Original Article

The Effect of miR-106b-5p Expression in The Production of iPS-Like Cells from Mice SSCs during The Formation of Teratoma and The Three Embryonic Layers

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Abstract
Objective: According to the mounting data, microRNAs (miRNAs) may play a key role in reprogramming. miR-106b is considered as an enhancer in reprogramming efficiency. Based on induced pluripotent stem cells (iPSCs), cell treatments have a huge amount of potential. One of the main concerns about using iPSCs in therapeutic settings is the possibility of tumor formation. It is hypothesized that a procedure that can reprogram cells with less genetic manipulation reduces the possibility of tumorigenicity.

Materials and Methods: In this experimental study, miR-106b-5p transduced by pLV-miRNA vector into mice isolated spermatogonial stem cells (SSCs) to achieve iPS-like cells. Then the transduced cells were cultured in specific conditions to study the formation of three germ layers. The tumorigenicity of these iPS-like cells was investigated by transplantation into male BALB/C mice.

Results: We show that SSCs can be successfully reprogrammed into induced iPS-like cells by pLV-miRNA vector to transduce the hsa-mir-106b-5p into SSCs and generating osteogenic, neural and hepatoblast lineage cells in vitro as a result of pluripotency. Although these iPS-like cells are pluripotent, they cannot form palpable tumors *in vivo*.

Conclusion: These results demonstrate that infection of hsa-mir-106b-5p into SSCs can reprogram them into iPSCs and advanced germ cell lineages without tumorigenicity. Also, a novel approach for studying the generation of iPSCs and the application of iPS or iPS-like cells in regenerative medicine is presented.

Keywords: Induced Pluripotent Stem Cells, Mir-106b, Spermatogonial Progenitor Cell, Transplantation, Tumorigenicity

Introduction

Yamanaka and colleagues were the first ones who produced adult fibroblasts into induced pluripotent stem cells (iPSCs) in a laboratory setting in 2006 providing the basis for substantial advancements in cell reprogramming technology (1, 2). Cell reprogramming has been used in developmental and stem cell biology and regenerative medicine fields for the last decade to investigate the potential of iPSCs in generating targeted cell types (3). In general, iPSCs may be produced *in vitro* from a variety of somatic cell types. Fibroblasts of mesodermal origins, endodermal hepatocytes, and ectoderm keratinocytes have been the most common cells used for this purpose till now (4-6). Spermatogonia stem cells (SSCs) are a type of testicular stem cell that has the ability to self-renew and differentiate into sperm cells. Current knowledge on biotechnology suggests that SSCs can be more efficient and safe in cell pluripotency studies over embryonic stem cell or adult somatic cell-based technologies. SSCs isolation from an individual's testicular tissue eliminates ethical and immunological concerns in cell treatments. As reported in previous studies, generating iPSCs from adult fibroblasts is required retroviral transduction of pluripotent stem cell genes such Oct4, Sox2, klf4, c-Mys (OSKM), results in retroviral infection and teratomas. In contrast, SSCs can be reprogrammed to iPSCs in a specific culture medium without the addition of oncogenes or the use of retroviruses. However, tumorigenicity has been reported in this method (7, 8). According to new research, SSCs may be self-reprogrammed in a feeder-free reprogramming technique. Furthermore, SSCs can express the octamer-binding transcription factor4 (Oct4), a key factor in sustaining pluripotency in stem cells (9).

MicroRNAs (miRNAs) are a type of small non-coding RNA that are functional in the self-renewal, pluripotency, and differentiation of human embryonic stem cells (hESCs). MiRNAs play a crucial role in animal development by targeting mRNAs and regulating genes post-transcriptionally (10). Some miRNAs, including miR-106 increased reprogramming efficiency. miR-106b was reportedly one of the miRNAs with the highest expression of OSKM. These factors are known as the main factors that can be used for reprogramming (11). miR-106b belongs to the polycistronic miR-106b25

cluster and is found inside an intron of the *MCM7* gene; It can significantly improve iPSC efficiency and boost the reprogramming process by targeting Tgfbr2 and p21 (12, 13).

