Int J Clin Exp Pathol 2017;10(3):3251-3257
www.ijcep.com /ISSN:1936-2625/IJCEP0047558

Original Article

Notch pathway and matrix metalloproteinases in the repair of cartilage injury with bone marrow mesenchymal stem cells

Wenqi Gu, Xin Ma, Ziquan An, Yuan Sun, Xiangguo Cheng, Youshui Gao, Bin Hu, Yebin Qian, Yanjie Mao, Gen Wen, Wanjun Liu

The Sixth People's Hospital, Shanghai Jiaotong University, Shanghai, China

Received December 28, 2016; Accepted January 25, 2017; Epub March 1, 2017; Published March 15, 2017

Abstract: Objective: The expressions of Notch, MMP-1 and MMP-13 were detected during the repair of cartilage injury with bone marrow mesenchymal stem cells (BMMSCs), aiming to explore the interaction among Notch pathway, MMP-1 and MMP-13 in the cartilage matrix metabolism. Methods: Pluronic F-127 and allogeneic BMMSCs were used to construct the tissue engineering complexes which were then implanted into the knees of rabbits with knee osteochondral defect. Results: Low expressions of Notch 1, Jagged 1, MMP-1 and MMP-13 were observed in normal cartilages. The Jagged 1 and Notch 1 expressions increased significantly at 1 month after implantation as compared to normal control group ($P<0.05$) and thereafter returned to normal level at 2 months. At 3 months, their expressions increased to a certain extent again, and dramatically increased at 6 months ($P<0.05$). MMP-1 and MMP-13 expressions began to increase at 3 months after implantation, and markedly increased at 6 months ($P<0.05$). Conclusion: Notch signal is responsible for the active cell proliferation during the early repair of cartilage injury with BMMSCs and may induce the differentiation of BMMSCs into chondrocytes in which the expression of molecules in Notch pathway reduces, leading to the channel closure. In the late repair phase, MMP-1 and MMP-13 expression increase significantly, accompanied by the significant elevation of Jagged 1 and Notch 1 expression. This implies that Notch pathway may regulate the expression of MMP during the repair of cartilage injury with BMMSCs, affecting the biomechanical properties of repaired cartilages.

Keywords: Bone marrow mesenchymal stem cells, notch pathway, matrix metalloproteinases, cartilage injury

Introduction

The repair of cartilage injury with bone marrow mesenchymal stem cells (BMMSCs) has achieved favorable efficacy in clinical practice, but the reconstructed cartilages have poor biomechanical properties and are susceptible to degeneration [1]. Notch is an important signaling pathway and play important roles in the regulation of proliferation and differentiation of chondrocytes, the maintenance of chondrocyte phenotype and the metabolism of extracellular matrix [2]. On the other hand, matrix metalloproteinases (MMPs) are the major enzymes responsible for the degradation of ECM [3]. However, the expression of molecules in Notch pathway and MMPs and their interaction in the regulation of ECM of tissue engineered cartilages are still unclear. In the present study, Pluronic F-127 temperature curing hydrogel and allogeneic bone marrow mesenchymal stem cells were used to construct tissue engineering complexes, which were then implanted into knee joints of rabbits with osteochondral defect at knee joints. Immunohistochemistry, Western blot assay and real time PCR were employed to detect the expression of Notch 1, Jagged 1, MMP-1 and MMP-13 in the injured cartilages after repairing. Our study aimed to explore the interaction between Notch pathway and MMPs in the cartilage repair and ECM metabolism in the cartilage, which may provide a new way for the repair of cartilage injury and the resolution of poor biomechanical properties of tissue engineered cartilages.

