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mirna-27a Targets Sprouty Homolog 2 Expression to Affect Growth of Blood Vessels into Degenerated Intervertebral Disc

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ABSTRACT This study aimed to evaluate the effects of miRNA-27a-targeting sprouty homolog 2 (SPRY2) on nucleus pulposus cell (NPC)-induced angiogenesis of human microvascular endothelial cells (HMEC-1) in degenerated intervertebral disc. Intervertebral disc tissues were collected from patients with scoliosis (control) and intervertebral disc degeneration (IDD). HMEC-1 cells were divided into control, negative control, sh-miR-27a, miR-27a, SPRY2 and miR-27a + SPRY2 groups. The invasive and angiogenic abilities of HMEC-1 cells were detected through Transwell and tube formation assays. TGF-β1 levels in NPCs and mixed medium were detected by enzyme-linked immunosorbent assay. MiR-27a expression in the intervertebral disc tissue of IDD group significantly exceeded that of the control group. In the SPRY2 group, the number of invading HMEC-1 cells decreased ($P<0.05$). Compared with the miR-27a group, the miR-27a + SPRY2 group had weakened invasive and angiogenic abilities, and decreased TGF-β1 expression ($P<0.05$). MiR-27a promotes NPC-induced angiogenesis of HMEC-1 cells through targeted inhibition of SPRY2 expression.

INTRODUCTION

Intervertebral disc degeneration (IDD) is typified by clinical symptoms such as pain in the neck, shoulder and waist, and seventy-five to eighty-five percent of the elderly suffer from lumbago and backache caused by IDD (Munir et al. 2018; Lyu et al. 2021). The clinical treatment methods for IDD mainly include symptomatic therapies (such as analgesia and radiofrequency ablation) and surgery (Buser et al. 2019). However, the underlying cause for IDD-induced changes in biological behaviours cannot be solved by the currently available methods (Meisel et al. 2019). From the perspective of treatment, the real problem is the pathological pain caused by IDD, rather than degeneration itself. Due to the unclear pathophysiological mechanisms of lumbago and backache induced by IDD, spine surgeons have been challenged in clinical diagnosis and treatment. Therefore, it is urgent to effectively relieve and even to inhibit the pain at the molecular level from the pathogenesis of IDD in the field of spinal surgery.

IDD is accompanied by changes in the composition of extracellular matrix, formation of fibrous ring fissure as well as ingrowth of blood vessels and nerves (Liu et al. 2017; Zhao et al. 2017; Waldenberg et al. 2018). There are no blood vessels and nerves in normal intervertebral disc tissues (Sun et al. 2017). Nevertheless, during the repair of intervertebral disc micro-injury, abnormal blood vessels and nerves grow into the repair region, leading to hyperalgesia (Vincent et al. 2019). Thus, vascular ingrowth into the intervertebral disc has a great impact on the progression of IDD. It is well documented that miRNAs played important roles in neurodegenerative and osteoarticular degenerative diseases. However, these studies have focused on the effects of miRNAs on cell apoptosis, proliferation, extracellular matrix degradation and inflammatory response (Kung et al. 2017), but the role of miRNAs in the angiogenesis of IDD has rarely been studied hitherto. MiR-27a is expressed in different tissues of the human body, and it is abnormally expressed in various diseases (Scheibner et al. 2012). Also, miR-27a plays an important role in tumour cell apoptosis. It promotes the cleavage of caspases, induces the apoptosis pathway regulated by caspases, and facilitates apoptosis by inhibiting Bcl-2 (Zhu et al. 2020). Recently, miR-20a, miR-17-5p, miR-34, miR-27a, miR-15a and miR-29 have been reported to regulate cell apoptosis (Bartel 2004). However, the regulatory mechanism of miRNA for IDD remains largely unknown. Given that miR-27a is a crucial part of the apoptotic pathway, the researchers hypothesised that miR-27a participated in the process of IDD.

