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Original Article

Osteosarcoma cells induce differentiation of mesenchymal stem cells into cancer associated fibroblasts through Notch and Akt signaling pathway

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Abstract: Cancer-associated fibroblasts plays an important role in tumor progession, growth and metastasis. However, the mechanisms of mesenchymal stem cells differentiation to cancer-associated fibroblasts induced by tumor cells remains unclear. This study aimed to explore whether osteosarcoma cells can also induce BMSCs into CAFs through cells co-culture system, and identify the probably signailing pathway that mediates differentiation of BMSCs to CAFs. The results showed that osteosarcoma cells can induce BMSCs to exhibit the typical characteristics of CAFs, with increased expression of α-SMA, the most reliable marker for CAFs. Morever, we also reveals that Notch signaling acts upstream of Akt signaling in HBMSCs-TAFs differentiation.

Keywords: Mesenchymal stem cell, osteosarcoma cell, cancer-associated fibroblasts, notch signaling pathway, akt signaling pathway

Introduction

Osteosarcoma (OS) is one of most malignant tumor in skeletal system [1]. Although OS incidence is lower than liver cancer, gastric carcinoma and colon caner, but OS with poor prognosis and high lethality that attracted most reseachers attention [2, 3]. The most obvious characteristics of OS is high metastasis rate and strongly invasive ability [4]. Cancer metastasis and invasive is a complex and multistep process, including epithelial-mesenchymal transition (EMT), lymphatic vessels, forming viable secondary cancer, and so on [5, 6].

There are many studies showed that stromal cells in cancer microenvironment exert the effect on tumor progression, angiogenesis, metastasis and invasive [7, 8]. Cancer-associated fibroblasts (CAFs) are most frequnt componet of tumor stroma, which have been reported to participate in solid tumor progession, growth and metastasis [9-11]. It is increasingly appreciated that Mesenchymal stem cells (MSCs) can attraced by malignant solid tumors into their microenvironment, and then become CAFs, contributing to tumor cell angiogenesis and metastasis [12-14]. However, the mechanisms of MSCs to CAFs is still poorly understood. To the best of our knowledge, most current research in MSCs to CAFs field have shed light on extracellular matrix proteins or cytokines involved in this process [15, 16]. Little information concerning the role of tumor microenvironment.

In this study shows that osteosarcoma cells can direct induce differentiation of bone marrow mesenchymal stem cells (BMSCs) to cancer-associated fibroblasts (CAFs), with an *in vitro* direct co-culture of osteosarcoma cells and BMSCs. Moreover, Notch and Akt signaling pathway play a critical role in regulate the process of differentiation of BMSCs into CAFs. More interestingly, we also note that Notch signaling acts upstream of Akt signaling in HBMSCs-TAFs differentiation. These findings represent a novel mechanism for differentiation of BMSCs to CAFs and provide a potential target for osteosarcoma treatment.

Materials and methods

Cell lines and cell culture

Human OS cell lines MG-63 and U2OS were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cul-

tured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin. Human bone marrow mesenchymal stem cells (HBMSCs) were purchased from ATCC and cultured in HBMSC-GM (Gibco). Cells were kept at 37°C with 5% humidified CO_2. MG-63 and U2OS cell maintained in the medium and passaged 2-3 days, cells were passaged twice prior to use.

Exposure of OS cells to HBMSCs culture

HBMSCs were seeded in 12-well plates (Corning Costar Co., NY, USA) for 24 h. MG-63 and U2OS were grown in RPMI-1640 with 10% FBS culture medium, when the cells were cultured near to 80% confluency, they were switched to serum free medium, after 24 h, the medium was harvested and centrifuged at 3000 rpm for 20 min. The OS cells was direct loaded onto the HBMSCs cultures. The co-culture system was maintained for 72 h, the ratio of MG-63 and U2OS cell in co-culture system were determined by flow cytometry. The OS cells and HBMSCs were cultured separately at the same time, and then the same ratio in co-culture system of OS cells and HBMSCs were mixed and used for control. The co-culture system were treated with 30 μM γ-secretase inhibitor DAPT (Cayman Chemical, Ann Arbor, USA) or 10 μM Akt phosphorylation inhibitor LY2940023 (Kang Chen Bio-tech, Shanghai, China).

