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Exogenous miR-29b Reduces DNA Methylation and Apoptosis in Transgenic Cells

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ABSTRACT

Background: MicroRNA-29b (miR-29b) is a 22-base pair long nucleotide, that post-transcriptionally affects the processes like proliferation, differentiation and apoptosis by changes in DNA methyl transferes expressions (DNMTs family gene), which regulate DNA methylation.

Methods: This study aimed to investigate the effect of miR-29b mimic and inhibitor on buffalo fibroblast cells containing the human insulin (*hINS*) gene. Transgenic buffalo cell line containing (*hINS)* gene was treated with 40 nM miR-29b mimic and inhibitor by lipofectamin-3000.

Result: miRNA-29b mimic treated cells showed a significant decrease (P<0.05) in expression of *DNMT1, DNMT3A, DNMT3B* genes but did not find any significant change (P<0.05) in *HDAC1* expression as compared to control. Whereas, miR-29b inhibitor-treated cells revealed significantly increased (P<0.05) the expression of *DNMT1, DNMT3A, DNMT3B* and *HDAC1* genes as compared to control. These miR-29b mimic-treated cells had significantly increased (P<0.05) expression of *BCL-XL* and *MCL-1* whereas miR-29b inhibitor-treated cells showed a significantly decreased (P<0.05) expression of *MCL-1* while *BCL-XL* showed no significant (P<0.05) change in expression. These findings indicate that treatment of miR-29b mimic on transgenic cells reduces the DNA methylation which helps in the reduction of apoptosis levels in cells. miR-29b mimic reduces the methylation status and help in nuclear reprogramming of transgenic cells. Thus in future, these cells can be a better choice as a nuclear donor for efficient animal cloning.

Key words: Epigenetic reprogramming, miR-29b mimic/inhibitor, Riverine buffalo, Transgenic.

INTRODUCTION

MicroRNAs (miRNAs/miR) are a class of short, non-coding, single-stranded, 22-24 nucleotides long RNAs, that affect transcriptional and post-transcriptional regulation of gene expression (Rashmi *et al.,* 2019). Emerging evidence shows that more than hundred miRNAs are regulated by epigenetic mechanisms and about one-half of them are modulated by DNA methylation (O'Brien *et al.,* 2018). Epigenetic regulates gene activity that does not involve a change in the DNA sequence however, there are modifications to the chromatin (Humphries *et al.,* 2019). DNA methylation does not always take place alone but often occurs in the presence of other epigenetic modifications, such as histone modification, which constitutes the second major epigenetic regulatory system of miRNAs. DNA methylation at the fifth carbon of cytosine residues in cytosine phosphate guanine (CpG) and non CpG dinucleotide sites is a key mechanism for epigenetic modification in eukaryotes. During gametogenesis and early embryogenesis, global DNA methylation patterns are dynamically reprogrammed (Gruber and Zavolan, 2013). This process is established and maintained by DNA methyl transferases. *DNMT 3A/3B* enzymes are accountable for de novo methylationand *DNMT1* is responsible for the maintenance of methylation (Garzon *et al.,* 2009). Abnormal activities of these enzymes can be detrimental to the fetal development (Song *et al.,* 2017).

miRNA expression is tissue-specific and depends on the cellular context, histone modification might regulate distinct subpopulations of miRNAs in different of cell types. Expression of miRNAs is tightly regulated in a developmental-stage-dependent, as well as in an organ-dependent manner (Kloosterman and Pasterk, 2006; Takada *et al.,* 2006). These observations suggest the existence of a regulatory circuit between epigenetic modulation and miRNAs, which could have a significant effect on transcription. Mature miRNAs form part of an active RNA-induced silencing complex (RISC) containing dicer and many associated proteins. This complex acts as a regulator of numerous biological processes by either triggering the degradation of the target mRNAs or suppressing their translation through incomplete base-pairing to the 3' untranslated region (O'Brien *et al.,* 2018). Although the functioning patterns and target genes of most miRNAs are still unknown. Diverse miRNAs have been reported to