According to reports from several laboratories, Yamanaka-induced pluripotency cells have the capacity to generate teratomas (14, 15). Probably, this specification is related to extensive genetic manipulation and the use of multiple viral vectors. Tumorigenicity has led to the limitation of the application of iPSCs in medicine, so a technique that can reprogram cells with less genetic manipulation reduces the possibility of tumorigenicity. According to the capacity of SSCs in converting to pluripotent cells and features of miRNAs mentioned above, we investigated the ability of reprogrammed SSCs into iPS-like cells by pLV-miRNA vector to transduce the hsa-mir-106b-5p into SSCs and generating three germ layers (ectoderm, endoderm, and mesoderm). Furthermore, we studied the capability of these iPS-like cells in tumorigenicity by measuring the size and pathology of tumors caused by the subcutaneous injection of cells into mice.

Material and Methods

Animals

Male BALB/C mice 8-10 weeks old and weighing 18-24 g, were treated with cyclosporine in a dose of 10 mg/kg per day by gavage. Transplanted mice received cyclosporine until two weeks after transplantation. Mice were maintained under sterile conditions on a 12-hour light-dark cycle at a constant temperature. Food and drink were freely available. All experiments were approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences and following the Declaration of Helsinki (IR.SBMU.REC.1398.072).

Isolation and culture of SSCs

For the isolation of SSCs, testis of BALB/C mice suppressed by cyclosporine were collected and also isolation was performed by enzymatic digestion as described in our previous study. Spermatogonia cells were cultured for one week. According to the previous protocols explained in our earlier study, an antibody directed against promyelocytic leukemia zinc finger (PLZF) was used to identify the SSCs. Cells were identified with primary and secondary antibodies that were labeled with fluorescent reagents (16).

The pLV-miRNA vector production in bacteria

The pLV-miRNA vector, which carried the hsa-mir-106b lentivirus and comprised green fluorescent protein (GFP) in infected *E. coli* BL21, was utilized to generate iPS-like cells (mir-p081, Biosettia, San Diego, CA, USA). The *E. coli* BL21 colony was cultured in 5 ml of Lysogeny broth (LB) medium (Sigma-Aldrich, USA) for 16 hours in a 37°C shaker incubator at 180 rpm. To determine the presence of vector in the bacteria, it was cultured for 24 hours in LB agar medium containing 100 μg/ml ampicillin. Vector purification from *E. coli* BL21 colonies was deployed by GF-1 Plasmid DNA Extraction Kit (Vivantis, Malaysia) instructions.

The quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) technique was applied to ensure the accuracy of the extracted pLV-miRNA vector. The 16s-RNA as a bacterial reference gene and mir-106b primers are listed in Table 1. The sequences of forward and reverse primers were designed by using GeneRunner software. The cDNA was synthesized according to the manufacturer's instructions (Fermentas, USA). PCR products were electrophoresed on 1% agarose gel along with 1 kb DNA Ladder Marker and examined.

Transduction of the hsa-mir-106b-5p vector to SSC colonies

To vector transduction, SSCs are inserted into the cells of a cell culture well. Then, in a microtube, 500 μl of culture media and 7.5 μl of lipofectamine 3000 (Invitrogen, USA) are combined. In a separate microtube, 500 μl of media is combined with 0.5 μg of plasmid and 7.5 μl of lipofectamine 3000. The contents of the two microtubes were combined and incubated at room temperature for 10 to 15 minutes. Finally, 250 μl of the prepared sample was pipetted into each plate housing, and the cells were incubated for three days at 37°C. It is important to validate the increase in miRNA after transduction of the virus carrying the hsa-miR-106b-5p gene into the cells. A fluorescent microscope was used to confirm viral transduction since the plasmid contains a GFP tracer reagent (Olympus BX51).

Table 1: The sequences of primers used for evaluation of the extracted pLV-miRNA vector

Primer	Primer sequence (5´– 3´)
16s-RNA	F: ACTCCTACGGGAGGCAGCAG
	R: ATTACCGCGGCTGCTGG
Stem loop	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAANNNNNN
miR106b	F: ACUGCAGUGCCAGCACTT
	R: GGCAAAGTGCTTACAGTGC

Examination of iPS-like cell differentiation into all three germ layers *in vitro* phase

Differentiation into three germ layers was performed in two groups of cells: hsa-mir-106b-5p induction and without-hsa-mir-106b-5p induction as control group (treated with the empty vector). Both experimental groups were placed in a hanging drop culture, a concentration of 5×10^4 cells/ml suspension was prepared to soak in 20 µl drops to produce the embryoid body (EB). Procedure includes holding cells in a droplet of culture medium and turning the microplate upside down to produce 3D spheroids. Surface tension forces and gravity are two factors that keep cells suspended (17). At the end of the procedure in each experimental group, the differentiation of iPS-like cells was observed in 5 randomly chosen fields under a fluorescence microscope.