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Objectives

The aim of this study was to provide new ideas and targets for IDD therapy in clinical practice by assessing the effects of miRNA-27a-targeting SPRY2 on human nucleus pulposus cell (NPC)-induced angiogenesis of human microvascular endothelial cells (HMEC-1) in degenerated intervertebral disc.

MATERIAL AND METHODS

Materials and Reagents

Intervertebral disc tissues were collected from 5 patients with scoliosis (control group) and 5 patients with IDD (IDD group) who underwent surgery in the hospital from May 2017 to May 2018. Scoliosis and IDD were classified as Pfirrmann grade I and IV, respectively (Pfirrmann et al. 2001). This study was approved by the ethics committee of the hospital, and the patients and their families voluntarily signed informed consents.

Human NPCs and HMEC-1 cells were purchased from Cell Bank, Shanghai Institutes for Biological Sciences (China). MiR-27a inhibition and overexpression lentiviruses, and SPRY2 inhibition lentivirus were purchased from Shanghai GenePharma Co. Ltd. (China). Dulbecco's modified Eagle medium (DMEM)/F12, DMEM and foetal bovine serum (FBS) were bought from Gibco (USA). Reverse transcription kit was offered by Sigma (USA). Lipofectamine-3000 transfection reagent and luciferase assay kit were bought from Vector (USA). Trizol kit was provided by TaKaRa (Japan). Digoxin-labelled miR-27a probe was purchased from Exiqon (USA). Fluorescence in situ hybridisation (FISH) kit was provided by Guangzhou BersinBio Co. Ltd. (China). Transwell assay kit was obtained from BD (USA). Matrigel matrix was offered by Corning (USA). Transforming growth factor-β1 (TGF-β1) antibody was obtained from Abcam (USA), and TGF-β1 enzyme-linked immunosorbent assay (ELISA) kits were bought from Shanghai MLBio Co. Ltd. (China).

Cell Culture and Transfection

NPCs and HMEC-1 cells were cultured with DMEM/F12 and DMEM containing ten percent FBS respectively, and the medium was replaced once every 2 days, followed by passage upon reaching about ninety percent confluence. NPCs in the logarithmic growth phase were transfected with lentiviruses using Lipofectamine-3000 transfection reagent according to the manufacturer's instructions. The cells were divided into control group (untransfected), negative control (NC) group (transfected with empty lentiviral vector), sh-miR-27a group (transfected with miR-27a inhibition lentivirus), miR-27a group (transfected with miR-27a overexpression lentivirus), SPRY2 group (transfected with SPRY2 overexpression lentivirus) and miR-27a + SPRY2 group (transfected with miR-27a and SPRY2 overexpression lentiviruses).

Screening of Differentially Expressed miRNAs by miRNA Microarray

Nucleus pulposus tissue was cut into pieces, added into liquid nitrogen and ground to powder. Small RNA (<200 nt) and large RNA (>200 nt) were extracted and isolated according to the instructions of E.Z.N.A.® miRNA kit. The detailed processes included separation of small RNA and large RNA, small RNA purification and large RNA purification.

Fluorescence labelling and hybridisation assay were performed for miRNAs in each group of samples using Agilent human miRNA microarray V19.0 according to the standard procedures of miRNA complete labelling and Hyb kit. The microarray results were scanned using Agilent microarray scanner, and analysed by GenePix Pro 6.0 software.

Detection of miR-27a and SPRY2 mRNA Expressions in Tissues by RT-qPCR

Total RNA was extracted by the Trizol method. Primer sequences: miR-27a (FP: 5'-GCAGGGCTTAGCTGCTTG-3', RP: 5'-GTGCAGGGTCCGAGGT-3'); SPRY2 (FP: 5'-GAGGCCAGAGCTCAGAGTGGCAACGGGTCG-3', RP: 5'-TGTCGGCTTTTCAAAGTTCCTAGGGGGGAC-3'); β-actin (FP: 5'-GCCCATCTATGAGGGTTACGC-3', RP: 5'-GCTTTAGCCACGCTCGGTC-3'). PCR conditions were pre-denaturation at 75°C for 2 minutes, followed by 40 cycles of denaturation at 90°C for 5 minutes, annealing at 60°C for 60 seconds and extension at 72°C for 30 seconds. Relative expression level was represented as $2^{-\Delta\Delta CT}$. Each sample was tested three times independently.

