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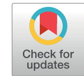
Epigenetic status of buffalo fibroblasts treated with sodium butyrate a chromatin remodeling agent



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ABSTRACT

The somatic cells having higher levels of DNA methylation and reducing it in donor cells before nuclear transfer (NT) by treating them with chemicals, may improve cloning efficiency of NT embryos by providing donor cells with similar epigenetic characteristics as in vivo embryos. Therefore, the present study was planned to understand mechanism of epigenetic changes in donor cells (buffalo fibroblasts) treated with different concentration of sodium butyrate (NaBu)-a chromatin remodeling agent. The cultured fibroblasts purity and lineage were confirmed by fibroblast specific protein and gene markers (Vimentin, Tubulin and Cytokeratin) at different passages using immuno-staining and *q*PCR respectively. The buffalo fibroblast cells were treated with 1, 3 and 5 mM of NaBu and observations were taken on their morphological changes, population doubling time and cell proliferation after 48 h of treatment. The epigenetic changes were observed using acetylation (H3K9ac) and methylation (H3K27me3) markers expression. The fibroblast cells derived from new born ear tissue started emerging and anchoring to cell culture flasks within 24 h and showed spindle-shaped morphology. The confluent culture was cryopreserved at different time interval. The post thaw culture behavior of the cryopreserved cells was also observed at different time interval and passages. The morphology of NaBu treated cells were changed with increase of dosages of NaBu treatment. The increase population doubling times and decreases the proliferation rate in the dose dependent manner. The NaBu treatment showed that the significantly increased the acetylation (H3K9ac) and methylation (H3K27me3) level over the control. Based on the observations of fibroblast characterization as well as epigenetic modifications of these cells after treatment with NaBu, results suggest that the cells may provide a useful approach for better epigenetic reprogramming in SCNT embryos.

1. Introduction

Somatic cells have been shown that higher levels of DNA methylation than either gametes or early embryos. The highly methylated somatic donor cells are used in nuclear transfer (NT) to generate cloned embryos, which in turn, have been shown to be hyper-methylated (Dean et al., 2001; Beaujean et al., 2004). Hence, the incomplete epigenetic remodeling of the somatic nucleus appears to be incompletely reprogrammed in cloned embryos during pre-implantation development (Enright et al., 2003; Campbell and Alberio, 2003). Epigenetic modifications of the somatic cells genome involve DNA methylation, histone modifications, and chromatin remodeling (Yang et al., 2007a,b; Saini et al., 2016, 2017). Previous studies also suggested that even subtle changes in histone acetylation and histone H3K9 methylation are beneficial to make donor cells more suitable for reprogramming (Santos et al., 2003; Yang et al., 2007a,b; Saini et al., 2017). Acetylated form of the histone H3-lysine 9-lysine 14 (acH3K9/14) is associated with euchromatin configuration of DNA (Rice and Allis, 2001). The likely consequence and relative success of cloning may based on the expression of histone marks present in the donor cell population before SCNT, which might provide the basis of a prognostic signature for the future evaluation and checking the risk assessment of donor cells prior to nuclear transfer, and thus increase future cloning success and mitigate the incidence of abnormal development (McLean et al., 2010).

Therefore, modifying the epigenetic status of donor cells may be considered as one of the most important facet for improving the cloning success rates in farm animals (Selokar et al., 2013; Saini et al., 2016; Saini et al., 2017). In this regard, several studies has been conducted to modify epigenetic status through histone deacetylase (HDAC) inhibitors such as the trichostatin A (Iager et al., 2008; Li et al., 2008; Wang et al., 2011; Saini et al., 2016), Scriptaid (Zhao et al., 2009; Zhao et al., 2010; Whitworth et al., 2011; Panda et al., 2012), suberoyl anilide hydroxamic acid (Ono et al., 2010), m-carboxycinnamic acid bishydroxamide (Dai et al., 2010), and Oxamflatin (Su et al., 2011) to improve the

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developmental competence of SCNT embryos.

Sodium butyrate (NaBu) is an another HDAC inhibitor molecule exert effects on cultured mammalian cells including inhibition of proliferation, induction of differentiation and induction or repression of gene expression (Kruh, 1981). The treatment of cells results in histone hyper-acetylation, and butyrate itself inhibits class I histone deacetylase activity (Candido et al., 1978), specifically HDAC1, HDAC2, HDAC3, and HDAC8. Butyrate used as essential vehicle for determining the role of histone acetylation in chromatin structure and function. Histone acetylation modifies chromatin structure through binding of transcription factors and polymerases and hence begins the transcription. The modulation of gene expression through core histone acetylation is one of the most relevant means by which cell function and DNA methylation are epigenetically regulated (Canani et al., 2011; Biancotto et al., 2010). NaBu has previously been used to treat the rabbit fibroblast which increased the levels of histone acetylation resulted into improved development of cloned embryos to blastocyst but not to term (Yang et al., 2007a,b). In another study, Kumar et al. (2007) also observed that NaBu induced histone hyper-acetylation in the porcine fetal fibroblasts and these treated cells served as more suitable donors for porcine SCNT. When bovine fetal fibroblasts was treated with NaBu, resulted in more than two fold increase in the rate of cloned blastocysts compared with that of untreated cells (Shi et al., 2003). These beneficial effects of NaBu on the developmental potential of cloned embryos reconstructed from treated donor cells merits further studies in the buffalo. Therefore, the present study was planned to understand mechanism of epigenetic changes in donor cells (buffalo fibroblasts) treated with different concentration of NaBu-a chromatin remodeling agent.

