The Effect of miR-106b-5p Expression in The Production of iPSC-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 iPSC-like cells. Then the transduced cells were cultured in specific conditions to study the formation of three germ layers. The tumorigenicity of these iPSC-like cells was investigated by transplantation into male BALB/C mice.

Results: We show that SSCs can be successfully reprogrammed into induced iPSC-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 iPSC-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 iPSC or iPSC-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 \textit{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 \textit{E. coli} BL21, was utilized to generate iPS-like cells (mir-p081, Biosettia, San Diego, CA, USA). The \textit{E. coli} BL21 colony was cultured in 5 ml of Lysogeny broth (LB) medium (Sigma-Aldrich, USA) for 16 hours at 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 \textit{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).

| Primer | Primer sequence (5’–3’) |
|--------|------------------------|
| 16s-RNA | F: ACTTCTACGGGAGGCAGCAG  
R: ATTACCGCGGCTGCTGG |
| Stem loop | GGTGGCTCTGTTGGCAGGTCACCAGGTTTACAGGCAAGCCAAAG |
| miR106b | F: ACUCAGCAGUGGCGACCTT  
R: GGCAAAAGTGCTTACAGTGC |
Examination of iPSC-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 iPSC-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% CO₂. 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).
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.

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.

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], hsa-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 iPSC-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 reprogramming 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 germine 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. iPSC-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.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.

References

1. Liu G, David BT, Trawczynski M, Fessler RG. Advances in pluripotent stem cells history, mechanisms, technologies, and applications. Stem Cell Rev Rep. 2020; 16(1): 3-32.
2. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; 126(4): 663-676.
3. Pires CF, Rosa FF, Kurochkin I, Pereira CF. Understanding and modulating immunity with cell reprogramming. Front Immunol. 2019; 10: 2809.
4. Corbett JL, Duncan SA. iPSC-derived hepatocytes as a platform for disease modeling and drug discovery. Front Med. 2019; 6: 265.
5. Lim SJ, Ho SC, Mok PL, Tan KL, Ong AH, Gan SC. Induced pluripotent stem cells from human hair follicle keratinocytes as a potential source for in vitro hair follicle cloning. PeerJ, 2016; 4: e2695.
6. Miller JD, Schlaeger TM. Generation of induced pluripotent stem cell lines from human fibroblasts via retroviral gene transfer. Methods Mol Biol. 2011; 767: 55-65.
7. Aponte PM. Spermatogonial stem cells: Current biotechnological advances in reproduction and regenerative medicine. World J Stem Cells. 2015; 7(4): 669.
8. Dym M, Kokkinaki M, He Z. Spermatogonial stem cells: mouse and human comparisons. Birth Defects Research (Part C). 2009; 87(1): 27-34.
9. Lee SW, Wu G, Choi NY, Lee HJ, Bang JS, Lee Y, et al. Self-reprogramming of spermatogonial stem cells into pluripotent stem cells without microenvironment of feeder cells. Mol Cells. 2018; 41(7): 631.
10. Wienholds E, Plasterk R H. MicroRNA function in animal development. FEBS Lett. 2005; 579 (26): 5911-5922.
11. Doeleman MJH, Feyen DM, de Vei J, Mestdagh CF, Sluijter JGP. Cardiac regeneration and microRNAs: regulators of pluripotency, reprogramming, and cardiovascular lineage commitment. Annu Rev Cell Dev Biol. 2016; 22: 79-109.
12. Li Z, Yang CS, Nakashima K, Rana TM. Small RNA-mediated regulation of iPS cell generation. EMBO J. 2011; 30(5): 823-834.
13. Fehlلهی علی دی، Garbicz F, Włodarski PK. The emerging roles of the poly-cis-tronic mir-106b-25 cluster in cancer—a comprehensive review. Biomed Pharmacother. 2018; 107: 1183-1195.
14. Gutierrez-Aranda A, Ramos-Mejia V, Bueno C, Munoz-Lopez M, Real PJ, Mácía A, et al. Human induced pluripotent stem cells develop teratoma more efficiently and faster than human embryonic stem cells regardless of the site of injection. Stem Cells. 2010; 28(9): 1568-1570.
15. Knoepfler PS. Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine. Stem Cells. 2009; 27(5): 1050-1056.
16. Hasani Fard AH, Mohseni Kouchesfahani H, Jalali H. Investigation of cholestasis-related changes in characteristics of spermatogonial stem cells in testis tissue of male Wistar rats. Andrologia. 2020; 52(9): e13660.
17. Velasco V, Shariati EA, Eslandyapour R. Microtechnology-based methods for organoid models. Microsyst Nanoeng. 2020; 6(1): 1-13.
18. Bakhshilizadeh S, Esmaeili F, Hoshmand F, Ebirahimi Hafshejani M, Ghasemi S. Neuronal differentiation of GFP expressing P19 embryonal carcinoma cells by depenylin, an anti-parkinson drug. J Shahrekord Univ Med Sci. 2014; 16.
19. Mazaheri Z, Movahedin M, Rahbarizadeh F, Amanpour S. Different doses of bone morphogenetic protein 4 promote the expression of early germ cell-specific gene in bone marrow mesenchymal stem cells. In Vitro Cell Dev Biol Anim. 2011; 47(6): 521-525.
20. Fakhr Aval S, Zarghami N, Mohammadi SA, Nouri M. Isolation of mesenchymal stem cells from human adipose tissue and comparison of miRNA-16 and miRNA-125b expression before and after differentiation into adipocytes and liver cells. Presented for the Ph.D., Tabriz. Tabriz University of Medical Sciences. 2017.
21. Ahmad N, Moghadasalal R, Ezzatizadeh V, Taghizadeh Z, Nassiri SM, Asghari-Vostokloueh ME, et al. Transplantation of mouse induced pluripotent stem cell-derived podocytes in a mouse model of membranous nephropathy attenuates proteinuria. Sci Rep. 2019; 9(1): 1-13.
22. Costoya JA, Hobbs RM, Barma M, Cattoretti G, Manova K, Sukhwani M, et al. Essential role of Ptx1 in maintenance of spermatogonial stem cells. Nat Genet. 2004; 36(6): 653-659.
23. Locher H, de Rooij KE, de Groot JC, van Doorn R, Gruis NA, Löwik WCA, et al. Mesothelial cell differentiation into osteoblast and adipocyte-like cells. J Cell Mol Med. 2011; 15(10): 2095-2105.
24. Frayre N, Krøispel F, Strahl N, Amini L, Schneid P, Bachmann S, et al. Herpetic differentiation of human induced pluripotent stem cells in a perfused three-dimensional multicompartment bioreactor. Biore. Open Access. 2016; 5(1): 235-248.
25. Pellicano R, Caviglia GP, Ribaldone DG, Altruda F, Fagoonee S. Induced pluripotent stem cells from human spermatogonial stem cells: potential applications. In: Birbrair A, editor. Cell sources for iPSCs. 1st ed. London: Academic Press; 2021; 15-35.
26. Yang M, Deng B, Geng L, Li L, Wu X. Pluripotency factor NANOG and human pluripotent stem cell behavior. Annu Rev Cell Dev Biol. 2016; 1568-1570.
27. Hasani Fard et al.
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33. Leonardo TR, Schultheisz HL, Loring JF, Laurent LC. The functions of microRNAs in pluripotency and reprogramming. Nat Cell Biol. 2012; 14(11): 1114-1121.

