to buffalo PGCs to dedifferentiate into EG-like cells (20 ng/mL is optimal) with many characteristics of pluripotent stem cells, including alkaline phosphatase (AP) activity, expression of pluripotency marker genes such as *OCT4*, *NANOG*, *SOX2*, *SSEA-1*, *CDH1*, and *TRA-1-81*, and the capacity to differentiate into all three embryonic germ layers. After chemically inhibiting pathways or components downstream of bFGF, data showed that inhibition of the PI3K/AKT pathway led to significantly lower EG cell derivation, while inhibition of P53 activity resulted in an efficiency of EG cell derivation comparable to that in the presence of bFGF. These results suggest that the role of bFGF in PGC-derived EG-like cell generation is mainly due to the activation of the PI3K/AKT/P53 pathway, in particular, the inhibition of P53 function.



## 1. Introduction

Primordial germ cells (PGCs) are embryonic precursors of the germ cell lineage, destined to form gametes. In mice, PGC precursors are initially a population of cells within the proximal margin of the epiblast at 6.5 days post-conception (dpc) [1]. At approximately embryonic day 7.25 (E7.25), a small cluster of 40–50 alkaline phosphatase (AP)-positive PGCs emerge at the posterior of the primitive streak near the base of the allantois [1]. Then PGCs migrate into the hindgut at E9 and enter from the dorsal body wall into the genital ridges at E10.0–E10.5, whereby they eventually colonize the genital ridges at E11.5 [2]. During this process, PGCs number about 25,000 at E13.5, at which time, in males, PGCs enter mitotic arrest, and, in females, germ cells enter meiosis [3].

Although the developmental potency of PGCs is restricted to the germline in normal development, PGCs can be induced to dedifferentiate into pluripotent cells *in vitro* when exposed to exogenous signaling molecules: leukemia inhibitory factor (LIF), stem cell factor (SCF), and basic fibroblast growth factor (bFGF) [4–6]. Embryonic germ (EG) cells possess pluripotency similar to embryonic stem cells (ESC), as they contribute to somatic and germ lineages after being introduced into blastocysts [7]. However, freshly isolated PGCs are incapable of participating in chimeras when introduced into blastocysts [5], indicating that PGCs are significantly different from EG cells and that some reprogramming events must occur during development.

The conversion of PGCs to EG cells is similar to nuclear reprogramming: some critical factors are required for PGC culture in the same way that cytoplasmic factors present in the egg cytoplasm contribute to reprogramming [8]. Research has confirmed that stem cell factor (SCF), kit ligand (KL), mast cell growth factor (MGF), leukemia inhibitory factor (LIF) and tumor necrosis factor-α (TNF-α) are important in the development of murine PGC *in vitro*

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[9–12], but these factors alone have little effect on dedifferentiation. Polypeptide factors SCF, LIF, and bFGF together promote PGC dedifferentiation into EG cells in mice [4], humans [6], swine [13], and buffalo [14], but SCF and LIF do not convert PGCs to EG cells. Rather, bFGF appears to be as critical as it is for human ESC self-renewal [15]. FGFs exert biological effects in an autocrine or paracrine fashion, interacting with transmembrane tyrosine kinase fibroblast growth factor receptors and promoting their dimerization and activation [16]. bFGF treatment during the first 24 h of primary culture has been reported to be sufficient for the derivation of mouse EG cell derivation but the manner in which bFGF affects EG cell formation is not well understood [5].

Two major pathways downstream of bFGF, bFGF/PI3K/AKT and bFGF/MAPK, are known to be involved in derivation, proliferation, self-renewal and pluripotency of PGCs. Activation of the PI3K/AKT signaling pathway can promote PGC dedifferentiation [17–21] and regulate ESC pluripotency [22]. Glycogen kinase 3 (GSK3) and tumor suppressor p53, which are downstream targets of the PI3K/AKT signaling pathway, are inhibited by activation of PI3K/AKT [23–25]. In cultured PGCs, PI3K/AKT negatively regulated p53 by enhancing MDM2 function and inhibiting phosphorylation of p53. AKT-mediated p53 inhibition is crucial to germ cell acquisition of pluripotency [18]. bFGF also activates several intracellular signaling components, such as mitogen-activated protein kinase (MAPK) and phospholipase Cγ [26]. In addition, the proliferation of PGCs in culture is supported by MAPK signaling [27], which suggests that the PI3K/AKT and MAPK signaling pathways promote EG cell derivation in a cooperative fashion. Forskolin and retinoic acid, which can replace bFGF during EG cell derivation, are reported to be mitogenic in addition to being survival factors for PGCs [28,29]. bFGF is also reported to be able to be replaced by trichostatin A (TSA), suggesting that epigenetics plays an important role in EG cell derivation [8].

