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Expression and secretory profile of buffalo fetal fibroblasts and Wharton's jelly feeder layers

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ABSTRACT

The present study examined the comparative expression and secretory profile of vital signaling molecules in buffalo fetal fibroblasts (BFF) and Wharton's jelly (BWJ) feeder layers at different passages. Both feeder layers were expanded up to 8th passage. Signaling molecules viz. bone morphogenetic protein 4 (BMP4), fibroblast growth factor 2 (FGF2), leukemia inhibitory factor (LIF) and transforming growth factor beta 1 (TGFB1) and pluripotency-associated transcriptional factors (POU5F1, SOX2, NANOG, KLF4, MYC and FOXD3) were immunolocalized in the both feeder types. A clear variation in the expression pattern of key signaling molecules with passaging was registered in both feeders compared to primary culture (0 passage). The conditioned media (CM) was collected from different passages (2, 4, 6, 8) of both the feeder layers and was quantified using enzyme-linked immunosorbent assay (ELISA). Concomitant to expression profile, protein quantification also revealed differences in the concentration of signaling molecules at different time points. Conjointly, expression and secretory profile revealed that 2nd passage of BFF and 6th passage of BWJ exhibit optimal levels of key signaling molecules thus may be selected as best passages for embryonic stem cells (ESCs) propagation. Further, the effect of mitomycin-C (MMC) treatment on the expression profile of signaling molecules in the selected passages of BFF and BWJ revealed that MMC modulates the expression profile of these molecules. In conclusion, the results indicate that feeder layers vary in expression and secretory pattern of vital signaling molecules with passaging. Based on these findings, the appropriate feeder passages may be selected for the quality propagation of buffalo ESCs.

1. Introduction

Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell masses (ICM) of preimplantation blastocyst stage embryo and maintain the potential to form the derivatives of the three primary germ layers *viz.* ectoderm, endoderm and mesoderm (Evans and Kaufman, 1981; Thomson et al., 1998). ESCs epitomize ideal tools for cloning (Stice and Keefer, 1993; Amit et al., 2000; Saito et al., 2003), gene targeting (Lohnes, 1999; Zheng et al., 1999), production of transgenic animals (Saito et al., 2003) and treating degenerative diseases (Fukuda and Takahashi, 2005; Lindvall and Kokaia, 2005). *In vitro* cultivation of ESCs requires a feeder layer support either biological or synthetic matrices that offer suitable culture milieu for these cells. Traditionally, human ESCs were cultured over mitotically inactivated mouse embryonic fibroblast feeder layer (Thomson et al., 1998). However, the drawbacks in

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using mouse embryonic fibroblasts are due to their limited proliferating ability, inter-batch variability and possible transmission of xenobiotic pathogens (Richards et al., 2003; Park et al., 2003). Therefore, focussed attempts have been made over the past few decades to employ homologous feeder cells system both in humans and animals (Richards et al., 2003; Amit et al., 2003; Verma et al., 2007; Kumar et al., 2011; Sharma et al., 2013). Till date, myriad feeder layer systems *viz.* mixed feeder cells (Kitiyanant et al., 2000; Cong et al., 2014), foreskin fibroblasts (Hovatta et al., 2003), adult marrow cells (Cheng et al., 2003), amniotic epithelial (Miyamoto et al., 2004), uterine endometrial cells (Lee et al., 2005), ear skin fibroblasts (Li et al., 2005), dermal fibroblasts (Tecirlioglu et al., 2010), oviductal cells (Sharma et al., 2013), amniotic fluid stem cells (Ma et al., 2014) *etc.* have been used in both humans and farm animals, but in spite of extensive efforts optimal culture conditions have not been established for propagation of quality ESCs. Recently, Dev et al. (2012) reported the culturing of amniotic fluid cells without feeder in water buffalo.