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participate in processes such as embryogenesis, embryonic development, stem cell pluripotency, differentiation, organogenesis, growth, cell proliferation and apoptosis (Gilchrist *et al.,* 2016). Recently, there is an increasing interest in miRNAs and their role in gene expression patterns. Several miRNAs presumably influence pre-implantation embryo development through DNA methylation (Sah *et al.,* 2020) and suggested that miRNA 29b participates in DNA methylation in different cells by regulating *DNMT3A/3B* expressions (Liang *et al.,* 2018). miR-29b is also involved in the regulation of DNA methylation during reprogramming and embryogenesis (Singh *et al.,* 2019). However, to the best of our knowledge, there are no reports available on the effects of miR-29b (mimics and inhibitors) on transgenic cells containing the human insulin gene. Therefore, the present study aims to investigate the effect of this miRNA on cultured buffalo fibroblast cells on the expression of some genes related to -epigenetics and -apoptosis.

MATERIALS AND METHODS

All the media and chemicals utilized in this study were procured from Sigma Chemical Co. (USA) and the plasticware was obtained from Nunc (Denmark), unless otherwise mentioned. Fetal bovine serum (FBS) was acquired from Gibco Life Technologies (USA).

Establishment of buffalo fetal fibroblast (BuFF) cell line

Female buffalo fetus obtained from slaughterhouse-based animal, was washed twice with normal saline fortified with antibiotics (gentamicin sulfate and penicillin-streptomycin). Then surface of the fetus was washed with 70% ethanol followed by several washings with antibiotic-fortified normal saline. Ear pinna tissue was washed 4-6 times with DPBS containing 50 µg/mL gentamicin sulfate. The biopsies were then cut with the help of a surgical blade into small pieces (~1 mm^3) which were then again washed 3-4 times with DPBS followed by the cell culture medium (DMEM supplemented with 2.0 mM L-glutamine,1% non-essential amino acids, 20% FBS and 50 µg/mL gentamicin sulfate). The explants were cultured into tissue culture flasks in a CO_2 incubator (5% CO_2 in air) at 37°C. The media was then replaced with a fresh medium every 3rd day until the fibroblast monolayer attained 30-40% confluence. Then cell monolayer was washed with DPBS and partially detached by trypsin-EDTA (0.25%) for 2 min to ensure fractional detachment of the fibroblasts while other cells, especially epithelial cells, remain attached to the culture flask. The sub-cultured cells were seeded in a new culture flask and anchored cells were allowed to grow till confluency. For characterization, passage- 3 cells were fixed for 1 h in 4% paraformaldehyde, permeabilized with 0.5%. Triton-X-100 for 20 min and blocked in 3% bovine serum albumin for 1 h. After blocking, cells were incubated for 1 h with primary antibody (mouse anti-cytokeratin, 1: 500 or anti-vimentin 1:500) diluted in blocking solution then with secondary antibody (goat anti-mouse IgG, 1:1000) conjugated with fluorescein isothiocyanate (FITC) for 1 h. Positive control was performed by incubating cells with mouse β-tubulin (1: 500) whereas for negative control the cells were incubated with secondary antibody only. Finally, cells were counterstained with Hoechst-33342. Fluorescence signals were detected with a fluorescence microscope (Nikon, Japan).

Transfection of buFF cells with pAcISUBC vector and their enrichment

An expression vector “pAcISUBC” was earlier constructed, had human insulin (*hINS*) gene beta-lactoglobulin (buBLG) promotor and buBLG 3’ UTR into pAcGFP-N1 (Clontech Laboratories Inc, USA) vector backbone (Kaushik *et al.,* 2014). Buffalo fetal fibroblast cells were transfected by nucleofection (AMAXA Biosystem, Germany) with pAcISUBC plasmid containing *hINS* gene. Transfected cells were cultured for the first 24 h in the culture medium containing 800 µg/mL G418 in 4 well culture plates with a change of medium every 48 h for 2-3 weeks to obtain transfected cell colonies. After 2-3 weeks of enrichment of transfected cells, transgene integration was confirmed by PCR amplification of hINS gene fragment as well as GFP expression was observed under the fluorescence microscope (Mehta *et al.,* 2019).