2. Materials and methods

The chemicals and culture media were obtained from the Sigma Aldrich (St. Louis, MO, USA), serum from Gibco (Grand Island, NY, USA), and disposable plastic ware from Nunc (Roskilde, Denmark), unless otherwise stated. Cell culture work was done at 38 °C with 5% CO_2 in air and maximum humidity.

2.1. Animal ethics

Animal experiments were conducted after following the guidelines laid down by Institute Animal Ethics Committee, ICAR-CIRB, Hisar, India.

2.2. Fibroblast cells culture and cryopreservation

The skin cells were derived from the ear tissue of new born female buffalo calf (NB-4862) available at the institute herd of ICAR-Central Institute for Research on Buffaloes. The confluent cultures were trypsinized by standard laboratory protocol to detach the cells from the adherent surface. The cells were cryopreserved in Dulbecco's modified Eagle's medium (DMEM) containing 10% dimethyl sulphoxide and 20% fetal bovine serum, in 1 mL cryovials through control freezing in Cryo −1 °C freezing container (Mr. Frosty, Nalgene) in deep freeze overnight. Next day the cryovials were plunged into liquid nitrogen for long time storage. For experimentation, cryopreserved cells were thawed, cultured, and maintained in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution in CO_2 incubator.

2.3. Immunofluorescence staining of somatic cells

The cultured cells were characterized by using immunofluorescence staining of vimentin and cytokeratin-18 as described earlier (Selokar et al., 2013). The marker of fibroblast specific protein, vimentin, and negative marker cytokeratin-18 (epithelial specific), while positive test marker, tubulin (present in both fibroblast and epithelial cells) were used. The expression of cytoskeleton marker proteins was detected by FITC labeled secondary antibodies under fluorescence microscope.

2.4. Real-time PCR of somatic cells

Total RNA of buffalo fibroblast cells was extracted using the RNA-aqueous kit (Ambion RNA micro kit-AM 3013). The RNA (100 ng) was then reverse transcribed by means of using first-strand cDNA synthesis kit (SuperScript III, Invitrogen) as per the manufacturer's instructions. Reverse transcription reactions were set up in a final volume of 20 μL. Before performing the reverse transcription reaction, 100 ng of RNA (in 2–4 μL vol.) was taken in nuclease free 0.2 mL micro centrifuge tube and 1 μL oligodT primer, 1 μL dNTP mix (10 mM) and 4–6 μL nuclease free water. This mixture was heated at 65 °C for 5 min to denature the RNA and then immediately quenched on ice for one min. Then to this mixture, 2 μL of 10 x RT reaction buffer, 1 μL reverse transcriptase enzyme, 2 μL 0.1 mM DTT and 1 μL RNAse, 1 μL $MgCl_2$ was added. The reaction mixture was incubated at 50 °C for 50 min, followed by heat inactivation of reverse transcriptase enzyme at 85 °C for 5 min. Then 1 μL RNAse H added and incubated at 37 °C for 20 min. The cDNA prepared was stored at − 20 °C until used. These samples were tested using SYBR green chemistry (Maxima SYBR Green Master Mix, Fermentas, USA) using qPCR (Step One Plus, Applied Biosystems, USA). The thermal cycling conditions: 50 °C for 2 min, 95 °C for 2 min, followed by 40 PCR cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 1 min. The 10 μL master mix contains 5 μL power @SYBR Green, 1 μL primer mix, 1 μL cDNA and 3 μL nuclease free water. Melting peaks were determined by melting curve analysis in order to ensure specific amplification. Three separate experiments were performed with three replicates for each gene (vimentin, tubulin, cytokeratin). The primers used in this study are indicated in Table 1.

2.5. Karyotyping of somatic cells

The chromosomal ploidy of cultured fibroblast cells were analyzed using karyotyping method as described earlier (Yadav et al., 2012).

2.6. Sodium butyrate treatments of fibroblast cells

After characterization of fibroblast cells were treated with the

Table 1
Sequences of oligonucleotide used for fibroblast cells characterization.

Gene name	Sequences (5′-3′)	Product Size (bp)	Annealing Temperature (°C)	Gene Bank Accession no.
Vimentin	CAAGTCCAAGTTTGCTGACCT (F) GTGACGAGCCATCTCTTCC (R)	264	58	NM_173969.3
Cytokeratin	CCCAGCAGATTGAGGAGAG (F) TTGACTTTGACATTCAGCAG (R)	300	58	NM_001192095.1
Tubulin	GTTGTATGGAAGCTCATTAGGGA (F) TAGTAGTCACAGTAGCTGAGGAG (R)	223	58	NM_001003900.1
β-Actin	ACCACACCTTCTACAACGAG (F) GAACATGATCTGGGTCATCTTC (R)	112	58	NM_001206502.1

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