MTT assay

Cells proliferation were detected by MTT assay. Cells were plated into 96-well plates with 5×10^4/well, and washed with PBS for twice, MTT (10 μl) was added into each well. The plates were incubated in 37°C for 1.5 h, and 150 μl DMSO was added to dissolve the formazan crystals. The Absorbance of each well was measured by Spectra Max Paradigm Multi-Mode Reader (Molecular Devices, Austria) at 565 nm. The results were presented as relative to contol, all experiments were performed in triplicate.

Cell invasion assay

Cell invasion ability was measured by a Transwell chamber assay (Costar, Pleasanta, CA, USA) according to the manufacturer's a instructions. Serum-free DMEM was added to the upper chambers and DMEM containing 10% fetal bovin serum was added to the lower chambers. After 48 h of cultured, cells were transferred to the upper chambers (1×10^5/Transwell). Cells were cultured for 24 h, and migrated to the lower surface cells were fixed by 90% alcohol and stained by 0.08% crystal violet.

Western blot

After treatment, cells were scraped in ice-cold PBS, and incubated on ice with lysis buffer containing 1 mM EGTA, 20 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, 30 mM sodium pyrophosphate, 25 mM β-glycerol phosphate, 1% Triton X-100, pH 7.4 for 30 min. The lysates of cells were centifuged at 12000 g at 4°C. 50 μg of supernatant proteins were subjected to 12% polyacrylamide gel, and then transferred to PVDF membrane (Millipore, Billerica, MA, USA). After blocking with fat-free milk in TBST at room temperature for 1 h, the primary antibodies was added and incubated at 4°C overnight. The proteins lable were visualized by exposing X-ray film using enhanced chemiluminescence (ECL) kit (Sigma), followed by incubation with secondary antibodies for 2 h at room temperature. The antibodies as following: α-SMA (1:1000; BOSTER, Wuhan, China), β-catenin (1:1000; Beyotim, Haimen, China), NF-κB (1:5000; Beyotim), Phosphorylation-Akt (Thr308) (1:500; Bioworld, Nanjing, China), total-Akt (1:500; Bioworld, Nanjing, China), GAPDH (1:5000; Sangon, Shanghai, China), secondary anti-mouse and anti-rabbit antibodies (1:500; Sangon, Shanghai, China).

Quantitative real time quantitative PCR

Cells were incubated in six well plates with 5×10^4/well, total RNA was isolated using Trizol reagent (Takara, Shiga, Japan) and used PrimeScript RT Master Mix (Takara) to synthesise cDNA according to manufacturer's protocol. Real time PCR was performed by StepOne PCR system (Applied Biosystems, UK). A total of 1 ml of cDNA was added to a final reaction volume of 22 μl containing 0.05 U/ml Taq, SYBR green and 0.2 mM specific primers. α-SMA F: 5'-TGGTATTGTGCTGGACTCTG-3'; R: 5'-CCATCAGGCAGTTCGTAG-3'. Hes1 F: 5'-GAGCACAGAAAGTCATCAAAGC-3', R: 5'-CGGAGGTGCTTCACTGTCAT-3'. Hey1 F: 5'-GATTGGCGGCCGCGCCACCATGAAGAGA-3', R: 5'-GTATGGGATCCTTAGAAAGCTCCGATCTCTGTCG-3'. GAPDH F: 5'-CGGAGTCAACGGATTTGGTCGTAT-3', R: 5'-AGCCTTCTCCAT-