Exogenous miR-29b treatment to transgenic BuFF cells

Transgenic buFF cells (10^5/well) were seeded in a 6-well plate 24 h before transfection. Transfection of miRNAs was done with lipofectamine-3000 (Invitrogen, USA), according to the manufacturer’s protocol. Briefly, miR-29 b mimics, inhibitorand scramble sequence (Ambion, USA), individually diluted to 40 nM in serum-free Opti-MEM and lipofectamine 3000 (10 µL/250 µL Opti-MEM) were incubated at room temperature for 15 min. After incubation, the two solutions were mixed thoroughly, incubated for 20 min and added to the cultured transgenic BuFF cells and kept in CO_2 incubator (at 37°C, >95% RH and 5% CO_2 in air). Thereafter, fresh complete medium was added following 4 h of incubation.

Expression of epigenetic and apoptosis-related genes

The miR-29b treated transgenic cells were harvested 48 h later from all three groups (miR-29b mimic-treated, miR-29b inhibitor-treatedand untreated control) and the effect of miR-29b was studied on some epigenetic-related (*DNMT1, DNMT3A, DNMT3B, HDAC1*) and apoptosis-related (*MCL-1, BCL-XL*) genes by qPCR. For this, total RNA isolation was performed using the Single Cell RNA Purification Kit (NORGEN, Canada) following the manufacturer’s protocol and cDNA was synthesized using SuperScript III Kit (Invitrogen, USA). Briefly, the reaction mixture consisted of 100 ng RNA, 1 µL oligo dT, 1 µL 10 mM dNTP mix, 1 µL random primers and 10 µL DNase-/RNase-free water. The mixture was incubated at 65°C for 5 min, followed by a cooling step on ice for 3 min. A master mix containing 4.5 µL of 5X First Strand Buffer, 1 µL of 0.1M

DTT and 0.25 µL (50 U) of SuperScript III RT was added. The reaction was performed using the following program: 25°C for 5 min, 50°C for 60 minand 70°C for 15 min. Subsequently, the cDNA was diluted 1:4 (v:v) with nuclease-free waterand gene amplification was carried out using Maxima SYBR Green Master Mix (Fermentas, USA) along with primer sets (Table 1). The thermal cycling conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s annealing at 60°C for 30 sand extension at 72°C for 30 s. The expression levels of the target genes were normalized using internal control genes, with *GAPDH* and *B-TUBULIN* (Shyam *et al.,* 2020).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 7 software. One-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's test was used to analyze the data and Student's t-test was employed for comparing the means of different groups. The presented data represent the mean value along with the standard error of the mean.

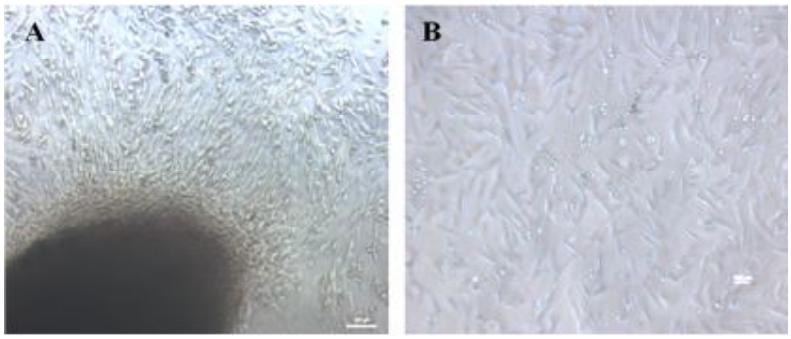
RESULTS AND DISCUSSION

Establishment and characterization of buffalo fetal fibroblast (BuFF) cells

The fibroblast cells from cultured ear tissues started to migrate after 3-5 days of culture (Fig 1A). When these cells were reached 30-50% confluency, then sub-cultured by partial trypsinization and cells were transferred to a new culture flask (Fig 1B). At passage-3, these cells showed the expression of vimentin (marker of fibroblast) and β-tubulin (positive control) while no expression was observed for cytokeratin-8 or cytokeratin-18 (epithelial cell marker), which revealed that cultured cells were fibroblast cells and had no cross contamination with epithelial cells (Fig 2).