Differentiation into the ectodermal derivative

Cells were transferred into a 12-cell gelatinized microplate containing α-minimum essential medium (α-MEM, Sigma-Aldrich, USA) with 3% fetal bovine serum (FBS, Gibco, UK). Toward induction of neural phenotype in the EBs in two weeks, the 5×10^{-7} molar concentration of retinoic acid (Sigma-Aldrich, USA) was administered. Two weeks later, a beta-tubulin marker (Santa Cruz, USA) was used to examine the differentiation of adult neural cells (18).

Differentiation into the mesodermal derivative

Cells were grown in a six-cell plate when the entire culture surface was covered with cells, and their media changed with a bone differentiation medium to generate osteogenic lineage cells as a mesodermal lineage in transduced cells. This medium included Dulbecco's Modified Eagle Medium (DMEM, Gibco, UK) with 10% bovine serum (Gibco, UK), 10 mM beta glycerol phosphate (Sigma-Aldrich, USA), 10 nM dexamethasone (Sigma-Aldrich, USA), and 50 g/ml ascorbic 3-phosphate (Sigma-Aldrich, USA), The cells were then put on mesenchymal cells by using a sampler. For 21 days, cells were cultured in a humidified 37°C incubator with 5% CO_2. Finally, immunocytochemistry was performed to validate the differentiation of cells using the alkaline phosphatase marker (Santa Cruz, USA) (19).

Differentiation into the endodermal derivative

Cells were cultured for 28 days in DMEM medium (Gibco, UK) containing 20 µl ascorbic acid (Sigma-Aldrich, USA) 20 µl, 10 ng/ml hepatocyte growth factor (HGF, Merck, Germany), 10 ng/ml oncostatin M (OSM, Sino Biological, China), 10% FBS (Gibco, UK). Immunocytochemistry was used to establish the presence of the albumin marker (Santa

Cruz, USA) that enabled the cells to differentiate into hepatocytes (20).

Tumorigenicity of cells

Cells were transplanted into 20 immunodeficient male BALB/C mice (4-6 weeks). Transplantation was applied in four groups (n=5): SSCs (negative control), iPSCs as a positive control were obtained by using the method described by Baharvand and colleagues (21). They were provided by Stem Cells Technology Research Center. hsa-mir-106b-5p control (SSCs with empty vector), and hsa-mir-106b-5p (SSCs infected with hsa-mir-106b-5p). 5×10^6 µl of culture medium was transplanted by subcutaneous injections into the loose skin over the mice back, and assessed tumor formation after eight weeks. Generation of tumors were measured by a caliper. The tumors were then isolated and stained for pathological examination using the hematoxylin and eosin technique.

Results

Confirmation of the nature of SSCs

Cell culture experiments were performed with male BALB/c mice testis. SSCs were isolated from seminiferous tubules of the testis and cultivated in DMEM conditions. In the testis, PLZF is a spermatogonia-specific transcription factor that is detected to identify SSCs (22). Immunocytochemistry analysis demonstrated high purity and proliferation of SSCs by the expression of the PLZF marker (Fig.1A).

Expression of miR106b in transfected bacteria

The presence of the mir106b was determined by the qRT-PCR method. Results indicated a significant expression in mir106b in the transfected *E. coli* group compared to the non-transfected *E. coli* group.
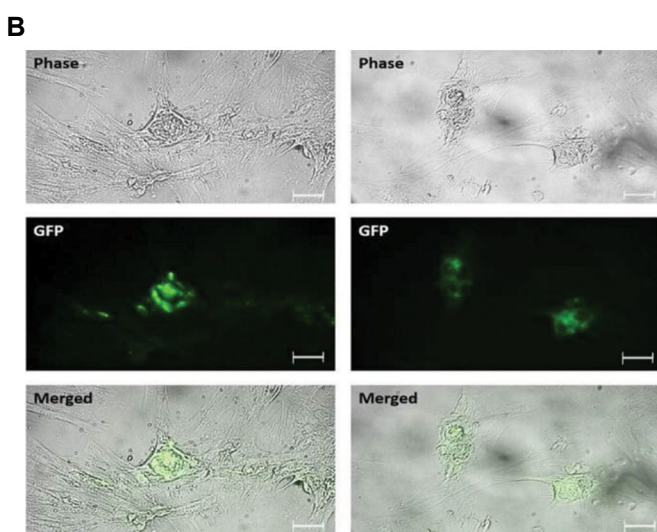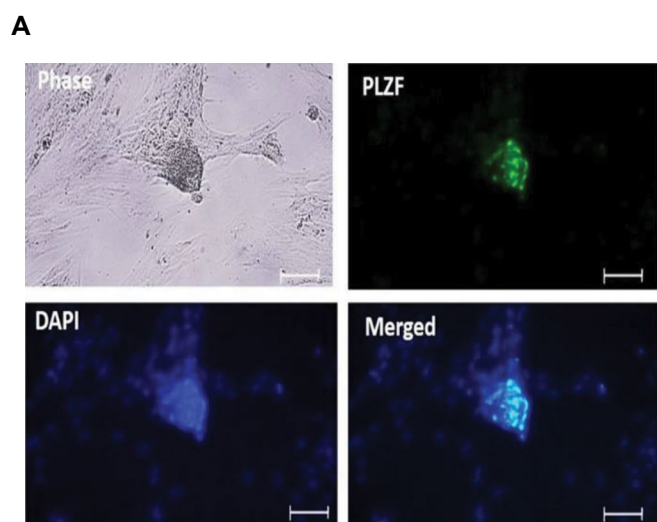
Confirmation of transduced cells