Detection of miR-27a Expression by FISH Assay

Digoxin-labelled miR-27a probe at the concentration of 1: 100 was used. Intervertebral disc tissue sections (5 μm-thick) were prepared routinely, and hybridised with miR-27a probe in accordance with the instructions of FISH kit. Five discontinuous visual fields were randomly selected under a microscope (400×). Then the mean optical density (MOD) of positive area was semi-quantitatively analysed using IPP6.0 software.

Verification of Relationship between miR-27a and SPRY2 by Bioinformatics Prediction and Dual Luciferase Reporter Assay

The binding fragments between miR-27a and SPRY2 were predicted using miRNA target gene database MiRanda. The SPRY2 fragments containing miR-27a binding sites were amplified by PCR, and then inserted into luciferase vector psi-CHECK to construct wild-type plasmid (psi-CHECK-SPRY2-wild). The nucleotide in binding fragments was mutated by gene mutation to construct mutant plasmid (psi-CHECK-SPRY2-mutant). Afterwards, the cells were co-transfected with miR-27a or NC and empty plasmids, psi-CHECK-SPRY2-wild and psi-CHECK-SPRY2-mutant respectively, and divided into control group, wild group and mutant group. Twenty-four hours after transfection, the luciferase activity (firefly luciferase activity/Renilla luciferase activity) was measured according to the kit's instructions.

Detection of Cell Invasion by Transwell Assay

Briefly, 5×10^4 NPCs were inoculated into a 24-well plate, and 1×10^4 HMEC-1 cells were inoculated into the upper transwell chamber, followed by culture until adherence. On the day of experiment, the conditioned medium of NPCs and complete DMEM were mixed (1:1) and added into the lower transwell chamber with HMEC-1 cells, followed by co-culture for 12 hours. The cells in the chamber were fixed with four percent paraformaldehyde for 15 minutes, rinsed with phosphate-buffered saline (PBS), stained with 0.1 percent crystal violet solution for 10 minutes and rinsed with PBS again. Then the images were acquired under an inverted fluorescence microscope and analysed using ImageJ software.

Tube Formation Assay of Vascular Endothelial Cells

Matrigel was thawed at 4°C overnight 1 day before the experiment, and diluted 1:1 with serum-free DMEM. Then the diluted Matrigel was added into a 24-well plate (300 μL/well), and solidified in an incubator with five percent CO_2 at 37°C for later use. After serum starvation overnight, HMEC-1 cells were digested and re-suspended with a mixture of conditioned medium of NPCs and complete DMEM (1:1). Then the HMEC-1 cell suspension was added into Matrigel-coated 24-well plate. Four hours later, the tube formation was observed under the microscope, and the images were analysed using ImageJ software.

Detection of TGF-β1 Level in Co-culture Medium by ELISA

The mixed medium of HMEC-1 cells was utilised as the sample. The level of TGF-β1 was measured using ELISA kit in accordance with the instructions. After reaction, the optical density was measured at 450 nm using a microplate reader.

Statistical Analysis

All data were statistically analysed by SPSS 19.0 software and plotted with GraphPad Prism 5.01 software. The comparisons between two groups were conducted by the t test. $P<0.05$ was considered statistically significant.

RESULTS

miRNA Microarray Results

Compared with the control group, the expressions of miR-27a, miR-551b, miR-1288 and miR-450b were up regulated remarkably (5.341, 3.215, 2.732 and 2.324), while those of miR-155, miR-510 and miR-492 were down-regulated notably (0.323, 0.387 and 0.416) in the IDD group (Table 1).