Table 1: Details of primers.

Gene	Sequence	Product size (bp)	Annealing tempe -rature (°C)	Acc. No
hINS	F-CAAGATCTGCCATGGCCCTGTGG	275	56	NM_000207
	R-CTTCTGCCGGGATCCCTCCAGGGC			
HDAC 1	F-ATCGGTTAGGTTGCTTCAATCTG	168	60	BT030718.1
	R-GTTGTATGGAAGCTCCATTAGGGA			
DNMT1	F-CTCAGAAGGGAGATGTGGAG	138	60	NM182651.2
	R-TAGTAGTCACAGTAGCTGAGGA			
DNMT3A	F-GTGCTGTCTCTATTCGATGG	188	58	NM001206502.1
	R-CCATTCCTGGATATGCTTCTG			
DNMT3B	F-AGCGGGACATCTCTCGGTTTT	174	60	NM181813.2
	R-TCCTATTGAACTCCAGGCAGTC	128	60	ENSBTAT00000008572
BCL-XL	F-TTGTGGCCTTTTTCTCCTTC			
	R-GATCCAAGGCTCTAGGTGGT			
MCL-1	F-TCGGAAACTGGACATCAAAA	128	58	ENSBTAT00000020159
	R-CCACAAAGGCACCAAAAGAA			
β-ACTIN	GAGAAGTCCGAGTTGAGTTTGGAA	191	60	NM001034035.2
	GGCTCGTAGTGCAAATGAAGAGT			
GAPDH	F-TCAAAGAAGGTGGTGAAGCAG	123	58	NM001034034.2
	R-CCCAGCATCGAAGGTAGAAG			



The scale bar represents 100 µm.

Fig 1: Migration of buffalo fetal fibroblast cells from tissue explant on day-5. (A); fetal fibroblast cells at 5th passage (B).

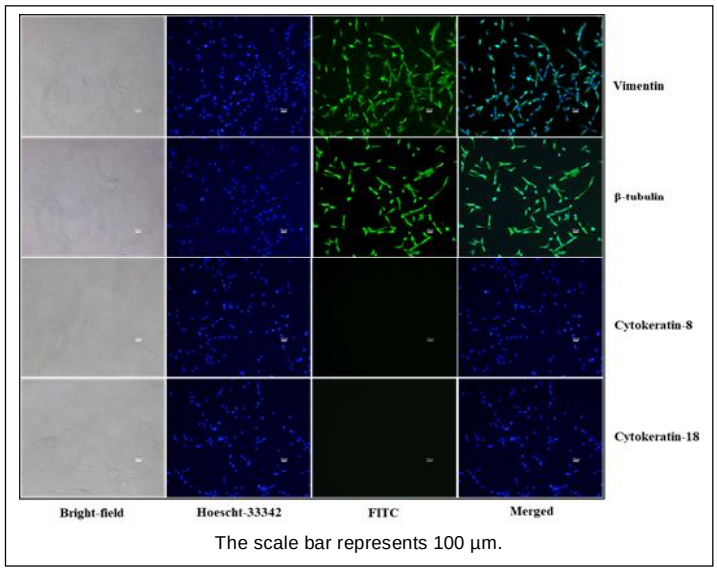


Fig 2: Immunostaining of buffalo fibroblast cells showing the expression of vimentin (fibroblast cell-specific); β-tubulin (all nucleated cells); but absence of cytokeratin-8 and -18 (epithelial cell).