Transduction of hsa-mir-106b-5p into SSCs confirmed by tracing the GFP protein reagent with a fluorescence microscope (Fig.1B).

Differentiation of iPSC-like cells into all three germ layers

Differentiation into the ectoderm derivative

The biomarker class III beta-tubulin is known for expresses in neural lineage cells (23). Therefore, a beta-tubulin marker was used to identify neurons in cultured cells. Immunocytochemistry analyzes revealed that the hsa-mir-106b-5p induction cell group differentiated into neurons, but there was no differentiation to neural cells in the without- hsa-mir-106b-5p induction cell group (Fig.2).

A

B

Fig.1: Confirmation of SSC nature as well as transfect. **A.** Immunocytochemical characterization of promyelocytic leukemia zinc finger (PLZF) in cultured spermatogonia stem cells (SSCs). Immunocytochemistry analysis of PLZF expression in the SSC (scale bar: 50 μm). **B.** Tracing the GFP protein reagent with a fluorescence microscope to confirm transduction. The phase contrast, fluorescent, and merged photomicrographs demonstrate the transduction of hsa-mir-106b-5p into SSCs (scale bar: 40 μm).
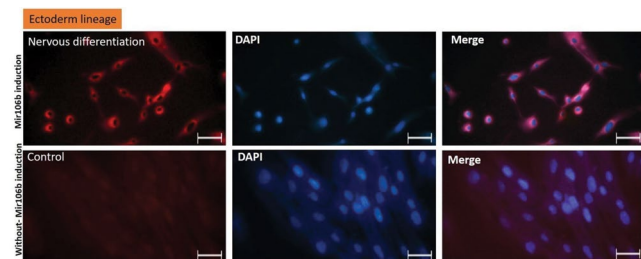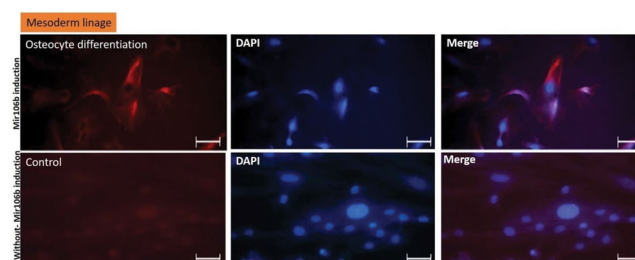

Fig.2: Fluorescent immunocytochemistry of beta-tubulin marker to detect ectodermal derivatives. hsa-mir-106b-5p induction group: differentiated nervous cells exhibited. Without-hsa-mir-106b-5p induction group: no differentiation detected (scale bar: 20 μm).

Differentiation into the mesoderm derivative

As shown by immunocytochemistry analyzes of alkaline phosphatase (ALP) in Figure 3, iPS-like cells

with induction of hsa-mir-106b-5p were differentiated to osteogenic lineage cells. ALP is a frequently used marker for observing cell lineages of the embryonic mesoderm such as osteogenic lineage (24). However, osteocyte differentiated cells were not detected in the group without hsa-mir-106b-5p induction.

Fig.3: Fluorescent immunocytochemistry of alkaline phosphatase marker to detect mesodermal derivatives. hsa-mir-106b-5p induction group: differentiated osteocyte cells exhibited. Without-hsa-mir-106b-5p induction group: no differentiation detected (scale bar: 20 μm).