Amassing evidences suggest that feeder layers putatively secrete several kinds of cytokines and growth components such as FGF, BMP4, TGFB1, Activin A, LIF, stem cell factor *etc.* (Beattie et al., 2005; Li et al., 2005; Prowse et al., 2007; Buhr et al., 2007; Eiselleova et al., 2008; Sharma et al., 2012; Hongisto et al., 2012). An amalgamation of all these factors and their interactions play a positive role and constitute an intricate stem-cell niche required for the maintenance of ESCs in an undifferentiated state. It has also been reported that, there is a difference in the pattern of growth factors and signaling molecules secreted by feeder cells (Eiselleova et al., 2008). Previous reports have elucidated the presence of alternate signaling pathways that play a vital role in maintaining self-renewal or differentiation in ESCs of different species. BMP4 coordinates with LIF to retain mESC in a pluripotent state under a serum-free culture condition (Ying et al., 2003) whereas FGF2 supplementation supported the undifferentiated growth of human ESCs (Xu et al., 2005). In buffalo, a combination of LIF and FGF2 is capable of supporting the prolonged self-renewal capacity of ESCs (Sharma et al., 2013). BMP4 has been registered to induce differentiation in buffalo ESCs, whereas the role of TGFB pathway may not be essential in maintaining pluripotency (Sharma et al., 2013a). Various factors like exogenous supplementation of culture media with growth factors as well as the mitomycin-C (MMC) treatment of feeder layer have shown to modify the expression or secretory pattern of various growth factors (Eiselleova et al., 2008; Sharma et al., 2012).

A comparative analysis of pluripotency regulating crucial players produced by homologous feeder layers may aid to decipher the secretory potential of these cells. In the present study, a comparison was made between two feeder cell types based on the expression profile and secretome analysis of important growth factors/signaling molecules (BMP4, FGF2, LIF and TGFB1) at different time points. To the best of our knowledge, this is the first report demonstrating the comparative investigation of key biomolecules in these two feeder types. Our findings may provide useful information regarding temporal alteration in secretory profile with the population doubling and furthering the optimal culture conditions for propagation of buffalo ESCs.

2. Materials and methods

2.1. Materials

All the chemicals and media used in this study were procured from Sigma–Aldrich Chemical Company (St. Louis, MO, USA) unless otherwise indicated. Gravid uteri were obtained from local slaughterhouse located at Bareilly (U.P.), India. Uteri were transported on ice under aseptic conditions.

2.2. Antibodies

Immunocytochemistry was performed using goat polyclonal antibodies BMP4 (Cat No # sc-6896, lot no # F1814), FGF2 (Cat No # sc-1390, lot no # F1113), LIF (Cat No # sc-1336, lot no # C2907), TGFB1 (Cat No # sc-146-G, lot no. # K0712), POU5F1 (Cat No # sc-8628, lot no # K1308), SOX2 (Cat No # sc-54517, lot no # D2805), NANOG (Cat No # sc-30328, lot no # E1706), KLF4 (Cat No # sc-48570, lot no # D2208), FOXD3 (Cat No # sc-27888, lot no # D2909), MYC (Cat No # sc-42, lot no # H1710) and donkey anti-goat IgG-fluorescein isothiocyanate (FITC) (Cat No # sc-2024, lot no # G1812) and donkey anti-goat IgG-Texas red (TR) (Cat No # sc-2783, lot no # C0513) (Santa Cruz Biotechnology®, Inc., Texas, USA).

2.3. Experimental design

The BFF and BWJ feeders were expanded up to 8th passage. Relative mRNA expression of *BMP4*, *FGF2*, *LIF* and *TGFB1* was evaluated in 2, 4, 6, 8 passages against passage 0 as calibrator. CM was harvested from passages 2, 4, 6, 8 and protein concentration of aforesaid proteins was assessed using ELISA. Based on the expression and secretory profile the passage exhibiting the optimal levels of signaling molecules was selected as the best passage. The effect of MMC treatment on the expression pattern of aforesaid key signaling molecules was evaluated in the best- selected passages of BFF and BWJ.

2.3.1. Study 1

Fetuses were processed for isolation of BFF and BWJ as previously described by (Sharma et al., 2013; Sreekumar et al., 2014) with some modifications. Briefly, fetuses were washed thrice with the pre-warmed normal saline solution (NSS). For the preparation of BFF feeder layer, ear biopsies were taken and skin was removed aseptically. The tissues were minced to the fine pieces and washed thoroughly in sterile phosphate-buffered saline (PBS) (Ambion, Life Technologies, CA, USA). For the preparation of BWJ feeder layer, umbilical cords were snipped from the fetuses using sterile BP blade. The surface of the cord was washed thoroughly and squeezed gently to remove the blood. Jelly fragments (approximately 1–1.5 mm) were harvested and washed in PBS. Both ear and jelly explants were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (D5796, Sigma) fortified with 15% fetal bovine serum (FBS)