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Downregulation of circBIRC6 and circCORO1C during differentiation of human cord blood-derived CD34 cells

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Research Article

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Abstract

Umbilical cord blood hematopoietic stem cells (UCB HSCs) transplantation has attracted the attention of many researchers due to the low probability of GVHD and HLA mismatching; however, the use of this resource for adults has been limited due to the inadequate number of $CD34^+$ HSC from any individual donor. Recent reports indicate that circular RNAs (circRNAs) are involved in stem cell properties. In this study, the functional role of several candidate circRNAs, including circCORO1C and circBIRC6 in $CD34^+$ HSCs was investigated. The results showed that the expression levels of circBIRC6 and circCORO1C and simultaneously, the expression of transcription factors such as NANOG and SOX2 were significantly downregulated during differentiation. Taken together, these findings suggested that circBIRC6 and circCORO1C were involved in the process of self-renewal of $CD34^+$ HSCs, and their expression is downregulated by differentiating these cells.

Introduction

Hematopoietic stem cell transplantation (HSCT) is a potentially new approach in the treatment of many diseases, including bone marrow (BM) failures and hematologic malignancies (Polak and Bishop 2006). Among all types of bone marrow transplants, umbilical cord blood hematopoietic stem cells (UCB HSCs) transplantation has attracted the attention of many researchers due to the low probability of GVHD and HLA mismatching; however, the use of this resource for adults has been limited due to the inadequate number of $CD34^+$ HSC from any individual donor (Gluckman 2009; Najem and Thu Minn 2011). An appropriate strategy for a successful transplant of UCB HSCs is their proliferation. Effective proliferation and increase in the level of proliferation of these cells require a reduced level of cell differentiation (Aggarwal et al. 2012; Zhang et al. 2019). Thus, the factors that lead to the self-proliferation of these cells must be well identified.

Considering that most HSCs are in the G0 phase of the cell cycle, or at the so-called quiescent state (Cheung and Rando 2013), entering the cell cycle in response to various stimuli will cause these cells to proliferate and differentiate (Aggarwal et al. 2011), resulting in epigenetic changes (Kosan and Godmann 2016) including the changes in the non-coding RNAs (ncRNAs) levels such as micro-RNAs (miRNAs) and circular RNAs (circRNAs), which can play different important roles in regulating the fate of HSCs (R et al. 2006; Beerman and Rossi 2014). CircRNAs are ncRNA molecules whose main characteristic is a closed-loop structure (Barrett et al. 2015). They are very stable and have a longer half-life than other ncRNAs due to their circular structure and lack of a free end (Jeck et al. 2013). In general, circRNAs are expressed in human cells and tissues in the tissue-/cell type-specific way (Xu et al. 2017). In some cases, it has been reported that their expression can be more than 10 times that of associated linear mRNA (Ivanov et al. 2015). circRNAs can play a sponge-like role for miRNAs and proteins, thereby regulating transcription and translation (Barrett et al. 2015; Du et al. 2016). Through these activities, circRNAs can play a vital role in regulating a variety of cellular processes, including proliferation and differentiation (Nicolet et al. 2018; Xia et al. 2018; Ren et al. 2020; Zhou et al. 2021). For instance, as

molecular sponges, circBIRC6 and circCORO1C suppress the function of the target miRNAs of the pluripotent genes such as NANOG, OCT4, and SOX2, thereby helping to maintain pluripotency in the ESC. The lack of expression of these two circRNAs inhibits pluripotency and stimulates ESCs differentiation (Yu et al. 2017). Considering the important role of circRNAs in various physiological processes, no basic mechanism is known regarding the exact role of circRNAs in $CD34^+$ HSCs. In this study, several candidate circRNAs, including circCORO1C and circBIRC6 associated with stem cells, were selected from circBase, and their expression levels in UCB CD34+ HSCs was investigated.

Methods

Sample preparation and isolation of mononuclear cells from human UCB

All experimental protocols were approved by the Medical Ethics Committee of Tarbiat Modares University. The umbilical cord blood was obtained from the parents who provided us with their written consent. Fresh umbilical cord blood from full-term newborns taken from the Blood Transfusion Organization of Iran was collected in sterile bags containing citrate-phosphate-dextrose (CPD) with a minimum volume of 80 ml to be used in this study. Separation of mononuclear cells (MNCs) from the three umbilical cord blood bags was performed with three independent replications. Each blood bag was added some hydroxyethyl starch (HES) separately to precipitate and reduce RBC. Is short, UCB depleted RBCs were slowly added to the medium-temperature ficoll (with a density of 1.077 g/ml; PAN Biotech, Aidenbach, Germany) in a 2:1 ratio and were separated using density-gradient centrifugation. Finally, the ring of MNCs was gently collected and washed twice with the PBS/EDTA solution.