Expression Levels of miR-27a in Intervertebral Disc Tissues

The results of RT-qPCR indicated that the expression level of miR-27a in the IDD group was 5.5 times that of the control group, which was consistent with the microarray results (Fig. 1).

Table 1: Differentially expressed miRNAs in intervertebral disc tissues

	miRNA	*Fold change (IDD group/ control group)*	*P value*
Up-regulated	miR-27a	5.341	<0.001
	miR-551b	3.215	<0.001
	miR-1288	2.732	<0.001
	miR-450b	2.324	<0.001
Down-regulated	miR-155	0.323	<0.001
	miR-510	0.387	<0.001
	miR-492	0.416	<0.001

IDD: Intervertebral disc degeneration.

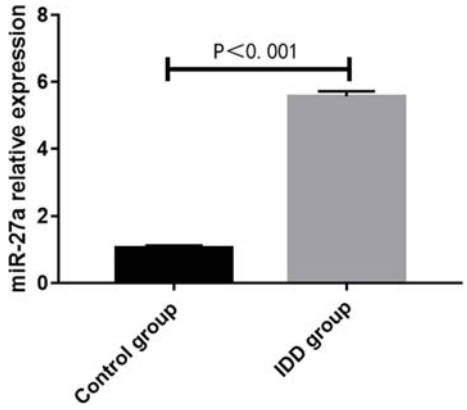

Fig. 1. Expression levels of miR-27a in intervertebral disc tissues

Targeted Regulatory Effects of miR-27a on SPRY2 Expression in NPCs

As shown in Figure 2A, the overexpression of miR-27a can significantly inhibit the expression of SPRY2 (P<0.05). After miR-27a was knocked down, however, the expression level of SPRY2 was elevated significantly (P<0.05). The TargetScan website predicted that there were complementary binding sequences between miR-27a and SPRY2 (Fig.2B). The luciferase reporter gene assay exhibited that miR-27a overexpression was able to significantly suppress the luciferase activity in the psi-CHECK-SPRY2-wild group (P<0.05), but it had no influence on the luciferase activity of the psi-CHECK-SPRY2-mutant group (P>0.05) (Fig. 2C), indicating that miR-27a regulated and inhibited SPRY2 expression in a targeted manner.

NPCs Affected the Invasion Ability of HMEC-1 Cells through miR-27a/SPRY2

In comparison with the NC group, the number of invading cells was raised significantly in the miR-27a group (P<0.05), but reduced in the SPRY2 group (P<0.05) (Fig. 3). Besides, the miR-27a + SPRY2 group had a significantly smaller number of invading cells than that of the miR-27a group (P<0.05). Collectively, overexpressing miR-27a prominently promoted the invasion of HMEC-1 cells, which was reversed through the overexpression of SPRY2.

