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Research paper

Bone morphogenetic protein 4 (BMP4) induces buffalo (*Bubalus bubalis*) embryonic stem cell differentiation into germ cells

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ABSTRACT

The aim of the present study was to investigate the effect of Bone morphogenetic protein 4 (BMP4) stimulation on differentiation of buffalo embryonic stem (ES) cells into germ lineage cells. ES cells were subjected to *in vitro* differentiation in floating and adherent cultures, under different BMP4 concentrations (20, 50 and 100 ngml^{-1}) for different culture intervals (4, 8 and 14 days). qPCR analysis revealed that BMP4 at a concentration of 50–100 ngml^{-1} for a culture period of 14 days led to maximum induction of germ lineage genes like *DAZL*, *VASA*, *PLZF* (PGC-specific); *SYCP*3, *MLH*1, *TNP*1/2 and *PRM*2 (Meiotic genes); *BOULE* and *TEKT*1 (Spermatocyte markers); *GDF*9, *ZP*2 and 3 (Oocyte markers). The expression levels of all the genes were significantly higher under BMP4 differentiation as compared to BMP4 + NOGGIN and spontaneously differentiated cultures. Immunocytochemical analysis of embryoid bodies (EBs) and monolayer adherent cultures revealed expression of PGC- (c-KIT, DAZL and VASA); Meiotic- (SYCP3, MLH1 and PROTAMINE1); Spermatocyte- (ACROSIN and HAPRIN); and Oocyte- markers (GDF9 and ZP4). Western blotting was positive for VASA, GDF9 and ZP4. Oocyte-like structures (OLS) obtained in monolayer differentiated cultures harbored a big nucleus and a ZP4 coat. They showed embryonic development and progressed through 2-cell, 4-cell, 8-cell and blastocyst-like structures. Global DNA methylation analysis showed significantly ($p < 0.05$) decreased levels of 5-methyl-2-deoxycytidine in EBs obtained in optimum differentiation medium. The expression of meiotic markers coupled with expression of spermatocyte and oocyte markers is an indication of post-meiotic progression into spermatogenesis and oogenesis, respectively.

1. Introduction

Primordial germ cells (PGC) are the progenitor cells of germ lineage which ensure continuity of life. Apart from the general interest in specification and formation of this highly specialized PGC lineage, elucidation of the molecular mechanisms underlying the special process of meiosis, genetic and epigenetic reprogramming of germ cell cycle is of vital importance. However, a number of difficulties exist in PGC research and their progression into germ cells under *in vitro* culture conditions. For example, the complex *in vivo* environment required for PGC development has not been adequately studied which renders the exact *in vitro* simulation impossible. Moreover, PGCs are limited in number and are deeply embedded within the embryo which further mitigates the extensive studies in this direction [1,2]. PGCs are also known to migrate during development, a process which to date has not been studied under *in vitro* conditions. In addition, the large-scale screening of potential inducers of PGC specification and their progression into germ cells is difficult to implement, thus further rendering the investigations incomplete [3]. Further, due to easy availability of biological resources, initial investigations into germ cell development have been conducted mostly in mice. The studies, however, warrant a caution because murine studies may not always translate to advances in higher mammalian germ cell development. This highlights the need to investigate germ cell signaling factors and their role in PGC specification, meiosis and mature germ cell formation either directly in the species of interest or in an

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ontogenetically closer one. In search of an alternative starting material that could replace PGCs, embryonic stem (ES) cells provide the promising candidates to investigate the developmental processes *in vitro* and thereby serve as a model to compliment the *in vivo* studies [3].

A number of studies have demonstrated that PGCs arise from a subset of epiblast cells on embryonic day 7.25 in mice. Bone morphogenetic proteins 4 and 8b (BMP4 and BMP8b) have been implicated in their formation [4–7]. Further studies found that embryos targeted for BMP4 deletion never form PGCs while BMP8b null mutants form no or very few PGCs [4,5], thereby implicating the main role to BMP4 protein in germ line establishment. BMPs trigger cellular responses by binding to heteromeric receptor complexes of type I (ALK1, ALK2, ALK3 and ALK6 (BMPRIB)) [8–10] and type II (BMPRII ACTRIIA and ACTRIIB) receptors [11–14]. Among type I receptors, ALK3 is the only one clearly shown to be expressed in epiblast while ALK2 is mainly expressed and functions in visceral endoderm during gastrulation [15]. After receptor binding, signal transduction is achieved by SMAD1 and SMAD5 proteins for BMP4 and BMP8b, respectively. These two BMP signaling pathways have been found to be sufficient requirements for PGC induction from cultured epiblasts and thus provides for different possible scenarios for the signaling pathway. Either BMP4 or BMP8b function as homodimers through separate receptor complexes or they function as heterodimers through the same receptor or both homo-or hetero-dimers are formed, both having the capability to activate the signaling pathway [5,16–18]. Irrespective of the pathway involved, BMP4 induces a subset of epiblast cells to express *FRAGILIS* and *BLIMP1* genes, thereby becoming PGCs. These PGCs, under the influence of BMP4 signaling, express c-KIT, DAZL and VASA proteins and undergo migration and proliferation to colonize the genital ridge. In the gonadal environment these PGCs undergo meiosis, under the influence of meiotic proteins like SYCP3, MLH1, PROTAMINES, TRANSITION PROTEINS etc, and subsequent germ cell development depending on the sex (XX or XY) of the embryo [4–6,19–22].