Separation of $CD34^+$ cells from MNCs separated from UCB

Separation of CD34+ HSCs was performed using a CD34 MicroBead Kit UltraPure human (Miltenyi Biotec, Bergisch Gladbach, Germany) and through positive selection according to the manufacturer's instructions. Thus, the MNCs were suspended in MACS Buffer. Then, 50µl of FCR Blocking Reagent and 50µl of CD34 MicroBeads were added and incubated for 30 minutes at 2-8 °C. The cell suspension was then centrifuged and re-suspended in the MACS Buffer. Next, it was passed onto LS Columns (Miltenyi Biotec) Associated with the magnetic field, and ultimately, the CD34+ labeled cells were separated from the column in the last step. The purity of the separated CD34+ HSCs was assessed using the flow cytometry.

$CD34^+$ HSCs differentiation

Differentiation of the CD34+ HSCs separated from USB was performed using semi-solid culture media based on methylcellulose (Miltenyi Biotec). This medium was based on 1% methylcellulose in the IMDM medium with 30% FBS, 1% bovine serum albumin, 2 mm L-glutamine, 0.1 mm Mercaptoethanol, and differential growth factors. A total of 1×103 CD34+ HSCs were cultured in this medium from day 0 to the appointed day (days 7 and 14) according to the manufacturer's instructions. After 7 and 14 days of

culture in a humidified incubator with 5% CO_2 and 37° C, the cells were examined under an inverted microscope and then separated from the semi-solid culture medium and got single.

Flow cytometry Analysis

The flow cytometry was performed to determine the percentage of CD34+ HSCs on days 0, 7, and 14. The cells were incubated for 30 minutes with CD34 monoclonal antibody conjugated to phycoerythrin (PE; Abcam, Cambridge, UK) and CD45 monoclonal antibody conjugated to Fluorescein isothiocyanate (FITC; Abcam, Cambridge, UK) in the dark at 4° C. The labeled cells were then analyzed by the flow cytometry (Attune NxT Flow Cytometer, Applied Biosystem, Foster City, CA) and using the Flow Jo 7.6.1 software (TreeStar, Ashland, OR, USA).

RNA isolation, cDNA synthesis and quantitative RT-PCR

Using RNX plus reagent (SinaClon BioScience, Tehran, iran), total RNA was extracted from newly isolated CD34+ cells from cord blood on day 0 and days 7 and 14 after differentiation, and its concentration was measured using NanoDrop One (Thermo Fisher Scientific). The extracted RNA was reversely transcribed using the Easy cDNA Synthesis Kit (parstous, tous industrial zone, iran). Then, qRT-PCR was performed using the SYBR Green method (Ampliqon, Odense, Denmark) and the ABL step-one device (Applied Biosystems). The expression level of the newly separated CD34+ HSCs gene (day 0) was used as the control. The HPRT gene was also used as the housekeeping, and the specificity of the products was confirmed through melting curve analysis. The $2^{-\Delta\Delta CT}$ formula was used to calculate the relative expression of circBIRC6 and circCORO1C genes in comparison with housekeeping gene. In addition, quantitative analysis of GATA-1 gene (specific marker of the erythroid line) and PU.1 gene (specific marker of differentiation to myeloid and lymphoid lines) as well as quantitative analysis of NANOG and SOX2 genes were used to conduct molecular evaluation of differentiated cells.

As presented in Table 1, the target genes primers were designed and examined using AlleleID 6 and Primer-BLAST. It should be noted that to design the divergent primers for the above mentioned circRNAs, the back splice junction (BSJ) region of the circRNAs was first obtained from the circular RNA interactome site. The BSJ region sequence was entered into AlleleID and the corresponding primer was designed by this software. The primer specificity was then examined by circprimer software as well. The location of divergent primers on the circRNA is shown schematically in Figure 1.