While the mechanism for murine PGC dedifferentiation into EG cells is gradually becoming better understood, little is known about the mechanisms underlying similar reprogramming processes in domestic animals, such as buffalo. For this reason, data to facilitate dissection of the underlying mechanisms by which bFGF initiates the derivation of EG-like cells from buffalo PGCs are here presented.

## 2. Materials and methods

### 2.1. PGC isolation and culture

This study was conducted in accordance with the State Key Laboratory for Conservation and Utilization of Subtropical Agro-bio-resources guide for the care and use of laboratory animals. Chinese swamp buffalo fetuses were obtained from a nearby slaughterhouse and genital ridges were dissected from each one (Fig. 1). Fetuses 30–80 dpc of age were selected. Isolated genital ridges were washed three times with phosphate-buffered saline (PBS). Cells were released by pricking and scraping the genital ridge with the pinhead of a 1 mL injector to produce a single cell suspension [14]. During primary culture, cells were seeded on 35 mm plates (Corning, Corning, NY) or 4-well plates (Nunc, Denmark), pre-coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO) for 30 min. After four to six days of primary culture, primary colonies with an embryonic germ cell-like phenotype were observed.

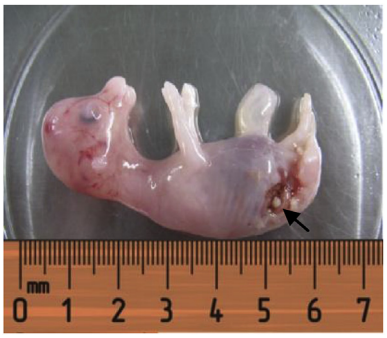


**Fig. 1.** Three-month old buffalo fetus. Arrows indicate genital ridge.

Primary colonies were passaged to 4-well plates prepared with mitomycin c-treated buffalo fetal fibroblast (BFF) feeder cells, and subsequently passaged every four to five days by mechanical methods (dissociation was performed with a scalpel). All cultures were maintained at 37 °C in 5% $CO_2$ with half the culture medium changed daily. PGCs and EG-like cells were cultured in stem cell medium [14]: DMEM (Invitrogen, Carlsbad, CA) supplemented with 20% ESC fetal bovine serum (FBS) (Hyclone, Logan, UT), 1% non-essential amino acids, 1 mM sodium pyruvate, 1 mM glutamine (Invitrogen, Carlsbad, CA), 0.1 mM 2-mercapto-ethanol, 20 ng/mL mouse recombinant leukemia inhibitory factor, 20 ng/mL mouse recombinant stem cell factor and 10, 20, or 40 ng/mL human recombinant basic fibroblast growth factor (Merck-Millipore, Boston, MA), which were added or not according to the experimental design. PD0325901 (final concentration 0.4 μM), CHIR99021 (final concentration 3 μM), LY294002 (final concentration 10 μM) and pifithrin-α (final concentration 10 μM, Stemgent, Boston, MA) were added to primary culture medium separately according to the experimental design. Control cells were cultured with an equal volume of dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO). After 5 days of primary culture, EG cell-like colonies were counted. BFFs were used as feeder cells, which were treated with 10 μg/mL mitomycin (Sigma-Aldrich, St. Louis, MO) for 3.5 h, and plated ($1 \times 10^5$ cells) in 4-well plates one day before use.

### 2.2. Alkaline phosphatase staining

AP activity in buffalo EG-like cells was measured by staining. After removal of the medium, cells were washed three times with PBS and fixed with 4% paraformaldehyde (PFA) for 30 min. After three washes with PBS, cells were stained with NBT/BCIP (Amresco, Solon, OH) for 15–30 min at room temperature. Primary multicellular colonies that were positive for AP activity were considered buffalo EG cell-like colonies [30].

### 2.3. Immunofluorescence

Briefly, passage 4 EG-like cells were fixed with 4% PFA, washed three times with PBS and then permeabilized with 0.1% Triton X-100 in PBS for 20 min, washed three times with PBS, and blocked 30 min with 1% bovine serum albumin (BSA) in PBS (blocking solution). The following primary antibodies were diluted in blocking solution and incubated with the samples overnight at 4 °C: SSEA-1 (1:250, all antibodies were from Cell Signaling Technology, Boston, MA., except where otherwise indicated), OCT4 (1:250), SOX2 (1:250), TRA-1-81 (1:250), CDH1 (1:250), and NANOG, TUBB3, cytokeratin-18, ACTA2 (1:200, Abcam, Cambridge, MA). The next day, samples were washed three times and incubated with secondary antibodies for 90 min at room temperature in the dark. Nuclei were counterstained with Hoechst 33342 (final concentration 5 μM, 6 min incubation). Immunostaining was assessed under a fluorescent microscope after three washes with PBS.

### 2.4. Reverse transcription-PCR

Total RNA was extracted from BFF, buffalo genital ridges, PGCs