Materials and methods

Reagents

Rabbit anti-human Jagged 1, MMP-1 and MMP-3 polyclonal antibodies (Santa Cruz), rab-

Table 1. Randomization of 16 New Zealand white rabbits

Group	1 month after surgery				2 months after surgery				3 months after surgery				6 months after surgery			
No	6	15	5	4	13	2	10	11	1	12	16	14	7	9	8	3

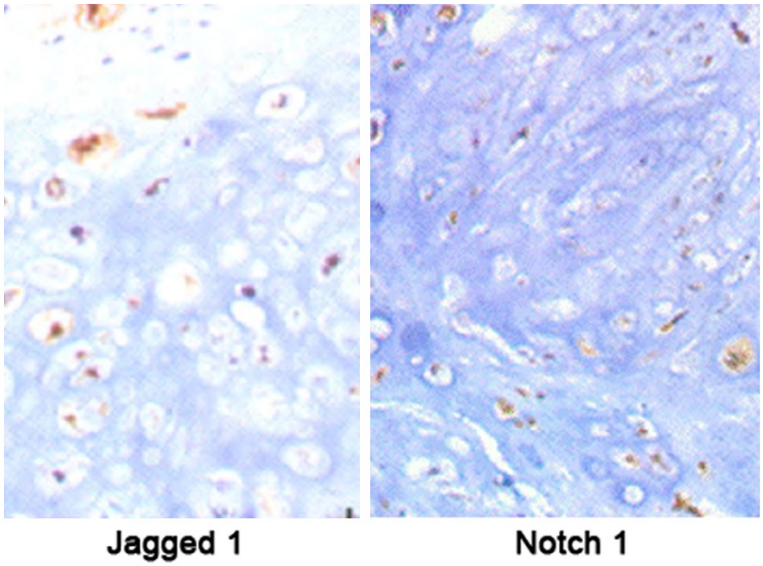

Figure 1. Immunohistochemistry for Jagged 1 and Notch 1 at 1 month after implantation (Immunohistochemistry; 200×).

bit anti-human Notch 1 polyclonal antibody (Abcam) and mouse anti-human β-actin monoclonal antibody (Shanghai Qiangyao Biotech Co., Ltd) were used in the present study.

Animal grouping and sample processing

Grouping: Healthy New Zealand white rabbits (n=16) were randomized into 4 groups and sacrificed at 1, 2, 3 and 6 months after surgery, respectively (**Table 1**).

Sample processing: Animals were anesthetized via ear vein with 3% sodium pentobarbital and then placed in a supine position. The hair at surgical site was removed, followed by sterilization. An incision was made at the internal side of the patella, followed by separation of soft tissues. The patella was pushed outward and the knee joint was bended to completely expose the femoral articular cartilage. The full thickness defects were made at the center of the cartilage with a drill, and then filled with complexes composed of Pluronic F-127 and BMSCs. The patella was reduced, and then the movement of the knee joint was checked. After flushing and hemostasis, the wound was closed.

Detection of Jagged 1, Notch 1, MMP-1 and MMP-13 expression by immunohistochemistry

The tissues were embedded in paraffin and cut into sections. After deparafinization, sections were blocked with 3% hydrogen peroxide to inactivate endogenous peroxidase. Antigen retrieval was done in a microwave oven, and then sections were blocked with normal calf serum. After incubation with primary antibody at 4°C over night, sections were treated with biotinylated secondary antibody at room temperature for 30 min. Visualization was done with DAB. After dehydration and mounting, sections were observed under a light microscope. In negative control, the primary antibody was replaced with PBS. Positive cells showed brown granules.

Detection of Jagged 1, Notch 1, MMP-1 and MMP-1 expression by Western blotting

Tissues were lysed in RIPA lysis buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS; addition of 0.1 g/L PMSF before use) for the extraction of total protein from the cartilage tissues. Protein concentration was determined with Coomassie brilliant blue method. Then, 100 μg of proteins was loaded for 8% SDS polyacrylamide gel electrophoresis at 4°C and 100 mA overnight, followed by transferring onto PVDF membrane. After blocking in 5% non-fat mild at room temperature for 1 h, the membrane was independently incubated with Jagged 1, MMP-1, MMP-13 (1:200), Notch 1 and β-actin (1:1000) at 4°C over night. After washing, the membrane was treated with horseradish peroxidase (HRP) conjugated IgG secondary antibody at 37°C for 2 h. Visualization was done with ECL kit, and the optical density (OD) of protein bands was determined with