Statistical analysis

All of the data were obtained with three independent replications. The comparison of the groups was done using one-way analysis of variance with GraphPad Prism6 soft. In all of the graphs, the range of error was plotted by calculating the mean and SD (standard deviation), and the $P\text{-value} < 0.05$ was considered significant.

Result

Flow cytometry analysis

The purity of CD34+ HSCs was assessed using flow cytometry on days 0, 7, and 14. The percentage of the CD34 cells was 87.9% immediately after isolation (Fig. 2a). The percentage of the CD34 cells cultured in the differentiation medium were 57.8% and 46.5% on days 7 and 14, respectively, (Figs. 2b, 2c), showing a statistically significant difference ($P < 0.05$).

$CD34^+$ HSCs differentiation

Figure 3 shows the CD34+ HSCs cultured under differentiation conditions after 14 days.

qRT-PCR

To investigate the role of circRNA in CD34+ HSC, a number of circRNAs, including circBIRC6 and circCORO1C associated with the stem cells, were first selected from circBase. After CD34+ HSC differentiation, qRT-PCR was performed on days 7 and 14, and the results were normalized with HSCs on day 0. The results of our study showed that the expression levels of circBIRC6 and circCORO1C were significantly downregulated on days 7 and 14 compared to the control group ($P < 0.0001$) (Figs. 4a, 4b). In addition, the qRT-PCR analysis showed that circRNA expression was often regulated during differentiation with the corresponding linear isoform, and this was the case for circBIRC6 and circCORO1C as well (Figs. 4c, 4d). Furthermore, the expression level of GATA-1 upregulated on day 7 compared with the control group ($P < 0.001$), and downregulated on day 14 compared to day 7 ($P < 0.001$) (Fig. 4g). On the other hand, the expression level of PU.1 upregulated on day 7 compared with the control group ($P < 0.001$) and downregulated on day 14 compared with day 7 ($P < 0.01$) (Fig. 4h). Besides, the expression of the two stemness transcription factors (STFs) including NANOG and SOX2 downregulated significantly during differentiation on days 7 and 14 ($P < 0.0001$) (Figs. 4e, 4f).

Discussion

In recent years, a large number of circRNAs has been identified in eukaryotes using specific circRNA bioinformatics algorithms and RNA sequencing (Kristensen et al. 2019; Wu et al. 2019). However, there is little information on the role of circRNAs in HSCs. On the other hand, HSCs are like ESCs in terms of having transcription factors such as NANOG, SOX2, and OCT4 as well as self-renewal properties. According to studies on ESCs, which represent the expression of circBIRC6 and circCORO1C and their relationship in the ESCs self-renewal process, assessing the expression of these factors in HSCs in vitro is of practical importance (Yu et al. 2017).

In this study, several candidate circRNAs, including circBIRC6 and circCORO1C, were selected from circBase (Glažar et al. 2014), which may regulate biological functions of HSCs. Having analyzed the qRT-PCR using divergent primers that specifically targeted the BSJ region, we detected the presence of circBIRC6 and circCORO1C in UCB HSCs. To identify whether these two circRNAs were functionally related to the self-renewal property, their expression patterns during differentiation were first examined. In

addition, quantitative analysis of GATA-1 and PU.1 genes as well as quantitative analysis of NANOG and SOX2 genes were carried out for molecular evaluation of differentiated cells. The expression levels of GATA-1 and PU.1 were upregulated on day 7 compared with the control group. The results confirmed the differentiation of HSCs into erythroid cells as well as myeloid and lymphoid cells. In contrast, the expression levels of GATA-1 and PU.1 downregulated on day 14 compared with day 7. Such a decrease in the expression level seemed essential for the final differentiation of the cells (Nutt et al. 2005; Suzuki et al. 2009). On the other hand, the expression levels of NANOG and SOX2 severely downregulated in differentiated cells. The expressions of NANOG and SOX2 in a cell confirmed the stemness of that cell, and the downregulation of the expression levels of these two factors confirmed the differentiation of HSCs as well. These results showed that with relevancy to STFs, circBIRC6 and circCORO1C may act considerably in the self-renewal of UCB HSCs.