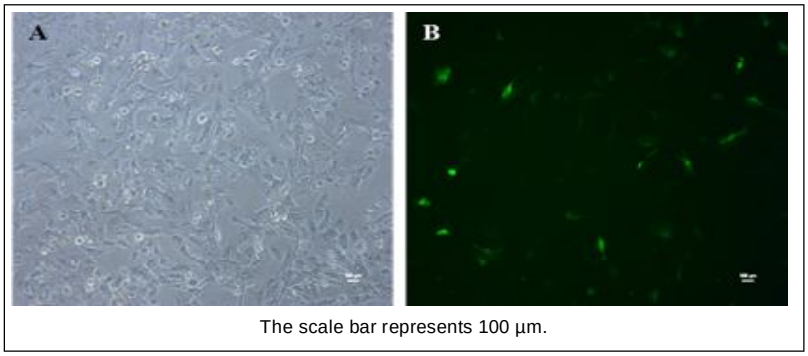


Fig 3: Transfected fibroblast cells after 48 h of nucleofection in bright light (A); and showing GFP expression in fluorescent light (B).

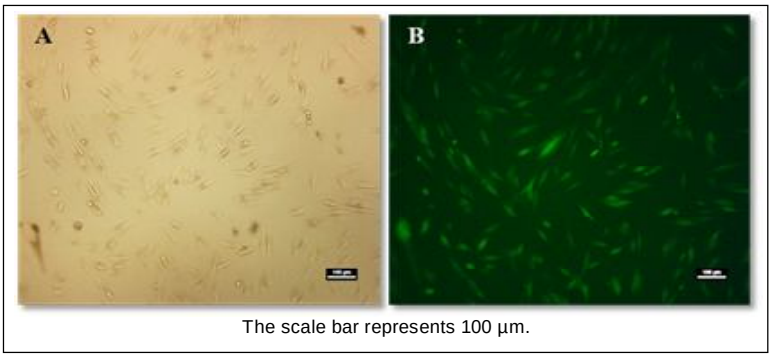


Fig 4: Enriched transgenic cells showing GFP expression at 5th passage. In bright light (A) and in fluorescent light (B).

Transfection of BuFF cells with pAcISUBC vector and their enrichment

Buffalo fetal fibroblast cells were transfected with pAcISUBC vector containing *hINS* gene and transfection efficiency was 5.15±0.49% and 13.31±0.48%, respectively observed by lipofectamine-3000 and by nucleofection using Lonza buffer (Fig 3). After the nucleofection, transfected cells were grown in selection media containing geneticin (800 µg/ml), only transfected cells survived in selection media. After 15-20 days of selection, a pure population of transgenic cells was obtained (Fig 4). Transfected cells showed normal morphology and GFP expression. RT-PCR of these cells showed a 275 bp human insulin gene fragment amplification, indicating transgene integration in cells in genome (Fig 5).

Exogenous miR-29b treatment to transgenic BuFF cells

After geneticin selection, transgenic cells were transfected with miR-29b mimics, inhibitor and scramble sequences. On culture, all three groups of cells showed normal morphology and growth patterns. Transfection of miR was confirmed by red fluorescence produced through TAMARA (5-carboxytetramethylrhodamine) dye tagged with scramble sequences (Fig 6).

Effect of miR-29b on gene expression in transgenic cells

Transgenic cells treated with miR-29b mimic, inhibitor and control were harvested after 48 h of treatment and total RNA was isolated and c-DNA was synthesized. The expression of *DNMT1, DNMT3A* and *DNMT3B* were significantly decreased (P<0.05) in miR-29b mimic treated transgenic cells, while in inhibitor-treated cells, the expression was significantly increased (P<0.05). The expression *HDAC1* gene had no significant difference (P<0.05) when treated with miR-29b mimic, whereas miR-29b inhibitor treatment showed a significant increased expression (P<0.05) as compared to control (Fig 7). The expression of apoptosis-related genes *BCL-XL* and *MCL-1* were increased significantly (P<0.05) in miR-29b mimic-treated cells, while in miR-29b inhibitor-treated cells showed significantly reduced (P<0.05) *MCL-1* expression, whereas, no significant change (P<0.05) in *BCL-XL* expression as compared to their control (Fig 8).