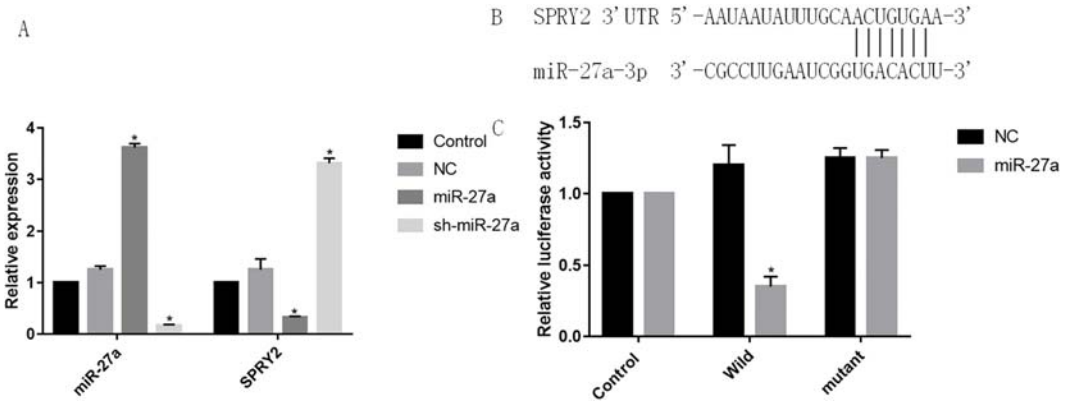

Fig. 2. Targeted regulatory effects of miR-27a on SPRY2 expression in NPCs. A: miR-27a and SPRY2 mRNA expressions detected by qRT-PCR. Compared with NC group, *P<0.05; B: TargetScan website prediction results; C: luciferase reporter gene assay results. Compared with NC group, *P<0.05. NC: negative control; NPC: nucleus pulposus cell; SPRY2: sprouty homolog 2

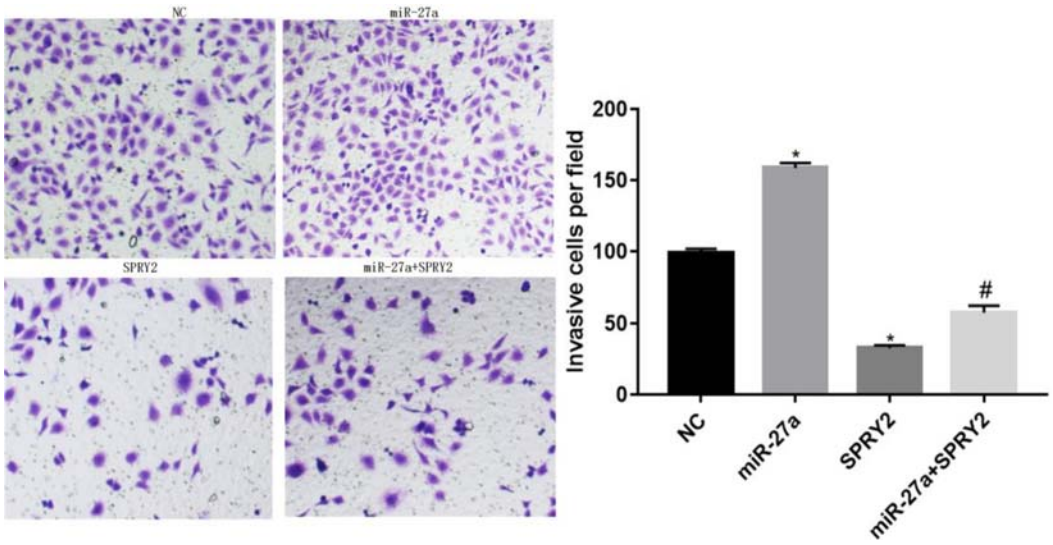

Fig. 3. Effects of miR-27a overexpression on HMEC-1 cell invasion. Compared with NC group, *P<0.05; compared with miR-27a group, #P<0.05. HMEC-1: human microvascular endothelial cell; NC: negative control

NPCs Affected the Angiogenic Ability of HMEC-1 Cells through miR-27a/SPRY2

HMEC-1 cells were cultured with the conditioned medium of NPCs undergoing different treatments. There was apparent tube-like structure in the miR-27a group compared with that in the NC group, with a significant difference (P<0.05), whereas no tube-like structure was observed in the SPRY2 group (Fig. 4). Moreover, the miR-27a + SPRY2 group had significantly decreased tube-like structure in comparison with the miR-27a group (P<0.05).