STFs such as OCT4, SOX2, and NANOG are essential for maintaining pluripotency in ESC (Ng and Surani 2011). In addition to STFs, ncRNAs including circRNAs have been reported to play an important role in maintaining pluripotency. For example, in the study by Chun et al., a subset of circRNAs enriched in ESCs was identified and it was shown that circBIRC6 and circCORO1C were functionally associated with the pluripotency state (Yu et al. 2017). This is consistent with the results of the present study in which the lack of circBIRC6 and circCORO1C expressions, but not their linear counterparts, inhibited pluripotency and stimulated ESCs differentiation (Yu et al. 2017). Chun et al. also found that circBIRC6 suppressed ESC differentiation through direct interaction with miR-34a and miR-145, and concluded that circBIRC6 acted as a sponge for these miRNAs. Furthermore, circBIRC6 had binding sites for several other miRNAs, including miR-92 and miR-103, which had previously been reported to play an important role in cell differentiation (Yu et al. 2017). It was also shown that as an RBP, ESRP1 enhanced circBIRC6 synthesis by interacting with adjacent introns of pre-BIRC6, and the ESRP1 synthesis was regulated via OCT4 and NANOG in ESCs (Fig. 5) (Yu et al. 2017). In the study by Yang et al., the role of CDR1as circRNA and several circRNAs, including circBIRC6, in the proliferation and differentiation of cord blood-derived mesenchymal stem cells were examined. The results showed that when the cells differentiated, the expression of several nuclear transcription factors such as OCT4, NANOG, SOX2, Sall4 and lin28 as well as the expression of CDR1as and circBIRC6 were downregulated (Yang et al. 2019b). The results of our study on HSCs also showed downregulation of STF and circBIRC6 expression during 14 days of differentiation. In general, molecular circuits are formed between STF and circRNA. In this study, we found that the expression of circBIRC6, circCORO1C, and STF changed in one direction. However, the regulatory mechanism of circBIRC6 and circCORO1C expression changes needs further study.

In addition, the expression of circBIRC6 and circCORO1C, as stemness genes, is upregulated in some cancers and can increase the progression and metastasis of cancerous tumors through the miRNA sponge. For example, circBIRC6 has been reported to act as a sponge for miR-4491 and mir-3918, promoting NSCLC (Non-Small Cell Lung Cancer) and HCC (Hepatocellular carcinoma) progression, respectively (Yang et al. 2019a; Jin et al. 2020). It has also been reported that as a sponge, circCORO1C binds to let-7c-5p, thereby increasing PBX3 expression, enhancing EMT, and ultimately promoting LSCC

(Laryngeal Squamous Cell Carcinoma) (Wu et al. 2020). In the near future, circBIRC6 and circCORO1C may be used as biomarkers for diagnosis and treatment of diseases such as cancer.

So far, a number of studies have shown that ncRNAs, including miRNAs, play an important role in stem cell proliferation and differentiation. For instance, miR-34a and miR-145 induce ESC differentiation by suppressing STFs expression (Xu et al. 2009; Choi et al. 2011). Mir-381 has been shown to regulate the proliferation and differentiation of neuronal stem cells by regulating Hes1 expression (Shi et al. 2015). Current studies have shown that most circRNAs play their biological role through miRNA sponges (Zhao et al. 2019; Chen et al. 2020; Huang et al. 2020; Zhang et al. 2021). However, the functions of circBIRC6 and circCORO1C in UCB HSCs via miRNA sponge should be investigated.

Conclusion

To date, no similar study has been conducted on the expression levels of circBIRC6 and circCORO1C on HSCs, and this is the first research conducted in this field. In the present study, we identified circCORO1C and circBIRC6, which were highly expressed in UCB CD34+ HSCs, and found that when HSCs differentiated, the expression of transcription factors such as NANOG and SOX2 and simultaneously, the expression of circBIRC6 and circCORO1C downregulated. These results confirm that these two circRNAs may be functionally involved in the self-renewal process of CD34+ HSCs and have practical significance. Hence, in future applied studies, the effect of manipulation on these genes can be investigated in order to achieve maximum proliferation without differentiation of umbilical cord blood stem cells.

Declarations

Acknowledgments

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Ethical disclosure

This study was approved by the Ethics Committee of Tarbiat Modares University of Medical Sciences.

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Informed Consent

Written informed consents have been signed by the participants.