The present study aimed to investigate the role of miR-29b mimic and inhibitor on transgenic buffalo fetal fibroblast cells (containing human insulin gene). miR-29b has been reported to reduce DNA methylation by regulating DNMTs in several types of cells (Zhang *et al.,* 2015; Wu *et al.,* 2022). Fabbri *et al*. (2007) reported that miR-29b targets both *DNMT3A* and *DNMT3B* in lung cancer leading to a reversal of aberrant DNA methylation. It also targets *DNMT3A* and *DNMT3B* directly and *DNMT1* indirectly to induce tumour suppressor gene repression and global DNA hypomethylation in acute myeloid leukemia (Garzon *et al.,* 2009). Takada *et al.* (2009) reported their role in the regulation of genomic DNA methylation in mouse primordial germ cells by targeting *DNMT3A* and *DNMT3B*. Treatment with miR-29b mimic and inhibitor modifies the epigenetics status of cloned embryos by decreasing the DNA methylation in different farm animals *i.e*. bovine (Liang *et al.,* 2018), buffalo (Singh *et al.,* 2019) and pig IVF embryo (Zhang *et al.,* 2018). Singh *et al.* (2019) reported that significantly lower expression of *DNMT3A* and *DNMT3B,* but not that of *DNMT1* were observed in buffalo blastocysts produced through SCNT in the mimic treatment group, compared with untreated controls. Similar results have been reported in another study in bovine SCNT blastocyst (Liang *et al.,* 2018). In mouse embryos also reported that miR-29b reduces the *DNMT3A* and *DNMT3B* expression and was the direct target gene of miR-29b (Movahed *et al.,* 2019). Our results agreed with these studies and suggest that treatment of transgenic cells with miR-29b mimic an effective approach for reducing DNA hypermethylation in transgenic cells. Due to global hypomethylation, plentiful gene expression is affected and cells may proliferate and maintain viability.

Apoptosis is also an important parameter for cellular health, proliferation and quality of the transgenic cell line.

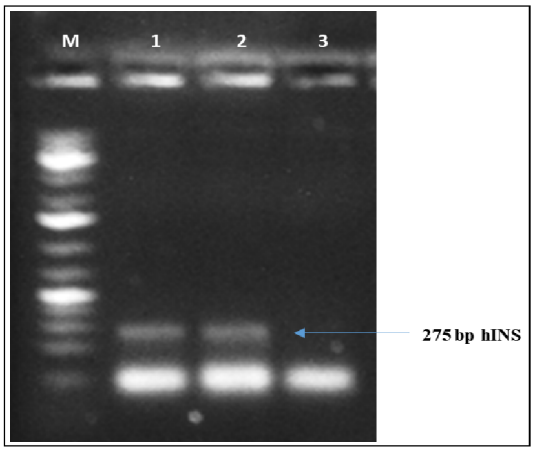


Fig 5: Amplification of human insulin gene (275 bp) in buffalo transgenic cell line (Lane-1 and -2); negative control (Lane-3) and 100 bp marker (Lane M).

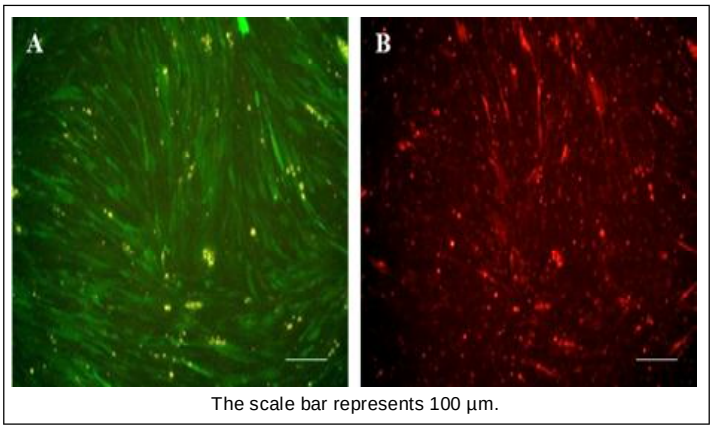


Fig 6: Transgenic cells showing GFP expression (A), expression of TAMARA dye labelled with scrambled sequence (B).