Differentiation into the endoderm derivative

Adult functional hepatocytes secrete albumin into the culture medium which is used as a marker to identify endoderm lineage cells (25). Immunocytochemistry analyzes in Figure 4 showed that at the completion of the procedure, in the hsa-mir-106b-5p induction cell group, hepatocytes produced intracellular albumin but were incapable to released albumin into the extracellular environment. As a result, the cells were hepatocyte-like and developed into hepatocyte lines. However, the differentiated cells were not functional.
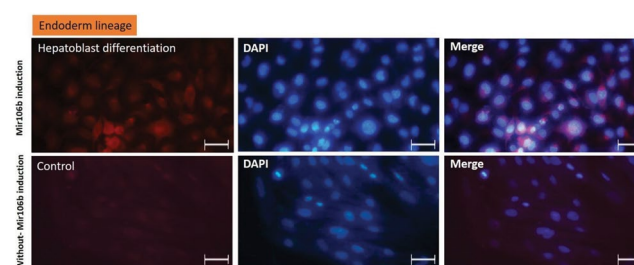

Fig.4: Fluorescent immunocytochemistry of Albumin marker to detect Endodermal derivatives. hsa-mir-106b-5p induction group: Albumin marker expressed in the hepatocyte-like cells but not detected in the secretion of the cells. Without-hsa-mir-106b-5p induction group: no differentiation detected (scale bar: 20 μm).

Tumor growth in mice

Four independent experiments were performed, each using a different colony of have transduced SSCs and none-transduced cells. In three experimental groups [the SSCs negative control)], has-mir-106b-5p control (empty vector), and hsa-mir-106b-5p (SSCs infected with hsa-mir-106b-5p) no palpable tumors were observed at the end of the experiment after two months. In contrast, the

iPSCs (positive control) formed palpable tumors at the site of injection in the mice back. Histological examination of tissue sections revealed tumorigenicity that accrued by iPSCS injection and the tumors grew 0.5×1×1 cm at week eight, when the animals were sacrificed (Fig.5).
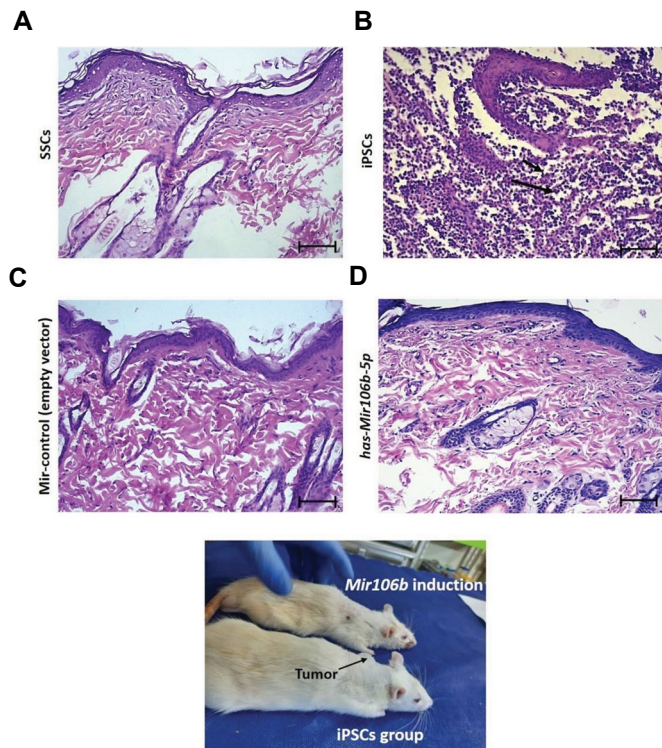
Fig.5: Histology of tumor formation in immunodeficient BALB/C mice following transplantation. **A.** In SSC group no teratomas were formed. **B.** Tumor formation (black arrows) in mice after transplantation of iPSCs **C.** In Mir-control, and **D.** Mir106b-5p groups no teratomas were formed. (Hematoxylin & Eosin staining, scale bar: 100 μm). SSC; Spermatogonial Stem Cell and iPSCs; Induced pluripotent stem cells.