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Table

Table 1. Primer Sequences, annealing temperature and product length used in this study

Primers	Primer Sequences	Annealing temperature, °C	Product length, bp
GATA-1/F	CAGCCTATTCCTCTCCCAAG	60	216
GATA-1/R	CTGCCCGTTTACTGACAATC		
PU.1/F	TTACCCCTATCTCAGCAGTG	60	113
PU.1/R	GCTCCGTGAAGTTGTTCTC		
SOX2/F	CTGATTCCAGTTTGCCTCTC	60	214
SOX2/R	CCGTCTCCATCATGTTGTAC		
NANOG/F	AATGGTGTGACGCAGGGATG	60	148
NANOG/R	TGCACCAGGTCTGAGTGTTC		
BIRC6/F	CTAACAGACGGGAGACATTTAC	60	175
BIRC6/R	CAAGGTTCATCAGTAGGTTCC		
circBIRC6/F	CTCCTCTAGTTGCTCAGATATAC	60	221
circBIRC6/R	CAGTGTCTAACAACAAGATTCC		
CORO1C/F	GGTGTTGCTGCCTTTCTATG	60	126
CORO1C/R	CTGAATGTGTTGAGGTAGTGG		
circCORO1C/F	CTCAGAGAGGGATGGGTTAC	60	134
circCORO1C/R	CATAGAAAGGCAGCAACACC		
ESRP1	TCCTCCCTCCTACACATTTC	60	197
ESRP1	GGAAGTAGCCAAGACTATTAGG		
circESRP1	AACTTCTTCCAAGGTTACCAG	60	102
circESRP1	CACAAATTGCTGAGGTAGTAC		
HPRT	CCTGGCGTCGTGATTAGTG	60	125
HPRT	TCAGTCCTGTCCATAATTAGTCC		

Figures

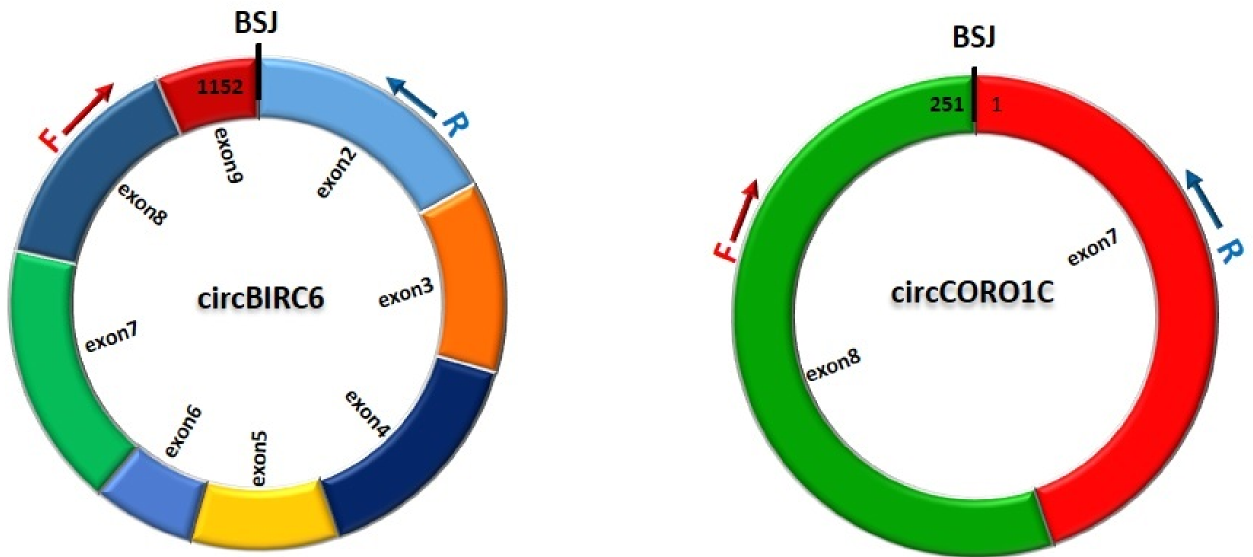

Figure 1

Location of the divergent primer on circRNA. Divergent primers are designed to amplify specific circRNAs, not their linear counterparts