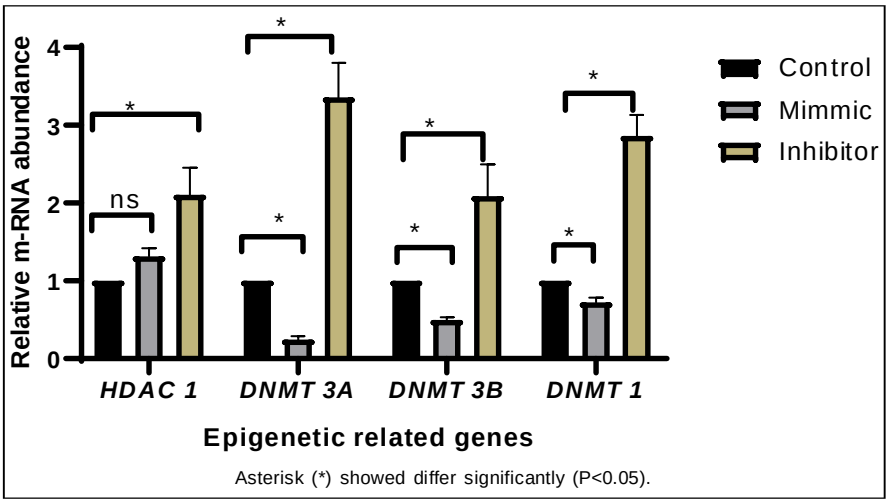


Fig 7: Relative mRNA abundance of some epigenetic-related genes in miR-29b treated transgenic buffalo fetal fibroblast cells.

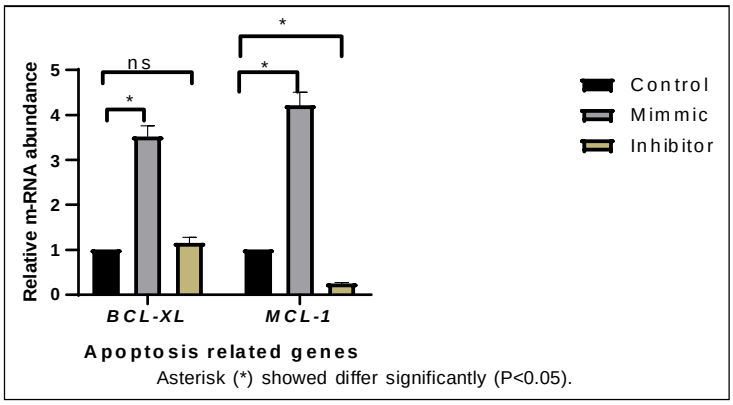


Fig 8: Relative mRNA abundance of some apoptosis-related genes in miR-29b treated transgenic buffalo fetal fibroblast cells.

Flavin *et al.* (2009) reported that miR-29b treated hepatocellular carcinoma cells significantly reduced the expression of the anti-apoptotic genes *BCL-2* and *MCL-1,* while in non-cancerous cells miR-29b plays the opposite role, inhibiting apoptosis in mammary epithelial cells (Yang *et al.,* 2016). In the present study, we found that the expression level of anti-apoptotic genes *BCL-XL* and *MCL-1* was significantly increased as compared to control when transgenic cells were treated with miR-29b. The expression level *MCL-1* was significantly increased as compared to the control while *BCL-XL* expression had no significant changes. This showed that miR-29b might inhibit apoptosis during cell proliferation and development.

CONCLUSION

The present study indicates that miR-29b mimic reduces both DNA methylation and apoptosis by altering the gene expression profile in transgenic cells. Since transgenic cells are used in animal cloning as donor cells, thus the use of miR-29b mimic may improve nuclear reprogramming and cloning efficiency.

Conflict of Interest

The authors declare no conflict of interest.

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