34. Lüningschrör P, Hausser S, Kaltschmidt B, Kaltschmidt C. MicroRNAs in pluripotency, reprogramming and cell fate induction. Biochim Biophys Acta Mol Cell Res. 2013; 1833(8): 1894-1903.

35. Lin SL, Chang DC, Lin CH, Ying SY, Leu D, Wu DT. Regulation of somatic cell reprogramming through inducible mir-302 expression. Nucleic Acids Res. 2011; 39(3): 1054-1065.

36. Nguyen PNN, Choo KB, Huang CJ, Sugii S, Cheong SK, Kamarul T. miR-524-5p of the primate-specific C19MC miRNA cluster targets TP53IPN1 and EMT-associated genes to regulate cellular reprogramming. Stem Cell Res Ther. 2017; 8(1): 1-15.

37. Tong MH, Mitchell DA, McGowan SD, Evanoff R, Griswold MD. Two miRNA clusters, Mir-17-92 (Mirc1) and Mir-106b-25 (Mirc3), are involved in the regulation of spermatogonial differentiation in mice. Biol Reprod. 2012; 86(3): 72.

38. Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, et al. Pluripotency of spermatogonial stem cells from adult mouse testis. Nature. 2006; 440(7088): 1199-1203.

39. Lim JJ, Kim HJ, Kim KS, Hong JY, Lee DR. In vitro culture-induced pluripotency of human spermatogonial stem cells. Biomed Res Int. 2013; 2013.

40. Lee AS, Tang C, Rao MS, Weissman IL, Wu JC. Tumorigenicity as a clinical hurdle for pluripotent stem cell therapies. Nat Med. 2013; 19(8): 998-1004.