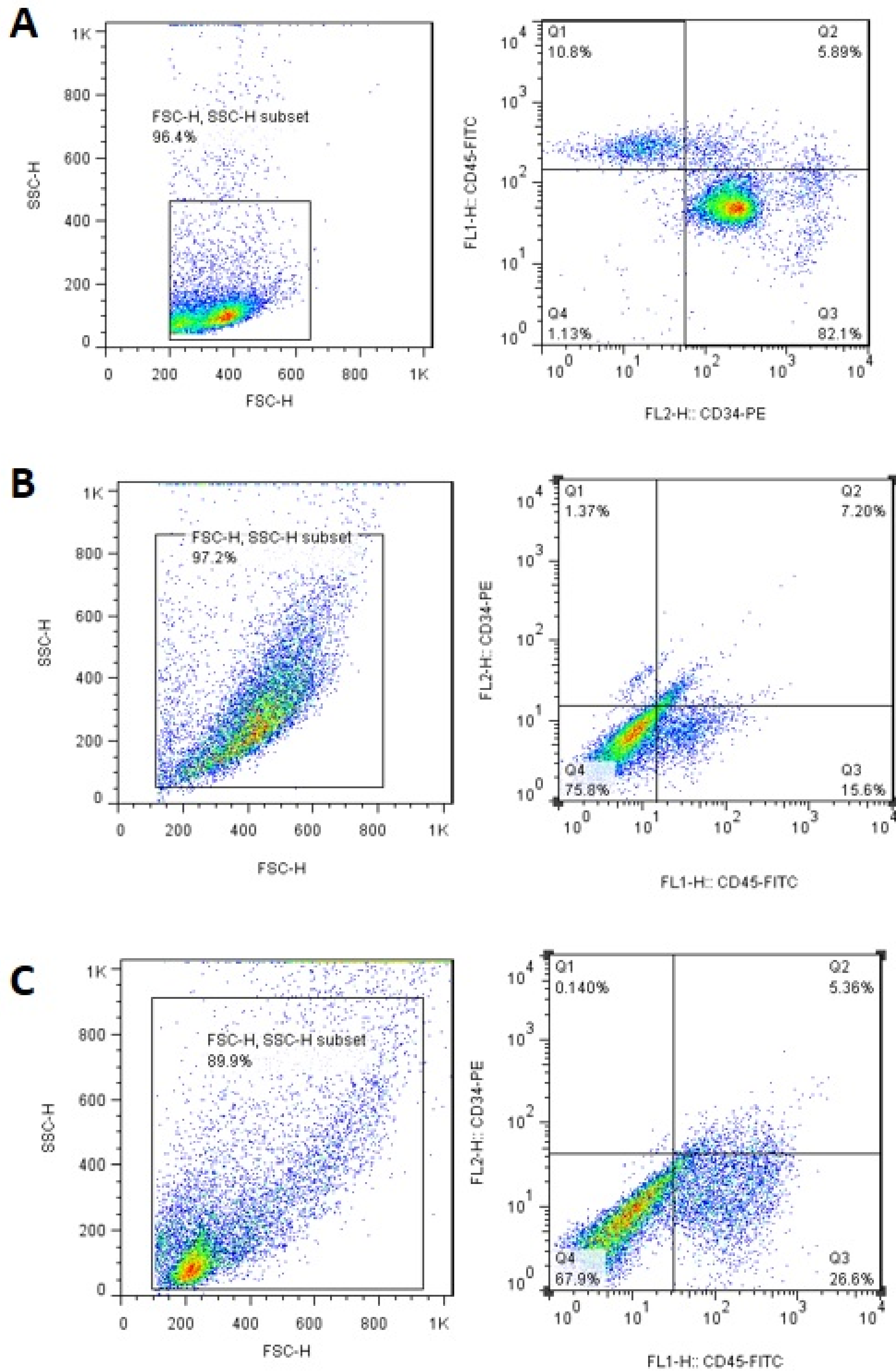

Figure 2

Purity of CD34+ HSCs using flow cytometry on days 0, 7, and 14. **a** Flow cytometry analysis of the cells isolated from MACS showed 87.9% expression of CD34 surface marker and 16.69% expression of CD45 surface marker. **b** Flow cytometry analysis of the isolated cells after culture in the differentiation medium on day 7 showed 8.57% expression of CD34 surface marker and 22.8% expression of CD45 surface

marker and on day 14 **c**, 5.46% expression of CD34 surface marker and 31.96% expression of CD45 surface marker. The reported data shows one of our replications (n = 3)

Figure 3

Morphology of hematopoietic colonies 14 days after culture in semi-solid culture medium based on methylcellulose in a humidified incubator with 5% CO_2 and 37° C (100x magnification). **a** CFU erythrocyte (CFU-E), **b** Burst-forming-unit erythrocyte (BFU-E), **c** CFU monocyte (CFU-M), **d** CFU granulocyte/ monocyte (CFU-GM), **e** CFU granulocyte/ erythrocyte /monocyte /megakaryocyte (CFU GEMM), **f** CFU granulocyte (CFU-G)