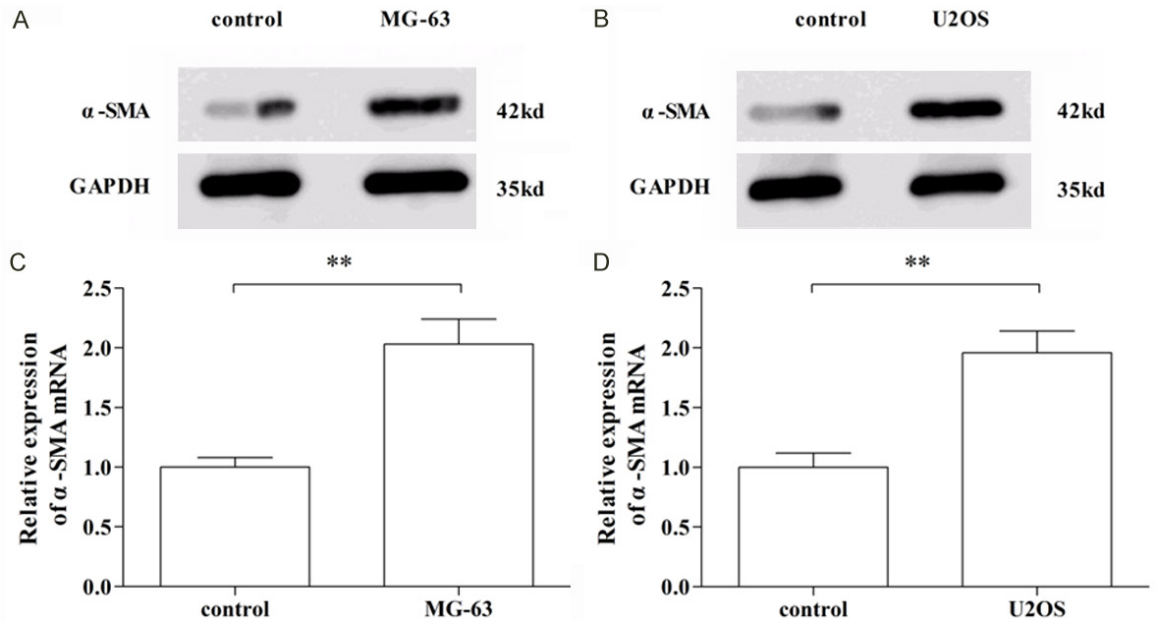

Figure 1. Expression of α-SMA in co-culture medium. A: Western Blotting determined the protein expressions of α-SMA induced by MG-63 cell. B: Western Blotting determined the protein expressions of α-SMA induced by U2OS cell. C: Real-time PCR determined the mRNA expressions of α-SMA induced by MG-63 cell; D: Real-time PCR determined the mRNA expressions of α-SMA induced by U2OS cell. $^{**}P<0.01$ compared to control. All experiments were performed at least three times. Data are presented as mean ± SD.

GGTGGTGAAGAC-3'. GAPDH served as as an endogenous control. The experiments were performed for triplicate, and relative expression of target gene was normalized by GAPDH using $^{\Delta\Delta}$Ct comparative method.

Statistical analysis

The related data of experiments were analyze by SPSS version 19.0 software (SPSS, Chicago, IL), and the data are presented as mean ± standard error, differences between groups was used student's *t*-test, A value of $P<0.05$ was considered statistically significant.

Results

Human OS cells induces expression of α-SMA in HBMSCs

Zhou et al. have documented that BMSCs were induced CAF phenotype and functional characteristics by prolonged exposure to tumor-conditioned medium [17]. To investigate whether osteosarcoma cells can also induce the differentiation of BMSCs into CAFs through cells co-culture system, BMSCs were either exposed to OS cells or mock treated, then we assayed the α-SMA expression in co-culture system, the most reliable marker for CAFs [18, 19]. As shown in **Figure 1**, the expression of α-SMA protein of BMMSCs in MG-63 and U2OS cells conditions medium was significantly increased when compered to control (**Figure 1A** and **1B**). In agreement with the Western blotting results, quantitative RT-PCR showed that α-SMA mRNA was also dramatically elevated in the conditions mentioned above (**Figure 1C**). These results indicated that BMMSCs treated with osteosarcoma cells undergo CAFs differentiation *in vitro*.

Human OS cells promotes proliferation and invasion of HBMSCs

Increased ability of proliferation and invasion is properties of tumor progression. Attieh et al. have reported that CAFs may participate in tumor progression [20]. For this reason, we investigated the effect of OS cells on proliferation and invasion of HBMSCs. As shown in **Figure 2**, the proliferation ability was significantly promoted induced by MG-63 and U2OS cell compared to control (**Figure 2A**), Moreover, OS cells could also significantly promote the invasion ability of HBMSCs (**Figure 2B**).