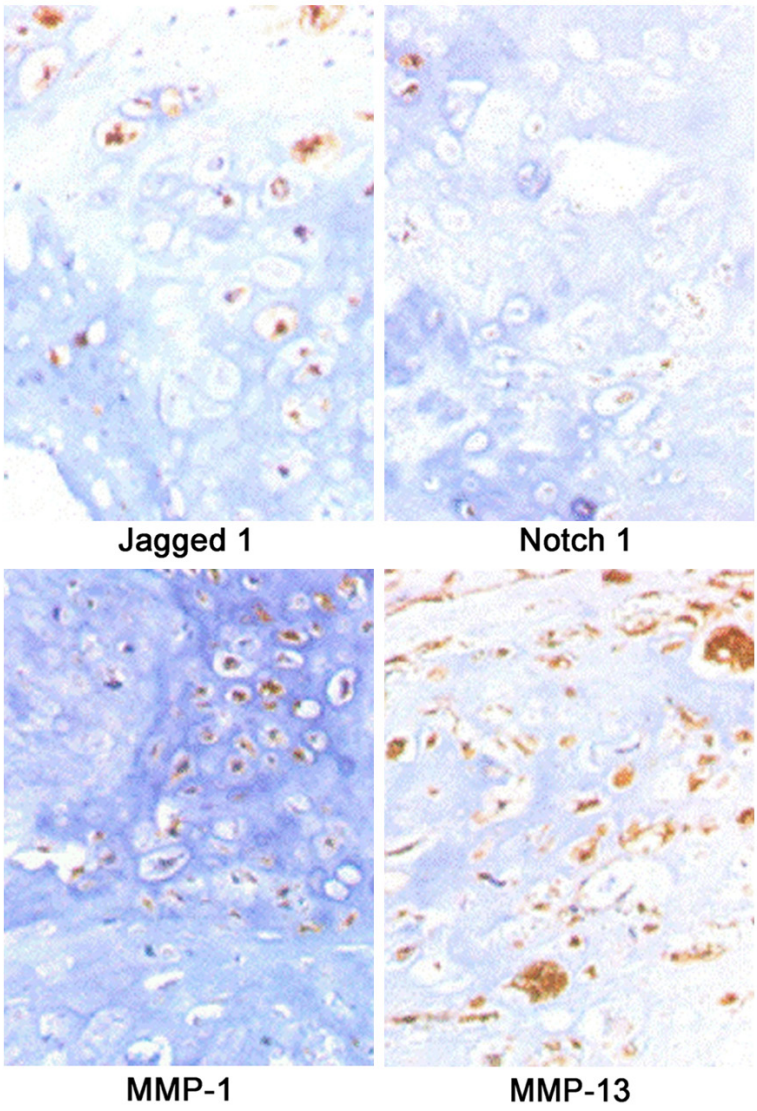

Figure 2. Immunohistochemistry for Jagged 1, Notch 1, MMP-1 and MMP-13 at 6 months after implantation (Immunohistochemistry; 200×).

gel analysis system as the protein expression. The protein expression of Jagged 1, Notch 1, MMP-1 and MMP-13 was normalized to that of β-actin as the relative expression of target protein.

Detection of Jagged 1 and Notch 1 mRNA expression by real time PCR

RNA prep pure kit was used to extract total RNA. A 20-μl mixture containing RNA was used for reverse transcription. The primers used for PCR were as follows: Jaded 1: 5'-AGA AGT CAG AGT TCA GAG GCG TCC-3' (forward), 5'-AGT AGA AGG CTG TCA CCA AGC CAA C-3' (reverse) (anticipated length: 112 bp); Notch 1: 5'-GTG GAT GAC CTA GGC AAG TCG-3' (forward), 5'-GTC TCC TCC TTG TTGTTC TGC A-3' (reverse) (anticipated length: 118 bp); 18S: 5'-CGC CGC TAG AGG TGA AAT TC-3' (forward), 5'-CCA GTC GGC ATC GTT TATGG-3' (reverse) (anticipated length: 149 bp). A 50-μl mixture was used for PCR as follows: predenaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 25 s and annealing at 60°C for 30 s. The Ct value was obtained and the relatively expression of target gene was calculated with $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistical analysis was performed with SPSS version 10.0. Data are expressed as mean ± standard deviation. Intergroup comparison was done with t test and a value of $P<0.05$ was considered statistically significant.