Figure 4

Mean fold changes in transcript levels of circBIRC6, circCORO1C, BIRC6, CORO1C, NANOG, SOX2, GATA-1 and PU.1 genes. Relative expression of the intended genes was performed by qRT-PCR in differentiated HSCs after culture (on days 7 and 14). The HPRT gene was used as housekeeping to normalize the data. The CD34+ HSCs gene expression level on day 0 was used as the control in qRT-PCR analysis. In all graphs, the fold change expression data was shown as mean ± SD (n = 3; $P < 0.0001$ = ****; $P < 0.001$ = ***; $P < 0.01$ = **; $P < 0.05$ = *)

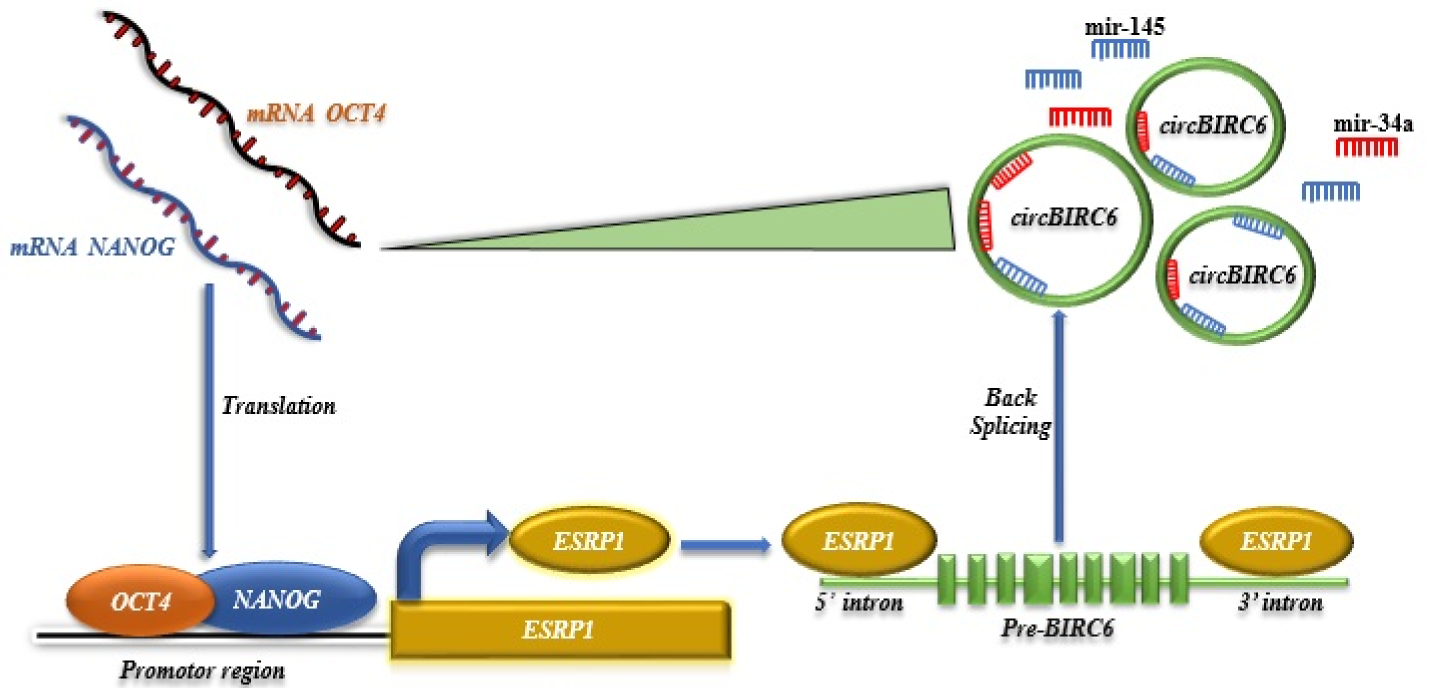

Figure 5

Regulatory network involved in controlling ESC pluripotency status. The interaction between ESRP1 and nuclear transcription factors including NANOG and OCT4 regulates the expression of circBIRC6, which acts as a sponge for mir-145 and mir-34a, to modulate the pluripotency status of ESC