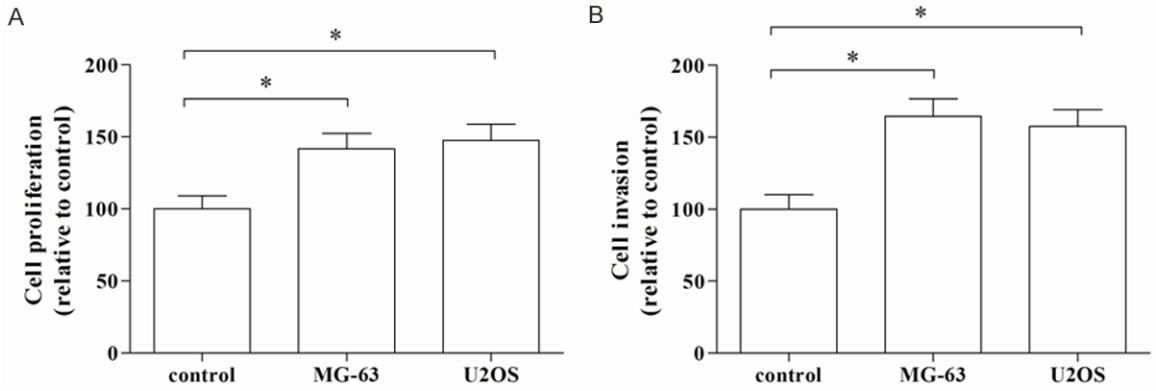

Figure 2. OS cells promotes proliferation and invasion of HBMSCs. A: MTT assay determined the cell proliferation induced by MG-63 and U2OS cell. B: Trans-Well assay determined the cell invasion induced by MG-63 and U2OS cell. *P<0.05 compared to control. All experiments were performed at least three times. Data are presented as mean ± SD.

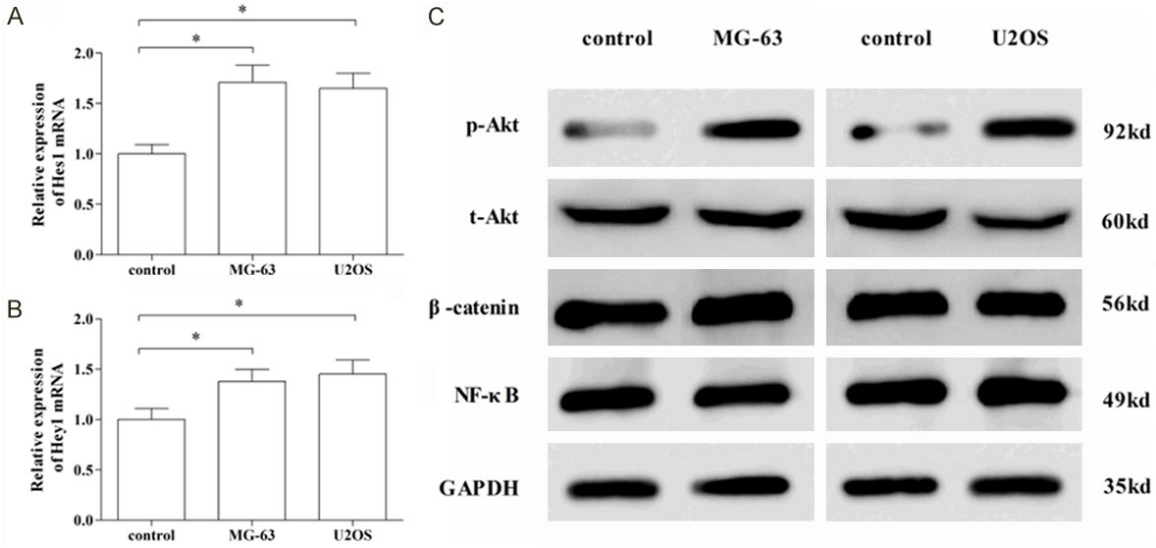

Figure 3. Activation of Notch and Akt signaling pathways in co-cultures of HBMSCs with MG-63 of U2OS cell. A: Real-time PCR determined the mRNA expressions of Hes1 in co-culture system. B: Real-time PCR determined the mRNA expressions of Hey1 in co-culture system. C: Western Blotting determined the protein expressions of p-Akt, t-Akt, β-catenin, NF-κB in co-cultures, GAPDH was used as control. *P<0.05 compared to control. All experiments were performed at least three times. Data are presented as mean ± SD.

Notch and Akt signaling pathways are activated in HBMSCs induced by human OS cells

Notch pathway is known as paly an improtant role in cell interaction mechanism [21]. To clarify the specific mechanism responsible for BMSCs to CAFs, we evaluated the activation of this pathways through Notch receptors. Quantitative RT-PCR results showed that HBMSCs conditioned in MG-63 and U2OS cell were dramatically increased the mRNA expression of the downstream Notch effector Hes1 and Hey1 (**Figure 3A** and **3B**).

There are accumulating amount of evidences demonstrating that α-SMA gene transcription is regulated by the interplay between multiple signal transduction pathways [22, 23]. To address this, Western blot was performed analysis against β-catenin, NF-κB, the phosphorylated form of Akt (p-Akt), which are indicators of Wnt, NF-κB, Akt pathway activation, respectively.