Results

A total of 16 rabbits were included for final analysis, and none died during the study. All the animals had no post-operative infection and presented normal activity, and stage I wound healing was observed in these animals. There were no deformity, redness and swelling, and the range of motion was normal. Patellar dislocation was found in the right knee of a rabbit, and manual reduction was administered without any other abnormality.

Immunohistochemistry for Notch 1, MMP-1 and MMP-13

Positive expression of Jagged 1 and Notch 1 was observed on the membrane of normal chondrocytes. At 1 month after implantation, the Jagged 1 and Notch 1 expression increased significantly as compared to normal knees (**Figure 1**). At 2 months after implantation, the

Table 2. Jagged 1, Notch 1, MMP-1 and MMP-13 protein expression

	Jagged 1	Notch 1	MMP-1	MMP-13
Normal cartilage tissues	0.158±0.045	0.119±0.012	0.251±0.079	0.412±0.038
1 month	0.316±0.073*	0.297±0.041*	0.248±0.023	0.392±0.026
2 months	0.172±0.021	0.210±0.017	0.312±0.019	0.346±0.061
3 months	0.373±0.019*	0.357±0.082*	0.506±0.026*	0.621±0.036*
6 months	0.563±0.012**	0.461±0.044**	0.857±0.079**	0.862±0.053**

Notes: *P<0.05 vs normal cartilage tissues; **P<0.01 vs normal cartilage tissues.

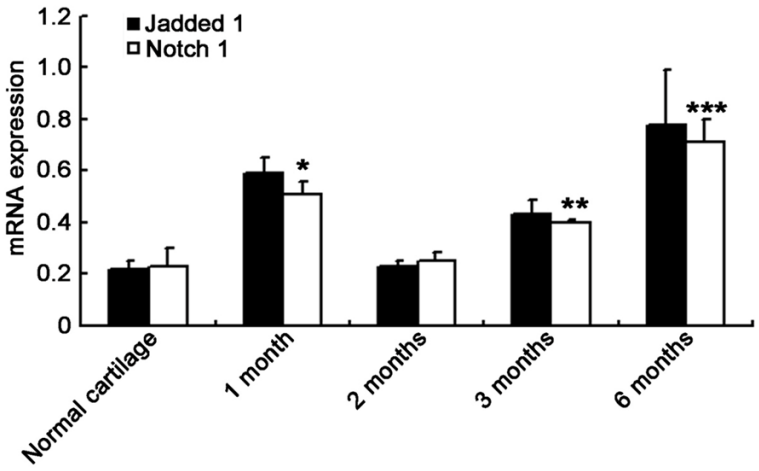

Figure 3. MRNA expression of molecules in Notch pathway. The mRNA expression of Jagged 1 and Notch 1 at 1 month after implantation was significantly higher than in normal cartilage tissues (*, P<0.05); the Jagged 1 and Notch 1 mRNA expression increased at 3 months (**, P<0.05) and significantly increased at 6 months after implantation (***, P<0.01).

Jagged 1 and Notch 1 expression returned to normal level, thereafter increased to a certain extent at 3 months and significantly increased at 6 months after implantation (P<0.05). Positive expression of MMP-1 and MMP-13 was found in the cytoplasm of normal chondrocytes. The MMP-1 and MMP-13 expression at 6 months after implantation was significantly higher than in normal chondrocytes (**Figure 2**).

Western blotting

The expression of Jagged 1, Notch 1, MMP-1 and MMP-13 was at a low level in normal cartilage tissues. At 1 month after implantation, the Jaded 1 and Notch 1 expression increased significant as compared to normal cartilage tissues (P<0.05), but it returned to normal level at 2 months, and thereafter increased to a certain extent at 3 months (P<0.05). The Jaded 1 and Notch 1 expression at 6 months after implantation was significantly higher than in normal controls (P<0.01). The MMP-1 and MMP-13 expression increased significantly at 3 months after implantation (P<0.05) and further increased at 6 months (P<0.01) (**Table 2**).