A number of culture strategies have been reported for differentiation of mouse and/or human embryonic stem cells into germ line cells [13,21,23–26]. In this study, we employed two most commonly used strategies: i) Differentiation in floating cultures *via* embryoid body (EB) formation, and ii) differentiation in monolayer adherent cultures in absence of feeder cells. We investigated the effect of BMP4 on differentiation of buffalo ES cells into germ lineage cells in a concentration-and-time dependent manner. This study is, as per our knowledge the first of its kind in farm animals, especially bubaline species and might provide a higher mammalian model to elucidate human PGC development and germ cell formation. The study will also provide for understanding genetics, epigenetics and biochemistry of bubaline gametogenesis and might be of some use in augmenting the reproductive performance of this poor breeding species, in addition to its future applications in transgenic animal production, elite animal conservation and propagation, and conservation of endangered species.

2. Materials and methods

2.1. Chemicals

All chemicals and media used in the present study were purchased from Sigma Aldrich (St. Louis, MO, USA) and the plastic ware from Falcon (Paignton, UK), unless stated otherwise.

2.2. Establishment and culture of buffalo ES cells

Three buffalo ES cell lines (48 + XX) were developed and characterized for pluripotency and self-renewal markers, stable karyotype and differentiation capability to all the three germ layers (ectoderm, mesoderm and endoderm) as described previously [27]. The cell lines were developed from *in vitro* fertilization (IVF) derived blastocysts and propagated on buffalo fetal fibroblast feeders in ES cell culture medium composed of KoDMEM + 15% Knock out serum replacer (KoSR), supplemented with 2 mM L-glutamine, 5 ng/ml basic fibroblast growth factor (bFGF), 1000 U/ml recombinant murine lukaemia inhibitory factor (rmLIF), 1X nonessential amino acids and 50 μg/ml gentamicin sulphate, at 38 °C in a 5% CO_2 incubator. The cell lines were maintained for more than 100 passages and were used for the present study at around 20–35 passage.

2.3. Differentiation into germ lineage cells

2.3.1. Differentiation in floating cultures

ES cell colonies were subjected to differentiation in static suspension cultures in low attachment 35 mm culture dishes. The static suspension culture (SSC) strategy was earlier found to induce greater differentiation across all the three germ layers (ectoderm, mesoderm and endoderm) than the alternate Hanging drop (HD) culture strategy [27]. The differentiation medium was composed of KoDMEM supplemented with 15% Knock out serum replacer (KoSR), 2 mM L-glutamine, 1X nonessential amino acids and 50 μg/ml gentamicin sulphate. BMP4 was exogenously added to the media at 20, 50 and 100 $ngml^{-1}$ concentration. Half of the media was changed every alternate day. The EBs formed in the three media formulations were collected at day 4, 8 and 14 to identify the optimum concentration-and-time for maximum germ lineage gene induction. EBs were also developed in spontaneously differentiated cultures (no exogenous BMP4 added) and collected after 14 day culture interval for the corresponding comparative studies.

2.3.2. Differentiation in monolayer adherent cultures

ES cell colonies were cut into small clumps in 1:8 split ratios. The clumps were washed thrice with Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} ($DPBS^{-}$) and treated with Trypsin-EDTA for 2–3 min to remove any attached feeder cells. This was followed by treatment with Accutase and repetitive pipetting, with a finely drawn glass pipette, till all the clumps dissociated into single cells. The cell pellet, obtained by centrifugation at 300 g for 5 min, was seeded onto MaxGel (1:200) coated 4-well dishes for differentiation under BMP4 stimulation in a manner similar to EB differentiation.

2.4. Transcriptional profiling of germ lineage gene induction

Total RNA was extracted from 100 randomly collected EBs and monolayer adherent cultures (5×10^6 cells), from each of the three media formulations, on day 4, 8 and 14 using RNeasy RNA extraction kit (Qiagen, Germantown, MD, USA) and employed for cDNA synthesis using SuperScript III first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA,USA). qPCR analysis was performed for quantification of such genes associated with: i) PGC-specification and proliferation like *OCT4*, *NANOG*, *DAZL*, *PLZF* and *VASA*; ii) Meiosis like *MLH1*, *SYCP3*, *TNP1/2* and *PRM2*; iii) Spermatocytes like *BOULE* and *TEKT1*; and iv) Oocytes like *GDF9* and *ZP2* and 3. The various primers used and their annealing temperatures and sequences are listed in Table 1. The optimized qRT-PCR reaction mixtures contained 2 μL (100 ng) cDNA template, 10 μL SYBR Green PCR Master Mix Buffer (2X), and 10 pmol each of forward and reverse primers in a total volume of 20 μL. The reactions were performed on CFX96 instrument (BioRad Hercules, CA, USA). *GAPDH* and *β-ACTIN* were used as the endogenous controls for each