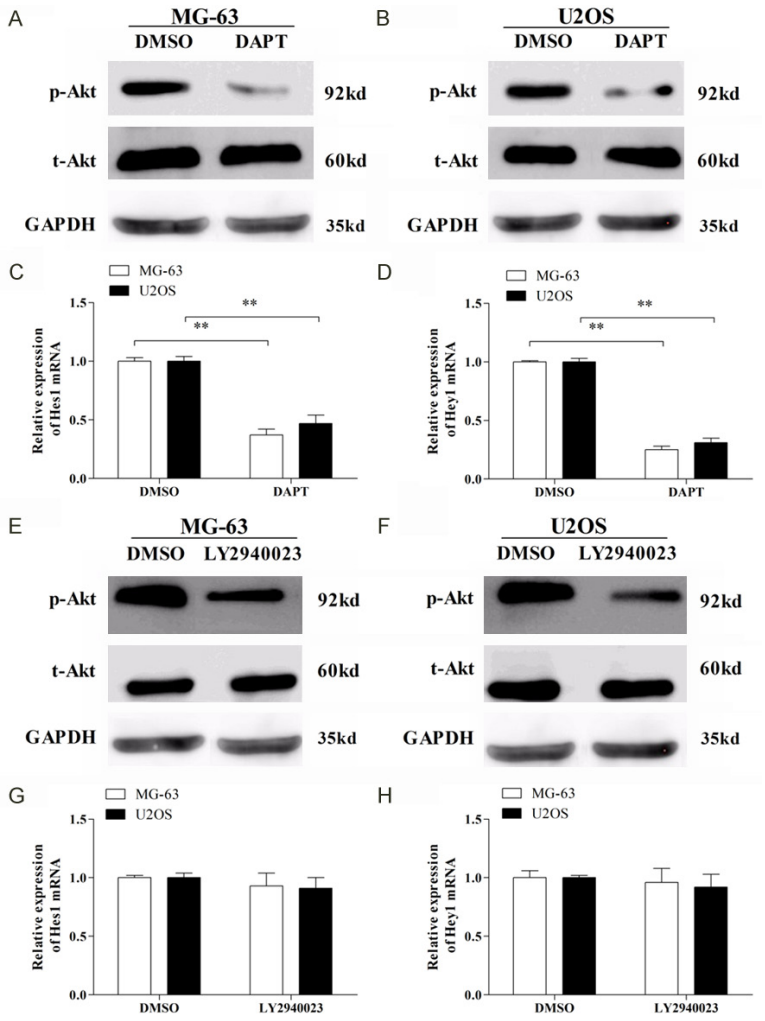

Figure 4. Akt signal acts the downstream of Notch signal in induced CAFs differentiation of HBMSCs. A: Western Blotting determined the protein expressions of p-Akt, t-Akt in co-cultures of HBMSCs and MG-63 were inhibited by DAPT treatment. B: Western Blotting determined the protein expressions of p-Akt, t-Akt in co-cultures of HBMSCs and U2OS were inhibited by DAPT treatment. C: Real-time PCR determined the mRNA expressions of Hes1 in co-cultures of HBMSCs and OS cells were inhibited by DAPT treatment. D: Real-time PCR determined the mRNA expressions of Hey1 in co-cultures of HBMSCs and OS cells were inhibited by DAPT treatment. E: Western Blotting determined the protein expressions of p-Akt, t-Akt in co-cultures of HBMSCs and MG-63 were inhibited by LY2940023 treatment. F: Western Blotting determined the protein expressions of p-Akt, t-Akt in co-cultures of HBMSCs and U2OS were inhibited by LY2940023 treatment. G: Real-time PCR determined the mRNA expressions of Hes1 in co-cultures of HBMSCs and OS cells were inhibited by LY2940023 treatment. H: Real-time PCR determined the mRNA expressions of Hey1 in co-cultures of HBMSCs and OS cells were inhibited by LY2940023 treatment.of HBMSCs and OS cells. GAPDH was used as control; $^{**}P<0.01$ compared to control. All experiments were performed at least three times. Data are presented as mean ± SD.

The results showed that the p-Akt was prominently increased in HBMSCs induced by two human OS cells, in contrast to no obvious changes in β-catenin and NF-κB (**Figure 3C**). These data suggested that human OS cells effects on HBMSCs could potentially be regulated by Notch and Akt signaling pathways.