MRNA expression of molecules in Notch pathway

The mRNA expression of Jagged 1 and Notch 1 at 1 month after implantation was significantly higher than in normal cartilage tissues (P< 0.05). At 2 months after implantation, the Jagged 1 and Notch 1 mRNA expression was similar to the normal level (P > 0.05). Thereafter, the Jagged 1 and Notch 1 mRNA expression increased slowly at 3 months (P<0.05) and significantly increased at 6 months after implantation (P<0.01) (**Figure 3**).

Discussion

Articular cartilages are the most common site that is affected by osteoarthritis, and the damage to articular cartilages is regulated by chondrocytes. Chondrocytes can synthesize a lot of growth factors and cytokines, and a variety of signaling pathways in the chondrocytes are responsible for the formation, rebuilding, homeostasis and post-injury repair of cartilage tissues. In case of osteoarthritis, any pathological factor may directly or indirectly affect the chondrocytes to induce a series of cascades, resulting in cartilage injury and disruption. To date, studies have confirmed that signal transduction play important roles in the occurrence and development of osteoarthritis, and Notch pathway is one of important one involved in the osteoarthritis.

Notch signaling pathway is composed of 4 receptors, 5 ligands and CSL protein. It is a highly conservative and responsible for the proliferation and differentiation of chondrocytes, the maintenance of chondrocyte phenotype, and the control of matrix metabolism in the cartilages [4-7]. The binding between ligand and receptor is required for the activation of Notch signaling pathway. It has been confirmed that there are 4 Notch receptors in mammalians: Notch 1, Notch 2, Notch 3 and Notch 4 [8]. Notch 1 receptor expression is observed in the articular cartilage of > 70% of adults [9, 10]. Karlsson et al [11] found that blocking of Notch signaling pathway could inhibit the proliferation of chondrocytes. In case of arthritis, the Notch signaling pathway is activated in the cartilages. Mahjoub et al [7] found the Notch signaling pathway was significantly activated in the injured cartilages as compared to normal cartilages. Karlsson et al [12] also confirmed the Notch signaling pathway was activated in the cartilages in case of osteoarthritis, and the expression of Jagged 1, HES5 and Notch 1 increased significantly. Jagged 1 is a key ligand in the activation of Notch signaling pathway and very important for the differentiation of mesenchymal stem cells into chondrocytes [12]. Jagged 1 protein may directly activate Notch signaling pathway to further increase the ligands in the Notch signaling pathway. Oldershaw et al [13] found Jagged 1 was highly expressed in early stage of cartilage formation, suggesting that Jaded 1 initiates the Notch 1 signaling pathway activation.

In the present study, Pluronic F-127 temperature curing hydrogel and allogeneic BMSCs were used to construct tissue engineering complexes which were then implanted into the injured knee joints of rabbits. Immunohistochemistry, Western blotting and real time PCR were employed to detect the expression of Jagged 1 and Notch 1 in the cartilages. Results showed Jagged 1 and Notch 1 expression was detectable on the membrane of normal chondrocytes, which was consistent with previously reported. Karlsonn et al [14] and Ustunel et al [9] also confirmed that Notch 1 receptor located in the superficial cells of articular cartilages of cows and mice. In the present study, the Jagged 1 and Notch 1 expression increased significantly at 1 month after implantation as compared to normal cartilage tissues. This indicates that the Jagged 1 mediated activation of Notch 1 pathway is a key event in the initiation of differentiation into chondrocytes, and the transient Notch signaling pathway activation causes the cascade reaction to activate downstream molecules and up-regulate key genes, leading to the differentiation into chondrocytes [15]. At 2 months after implantation, Jagged 1 and Notch 1 expression returned to normal level. These changes indicate the Notch pathway is inhibited at late stage of differentiation into chondrocytes, which assures the formation of chondrocytes. Oldershaw et al [16] also confirmed Notch signaling pathway was inhibitory on the formation of cartilages. Thus, in the late stage of differentiation, the inhibition of Notch signal pathway is helpful for the normal differentiation into and formation of cartilages. At 3 months after implantation, the Jagged 1 and Notch expression increased again and further increased at 6 months. This indicates that the expression of molecules in Notch signaling pathway increases significantly at the late stage of cartilage repair. The Jagged 1 and Notch 1 protein expression increased gradually, which was consistent with the increase in Notch 1 mRNA expression.