Discussion

Studies have shown that induced pluripotency SSCs can differentiate *in vitro* into the three germ layers including endoderm, mesoderm, and ectoderm. SSCs can conveniently be induced into pluripotent stem cells in a specified culture medium (26, 27). Shinohara and colleagues reported in 2004 that they had generated embryonic stem-like cells (ES-like) from mouse testis SSCs that were phenotypically resembling to ES cells (28). Reprogramming competence of SSCs qualified them into the proper cells for iPSCs in rodents and human research (29). These iPS cells produce teratomas after transplantation which is the most significant obstacle of using these cells in medicine. Our findings demonstrate that induction of pluripotency in SSCs can be achieved by inducing hsa-mir106b-5p. These iPS-like cells could establish embryonic lineages. Also, these cells did not show tumorigenicity, which is their privilege compared to other reprogrammed cells.

Tumor creation in iPSCs is related to the activation of some oncogenes such as c-Myc. Nakagawa et al. (30) reported that removing c-Myc from the reprogramming process resulted in the development of pluripotent cells but eliminated teratoma formation. We have observed SSCs can be successfully reprogrammed into iPS-like cells using procedures without multiple retroviral transductions. It can be concluded that hsa-mir-106b-5p does not target c-Myc gene, and we generated pluripotent cells without tumorigenic properties. Previously, it was reported that the miR-106b-5p cluster significantly increased during reprogramming stages, inhibiting this cluster decreases reprogramming efficiency, so it is a reprogramming activity regulator (12). In addition, studies showed that certain miRNA families, such as miR-17, miR-106, miR-520, miR-372, miR-195, and miR-200, are upregulated in human pluripotent stem cells (hPSCs) as compared to adult differentiated cell types. Moreover, miRNA clusters can promote reprogramming into iPSCs. The miR-106b-25 cluster is proven to be early active in the reprogramming of mouse embryonic fibroblasts (31-34). Lin and colleagues induced mir-302 into human hair follicle cells by an inducible vector; this procedure resulted in some human embryonic specific gene expression, such as NANOG, OCT3/4, SOX2 so these somatic cells were successfully reprogrammed to iPSCs (35). Nguyen and colleagues revealed that co-expression of miR-524-5p with OSKM factors in human somatic cells leads to generate iPSCs. According to their study, miR-524-5p initiates reprogramming by targeting ZEB2 and SMAD4 genes, which are epithelial-to-mesenchymal transition-related genes (36). Based on the functional role of mir-106b in cell reprogramming, the dosage of hsa-mir-106b, a naturally present miRNA in spermatogonia stem cells was enhanced in this technique by infecting the pLV-miRNA vector into isolated SSCs to generate iPSCs (37). Here, these iPS-like cells showed pluripotent characteristics and successfully differentiated into osteogenic cells (mesoderm derivative), neuronal cells (ectoderm derivative) and hepatocyte lineage (endoderm derivative) as a result of reprogramming. Isolated SSCs from adult mice have been shown to be capable of reprograming and differentiating into all three embryonic germ layers under three defined culture conditions, as well as generating teratomas in immunodeficient mice (38). Another study by Lim and colleagues reported *in vitro* expression of three germline markers in the EB-like structures in iPSCs of human SSCs (39). These findings authenticate that induction of pluripotency in SSCs can lead them to produce embryonic layers. Although pluripotency of iPS cells is a remarkable result in cell-based therapies, the tumorigenicity of this type of cells is still a concern for clinical applications (40). Based on findings in this study, transduction of hsa-mir106b-5p to SSCs by one vector and limited genetic manipulation led to reprogramming of SSCs into pluripotent cells without the capability of tumor formation. Through this method, there can be a new prospect at the generation of iPSCs and the application of iPS or iPS-like cells in regenerative medicine. This method can be an acceptable alternative

for techniques that are involved with substantial genetic manipulation. Besides, vectors as a viral basis element are not allowed to use considerably in the human body, so we can take advantage of them in a minimum of manipulation to produce pluripotent cells without tumorigenicity.

Conclusion

The results of this study showed that using this new method in infecting hsa-mir-106b-5p into SSCs, leads to reprogramming them and turning them into pluripotent cells. iPS-like cells differentiated successfully into germ layers as a result of pluripotency. On the other hand, iPSC-like cells do not cause tumors, which is a significant characteristic of iPSCs in medical applications. This reprogramming method provides a simplified and convenient way to convert SSCs into pluripotent cells with less ethical and immunological concerns in cell treatments.

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Authors' Contributions

A.H.H.F., Z.M., S.J.H.; Proposed and performed experimental works, and data collection. F.K.; Performed bioinformatics work. F.K., A.H.H.F.; Contributed to article writing and manuscript approving. All authors read and approved the final manuscript.

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