Notch signaling pathway trigger CAFs differentiation from HBMSCs via Akt pathway

DAPT is a γ-secretase inhibitor, which induced intramembranous cleavage of Notch receptor and sequence biological effects [24]. There are several reporters showed that α-SMA expression are regulated by Notch and Akt pathway through different modes of cross talk, which are either in simple up and downstream ways or mutually regulated ways [25, 26]. For the sake of verifying the epistasis between Notch and Akt pathway in HBMSCs to CAFs process induced by OS cells, we firstly measured the expression of p-Akt in HBMSCs with OS cells blocked by DAPT. As shown in **Figure 4**, the expression of p-Akt were significantly reduced in DAPT block system (**Figure 4A-D**). Then LY2940023 was added into co-culture system, we detected the mRNA expression of Hes1 and Hey1, two target genes of Notch signaling pathway. As shown in **Figure 4**, pharmacological inhibition of Akt pathway by Akt inhibitor LY2940023 (**Figure 4E** and **4F**), that no effect on Notch signaling molecules in HBMSCs (**Figure 4G** and **4H**), which clearly implies that Notch signaling acts upstream of Akt signaling in HBMSCs-TAFs differentiation.

Discussion

Cancer-associated fibroblasts are a predominant in tumor stromal cells and paly a vital role

in the progession and metastasis of tumor cells. However, the origins of CAFs in the tumor stroma remain poorly elucidated. Subramanian et al. reported that under long-term exposure to tumor medium, mesenchymal stem cells can induce differentiation to CAFs, as characterized by CAFs-like protein [27]. An important recent study explored that colon cancer cells can induce adjoining BMSCs to exhibit the typical characteristic of cancer-associated fibroblast [28]. In the present study, we showed that exposure of osteosarcoma cells can also induced CAFs-like phenotype from HBMSCs *in vitro*. This results is based upon that direct exposure of HBMSCs to different osteosarcoma cell lines induces increased expression of α-SMA, the most reliable marker for CAFs. In present experiment we also demonstrate that cell-cell interaction promotes the ability of proliferation and invasion of HBMSCs induced by OS cells. This conclusion is agreement with a previously published data that circulating MSCs are able to migrate to colon tumor cells and then promote the growth and metastasis ability of cells [29].

The differentiation of HBMSCs to CAFs is a multistep and complex biological process, which may involve epithelial-mesenchymal transition, bone marrow-derived progenitor, cell-cell contaction and cytokines [30-32]. However, little is known about the possible molecular mechanisms underlying transdifferentiation of BMSCs to CAFs. Notably, the present result here showed that HBMSCs are exposed to OS cells, concurrent activation of Notch and Akt signaling was observed in the co-culture system. Moreover, we also identify that blocking Notch signaling pathway by DAPT affects p-Akt expression in the co-culture medium, whereas there are no changes in expression of Hes1 and Hey1, two target genes of Notch signaling when treated with Akt inhibitor LY2940023. This phenomenon is clearly indicates that Akt signaling acts as intermediate step in Notch signaling induced upregulation of CAFs marker in HBMSCs. Viant and co-workers in their latest study shows that Notch activates Akt signaling by upregulated the expression of TGF-β, which induces the exression of phosphorylates Akt (p-Akt) [33]. However, in this study the elevated of p-Akt comes from osteosarcoma cells or HBMSCs was not fully investigated. More works will be needed to fully clarify the mechanisms of Notch-mediated Akt signaling activation in osteosarcoma cells and HBMSCs co-culture system.

In conclusion, our results suggest that osteosarcoma cell-derived Notch signal can induce CAFs differentiation of HBMSCs through activating the Akt signaling. However, it is uncertain whether this mechanisms exists in other tumor types. Yu et al. indicated that Notch-1 signaling pathway upregulates NF-κB transcriptional activity and induces the adhesion, migration and invasion of human breast cancer cell line MDA-MB-231 [34]. Giovannini et al. also reported that Notch signaling pathway is a potentially important mechanism in the regulation of metformin on macrophage polarization and the subsequent change of hepatoma cells [35]. Therefore, Notch and Akt signaling pathway seems not to be osteosarcoma cell specific, although the molecular mechanisms involved in this process may be not exactly the same in different tumor cells. These findings represent a novel mechanism for differentiation of BMSCs to CAFs and provide a potential target for osteosarcoma treatment.

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Disclosure of conflict of interest

None.

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