The expression of MMP-1 and MMP-13 is closely related to arthritis and cartilage degeneration. A meta-analysis of Zeng et al [17] confirmed that MMP-1 was highly expressed in case of arthritis. Abd-Allah et al [18] also draw similar conclusion and also speculated that MMP-1 could serve as a marker of arthritis. Özler et al [19] investigated the MMP-13 expression in late stage of arthritis, and their results showed MMP-13 expression increased significantly. This suggests that MMP-13 expression is closely related to the occurrence and development of arthritis and inhibition of MMP-13 expression might be able to delay the progression of arthritis [19, 20]. In the present study, MMP-1 and MMP-13 expression increased at 3 months after implantation, which was consistent with the changes in the expression of molecules in Notch pathway. Thus, we speculate that these may affect the biomechanical properties of cartilages after repair.

There were limitations in this study: 1) Our results did not elucidate the mechanism underlying the interaction between Notch pathway

and MMP-1/13, and more studies with elegant design are required; 2) BMSCs have potent capabilities of division and proliferation. Theoretically, there is the possibility of tumorigenesis. However, BMSCs are easy to collect, have potent proliferation, are easy to culture, have favorable plasticity and relatively stage genetic background. The collection of BMSCs is mini-invasive, and BMSCs transplantation has been confirmed to be safe and only induces mild rejection.

Taken together, Notch signaling pathway is activation for the cell proliferation in early stage of cartilage repair after implantation of tissue engineering complexes and may induce the differentiation of BMSCs into chondrocytes. During the differentiation of BMSCs into chondrocytes, Notch signaling pathway is inhibited. In late stage of cartilage repair, MMP-1 and MMP-13 expression increases, accompanied by elevated expression of Jagged 1 and Notch 1. These finding suggest that Notch signaling pathway may regulate MMP expression to affect the biomechanical properties of repaired cartilages after implantation of tissue engineering complexes containing BMSCs.

Acknowledgements

This study was supported by the Program of Shanghai Health and Family Planning Commission (20134186).

Disclosure of conflict of interest

None.

Address correspondence to: Wanjun Liu, The Sixth People's Hospital, Shanghai Jiaotong University, Shanghai, China. Tel: +86-21-3829773; E-mail: liuwanjunguk@163.com

References

[1] Hendren L, Beeson P. A review of the differences between normal and osteoarthritis articular cartilage in human knee and ankle joints. Foot (Edinb) 2009; 19: 171-176.

[2] Hardingham TE, Oldershaw RA, Tew SR. Cartilage, SOX9 and Notch signals in chondrogenesis. J Anat 2006; 209: 469-480.

[3] Sandy JD. A contentious issue finds some clarity: on the independent and complementary roles of aggrecanase activity and MMP activity in human joint aggrecanolysis. Osteoarthritis Cartilage 2006; 14: 95-100.

[4] Liu Z, Chen J, Mirando AJ, Wang C, Zuscik MJ, O'Keefe RJ, Hilton MJ. A dual role for NOTCH signaling in joint cartilage maintenance and osteoarthritis. Sci Signal 2015; 8: ra71.

[5] Sassi N, Gadgadi N, Laadhar L, Allouche M, Mourali S, Zandieh-Doulabi B, Hamdoun M, Nulend JK, Makni S, Sellami S. Notch signaling is involved in human articular chondrocytes de-differentiation during osteoarthritis. J Recept Signal Transduct Res 2014; 34: 48-57.

[6] Chen S, Lee BH, Bae Y. Notch signaling in skeletal stem cells. Calcif Tissue Int 2014; 94: 68-77.