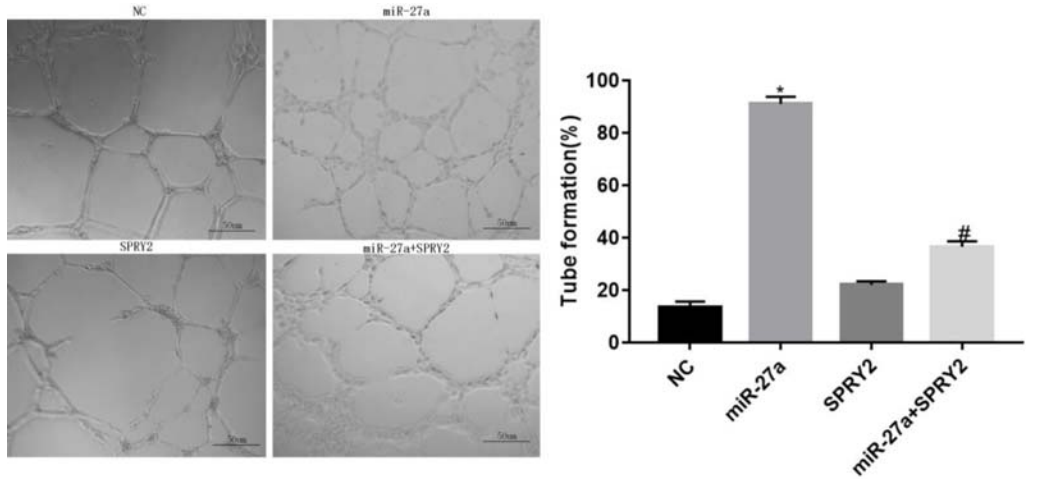

Fig. 4. Effects of miR-27a overexpression on HMEC-1 cell tube formation. Compared with NC group, *P<0.05; compared with miR-27a group, #P<0.05. HMEC-1: human microvascular endothelial cell; NC: negative control

Taken together, overexpressing miR-27a distinctly enhanced the angiogenic ability of HMEC-1 cells, which was apparently suppressed through the overexpression of SPRY2.

TGF-β1 Expressions in NPCs and Mixed Culture Medium

TGF-β1 was located in the cytoplasm of NPCs, and its expressions in both NPCs and culture medium were significantly higher in the miR-27a group than those in the NC group ($P<0.05$) (Fig. 5). After SPRY2 overexpression, the expressions of TGF-β1 in NPCs and culture medium were lowered significantly ($P<0.05$). Furthermore, the expressions of TGF-β1 in NPCs and culture medium were significantly lower in the miR-27a + SPRY2 group than those in the miR-27a group ($P<0.05$), demonstrating that miR-27a facilitated the expression and secretion of TGF-β1 in NPCs, which can be reversed by further overexpressing SPRY2.

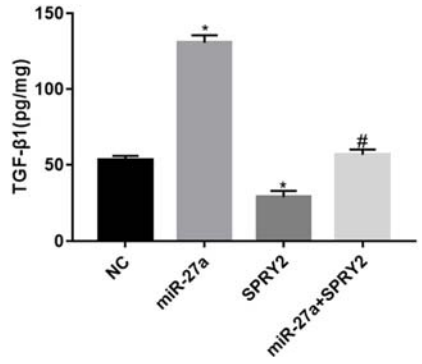

Fig. 5. TGF-β1 expressions in NPCs and mixed culture medium. Compared with NC group, *P<0.05; compared with miR-27a group, #P<0.05. NC: negative control; NPC: nucleus pulposus cell; TGF-β1: transforming growth factor-β1

DISCUSSION

The intervertebral disc is the largest organ without blood vessels and tends to be vascularised with the progression of IDD (Middendorp et al. 2017). Degenerated NPCs can induce the migration of vascular endothelial cells to trigger angiogenesis, but the molecular mechanism has not been clarified. A recent study revealed that miRNAs played various roles in IDD progression (Zhao et al. 2017). Hence, the role of miRNAs in inducing the neovascularisation of NPCs was mainly investigated in this study.

Several microarray studies have verified that the expression of miRNAs in degenerated NPCs is remarkably different from that in normal NPCs, and such differentially expressed miRNAs may control corresponding upstream and downstream pathways to participate in the degenerative process of NPCs, including the regulation of NPC apoptosis and proliferation (Meng et al. 2018). Moreover, they can regulate the disequilibrium of extracellular matrix homeostasis and the secretion of inflammatory factors through the matrix metalloproteinase family. According to a previous literature (Cao and Chen 2017), miR-27a exerted vital effects on IDD progression through inhibiting NPC apoptosis and promoting release of inflammatory factors by NPCs. Therefore, the differentially expressed miRNAs in intervertebral disc tissues in control and IDD groups were screened herein, and miR-27a was found to be significantly up-regulated in the IDD group. Furthermore, the expression level of miR-27a was elevated significantly in the IDD group ($P<0.05$), implying the importance of miR-27a to IDD.

SPRY2 is one of the crucial members of the Sprouty protein family. Its expression is down regulated in various tumour cells and closely associated with the abnormal proliferation of tumour cells (Jiang et al. 2017). In addition, SPRY2 is an important player in suppressing cell transformation and neovascularisation (Tremblay and Sirard 2017). Given that miRNAs exert their regulatory effects on a variety of diseases by inhibiting the expressions of target genes, miR-27a was overexpressed in NPCs in this study. As a result, SPRY2 expression was inhibited significantly ($P<0.05$). On the contrary, the expression of SPRY2 was raised after the knockdown of miR-27a ($P<0.05$). Moreover, SPRY2 was confirmed to be a target gene of miR-27a, verifying that miR-27a suppressed the mRNA translation of SPRY2 to induce the differential expression of SPRY2 in cells, thus regulating the activation of downstream pathways. To investigate whether miR-27a can function in the neovascularisation in degenerated intervertebral disc by inhibiting SPRY2 expression, HMEC-1 cells were cultured with the conditioned medium and complete medium of differently treated NPCs in this study. Overexpressing miR-27a in NPCs markedly induced the invasive and angiogenic abilities of HMEC-1

cells. However, on the basis of miR-27a overexpression, the overexpression of SPRY2 reversed the promoting effects of miR-27a in NPCs on the invasive and angiogenic abilities of HMEC-1 cells. Hence, NPCs can enhance the invasive and angiogenic abilities of HMEC-1 cells through miR-27a/SPRY2.

The formation of new capillaries may be a response of autologous damage repair after IDD. In the repair process, the vascular structure of new granulation tissues usually grows into the degenerated intervertebral disc along the fibrous ring fissures, accompanied by the release of massive growth factors and cytokines. For example, TGF-β1, bFGF and CTGF can stimulate further denaturation and differentiation of intervertebral disc cells (Sun et al. 2017). The members of the TGF-β family can facilitate neovascularisation, among which TGF-β1 is a multi-functional regulatory factor for differentiation and growth and has vital functions in inflammatory reaction and tissue repair (Chen et al. 2019). In this study, the expression level of TGF-β1 in the medium of NPCs and HMEC-1 cells was determined by ELISA. The expression of TGF-â1 rose remarkably in the miR-27a group compared with that in the NC group ($P<0.05$), while it declined evidently in the miR-27a + SPRY2 group in comparison with that in the miR-27a group ($P<0.05$), suggesting that NPCs can regulate the expression of TGF-β1 via the miR-27a/SPRY2 pathway to induce the invasive and angiogenic abilities of HMEC-1 cells.

CONCLUSION

NPCs can inhibit the expression of SPRY2, stimulate the production of TGF-β1 and enhance the invasive and angiogenic abilities of HMEC-1 cells through miR-27a, which lays a foundation for *in vivo* experiments and provides new targets for future research on IDD.

RECOMMENDATIONS

IDD can be treated by using MiR-27a, SPRY2 and TGF-β1 as potentially applicable targets.

ABBREVIATIONS

DMEM: Dulbecco's modified Eagle medium
ELISA: enzyme-linked immunosorbent assay
FBS: foetal bovine serum
HMEC-1: human microvascular endothelial cell
IDD: intervertebral disc degeneration
NC: negative control
NPC: nucleus pulposus cell
SPRY2: sprouty homolog 2
TGF-β1: transforming growth factor-β1

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