[7] Mahjoub M, Sassi N, Driss M, Laadhar L, Allouche M, Hamdoun M, Romdhane KB, Sellami S, Makni S. Expression patterns of Notch receptors and their ligands in human osteoarthritic and healthy articular cartilage. Tissue Cell 2012; 44: 182-194.

[8] Gering M, Patient R. Notch signalling and haematopoietic stem cell formation during embryogenesis. J Cell Physiol 2010; 222: 11-16.

[9] Ustunel I, Ozenci AM, Sahin Z, Ozbey O, Acar N, Tanriover G, Celik-Ozenci C, Demir R. The immunohistochemical localization of notch receptors and ligands in human articular cartilage, chondroprogenitor culture and ultrastructural characteristics of these progenitor cells. Acta Histochem 2008; 110: 397-407.

[10] Grogan SP, Miyaki S, Asahara H, D'Lima DD, Lotz MK. Mesenchymal progenitor cell markers in human articular cartilage: normal distribution and changes in osteoarthritis. Arthritis Res Ther 2009; 11: R85.

[11] Karlsson C, Jonsson M, Asp J, Brantsing C, Kageyama R, Lindahl A. Notch and HES5 are regulated during human cartilage differentiation. Cell Tissue Res 2007; 327: 539-551.

[12] Karlsson C, Brantsing C, Egell S, Lindahl A. Notch 1, Jagged 1, and HES5 are abundantly expressed in osteoarthritis. Cells Tissues Organs 2008; 188: 287-298.

[13] Oldershaw RA, Hardingham TE. Notch signaling during chondrogenesis of human bone marrow stem cells. Bone 2010; 46: 286-293.

[14] Karlsson C, Stenhamre H, Sandstedt J, Lindahl A. Neither Notch 1 expression nor cellular size correlate with mesenchymal stem cell properties of adult articular chondrocytes. Cells Tissues Organs 2008; 187: 275-285.

[15] Liu XW, Hu SS, Hu J. Relationship of Notch signaling pathway with articular cartilage development and arthritis. J Tissue Engineer Reconstruc Surg 2016; 12: 44-47.

[16] Oldershaw RA, Tew SR, Russell AM, Meade K, Hawkins R, McKay TR, Brennan KR, Hardingham TE. Notch signaling through Jagged-1 is necessary to initiate chondrogenesis in human bone marrow stromal cells but

must be switched off to complete chondrogenesis. Stem Cells 2008; 26: 666-674.
[17] Zeng GQ, Chen AB, Li W, Song JH, Gao CY. High MMP-1, MMP-2, and MMP-9 protein levels in osteoarthritis. Genet Mol Res 2015; 14: 14811-14822.
[18] Abd-Allah SH, El-Shal AS, Shalaby SM, Pasha HF, Abou El-Saoud AM, Abdel Galil SM, Mahmoud TA. Influence of Matrix metalloproteinase 1 and 3 genetic variations on susceptibility and severity of juvenile idiopathic arthritis. IUBMB Life 2015; 67: 934-942.
[19] Ozler K, Aktas E, Atay C, Yilmaz B, Arikan M, Gungor S. Serum and knee synovial fluid matrix metalloproteinase-13 and tumor necrosis factor-alpha levels in patients with late-stage osteoarthritis. Acta Orthop Traumatol Turc 2016; 50: 356-361.
[20] Ruminski PG, Massa M, Strohbach J, Hanau CE, Schmidt M, Scholten JA, Fletcher TR, Hamper BC, Carroll JN, Shieh HS, Caspers N, Collins B, Grapperhaus M, Palmquist KE, Collins J, Baldus JE, Hitchcock J, Kleine HP, Rogers MD, McDonald J, Munie GE, Messing DM, Portolan S, Whiteley LO, Sunyer T, Schnute ME. Discovery of N-(4-Fluoro-3-methoxybenzyl)-6-(2-(((2S,5R)-5-(hydroxymethyl)-1,4-dioxan-2-yl)met hyl)-2H-tetrazol-5-yl)-2-methylpyrimidine-4-carboxamide. A highly selective and orally bioavailable matrix metalloproteinase-13 inhibitor for the potential treatment of osteoarthritis. J Med Chem 2016; 59: 313-327.