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Direct contacts with colon cancer cells regulate the differentiation of bone marrow mesenchymal stem cells into tumor associated fibroblasts

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ABSTRACT

Tumor–stroma interactions are referred to as essential events in tumor progression. There has been growing attention that bone marrow-derived mesenchymal stem cells (BMSCs) can travel to tumor stroma, where they differentiate into tumor-associated fibroblast (TAF)-like cells, a predominant tumor-promoting stromal cell. However, little is definitively known about the contributors for this transition. Here, using an *in vitro* direct co-culture model of colon cancer cells and BMSCs, we identify that colon cancer cells can induce adjoining BMSCs to exhibit the typical characteristic of TAFs, with increased expression of α-smooth muscle actin (α-SMA). Importantly, the present data also reveals that activated Notch signaling mediates transformation of BMSCs to TAFs through the downstream TGF-β/Smad signaling pathway.

1. Introduction

Most solid tumors are composed of parenchymal tumor cells and a complex array of tumor stromal cells [1]. It is increasingly appreciated that stromal cells in the tumor microenvironment exert profound effects on neoplastic progression, tumor growth, angiogenesis and metastasis [2,3]. Tumor-associated fibroblasts (TAFs), also termed cancer-associated fibroblasts, are the most frequent component of tumor stroma [4]. They distinctively express α-smooth muscle actin (α-SMA) and have been reported to participate in important aspects of solid tumor progression [5,6].

Mesenchymal stem cells (MSCs) are a population of pluripotent progenitor cells that can be isolated from a variety of adult and fetal tissues, including bone marrow, adipose tissue, umbilical cord blood, placenta and amniotic fluid [7]. These cells can self-renew *in vitro* and have the potential to give rise to multiple mesenchymal cells, such as osteoblasts, chondrocytes, adipocytes, fibroblasts and myocytes [8,9]. Importantly, MSCs exhibit an innate tropism for inflamed or damaged tissues as well as tumor sites, which are likened to wounds that never heal, due to the close proximity of factors secreted by tumors and wounds [10,11]. There is an accumulating amount of evidence demonstrating that primary and metastatic tumors attract MSCs into their microenvironment, where they become TAFs, contributing to tumor stroma formation and affecting tumor cell survival and angiogenesis [12–14]. However, the underlying regulatory mechanisms that link MSCs to TAFs remain incompletely understood. To the best of our knowledge, the majority of current research in this field is devoted to defining which cytokines and extracellular matrix proteins are involved in this process [15,16]. Little information concerning the role of cell contact-dependent signaling is available.

This study shows that direct contact with colon cancer cells can stimulate differentiation of bone marrow mesenchymal stem cells (BMSCs) to TAFs, with an *in vitro* direct co-culture model of colon cancer cells and BMSCs. Specifically, Notch and TGF-β/Smad signaling pathways are demonstrated to synergistically regulate the differentiation of BMSCs into TAFs. These findings are novel and

Abbreviations: BMSCs, bone marrow-derived mesenchymal stem cells; TAF, tumor-associated fibroblast; α-SMA, α-smooth muscle actin; MSCs, mesenchymal stem cells; FBS, fetal bovine serum; PFA, paraformaldehyde; SE, standard error; GFP, green fluorescent protein; BMMSCs, murine bone marrow-derived mesenchymal stem cells; p-Akt, the phosphorylated form of AKT; p-Smad2/3, the phosphorylated form of Smad2/3.

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important to the understanding of stem cell biology and the developmental events that govern the initiation of TAFs from MSCs.

2. Materials and methods

2.1. Cell culture

DLD1 and SW480 (human colorectal cancer cell lines) were obtained from the Chinese Type Culture Collection and grown in RPMI-1640 (Gibco, Grand Island, NY, USA) containing 1× penicillin/streptomycin and 10% fetal bovine serum (Gibco). Human bone marrow mesenchymal stem cells were purchased from Chinese Biowit Technologies and cultured in HBMSC-GM (Biowit, China). Cells were kept at 37 °C in 5% humidified CO_2.

2.2. Lentiviral experiments

To select for DLD1 and SW480 stably expressing green fluorescence protein (GFP), pLVX-AcGFP1-N1 (Clontech), psPAX2 and pMD2.G were co-transfected into 293T cells at a ratio of 15:10:5 μg using the calcium phosphate transfection method. Thirty-six hours later the viral supernatant was collected and concentrated using 100 kDa ultrafiltration membranes (Millipore). Rapidly proliferating DLD1 and SW480 were infected with the concentrated virus in the presence of polybrene (8 μg/ml) for 24 h and then subjected to selection using 5 μg/ml puromycin. This method resulted in >90% infection efficiency, as determined by the percentage of cells labeled with GFP.

We utilized the lentivirus-based shRNA expression plasmid pLL3.7 to knockdown the endogenous expression of Jagged1 and DLL1 in the colon cancer cell line DLD1. shRNA oligonucleotides for Jagged1 and DLL1 were chemically synthesized, annealed and cloned into the PLL3.7 lentivector utilizing HpaI and XhoI restriction sites. Correct insertions of shRNA cassettes were confirmed by direct DNA sequencing. Recombinant lentivirus was generated by co-transfecting 293T cells with three plasmids: pLL3.7-Jagged1/DLL1 shRNA (experimental virus) or pLL3.7-control shRNA (control virus), plus psPAX2 and pMD2.G. The infection of colon cancer cells was performed according to the protocol above. Hairpin sequences in these shRNA constructs are depicted in Supplementary Table 1.

2.3. Co-culture assay

BMSCs were plated in 12-well plates (Corning Costar Co., NY, USA) with gelatin coated glass slides for immunofluorescence analysis or in 75 cm^2 tissue culture flasks (Corning Costar Co., NY, USA) for protein or RNA isolation. After 24 h, colon cancer cells were loaded directly onto the BMSCs cultures. The direct co-culture systems were maintained for 72 h in HBMSC-GM medium. Some of the co-cultures were treated with 30 μM γ-secretase inhibitor DAPT (Cayman Chemical) or 10 μM transforming growth factor-β type I receptor kinase inhibitor SB431542 (Cayman Chemical).

2.4. Immunofluorescence staining

After co-culturing, the cells were fixed in 4% paraformaldehyde (PFA) at room temperature for 30 min, then permeabilized with 0.3% Triton X-100 in PBS for 10 min. Next, the slides were blocked in 3% BSA (Sigma) for 1 h and then incubated with the primary antibody for α-SMA (1:100, Biotime, China) at 4 °C overnight. Anti-mouse-TRITC secondary antibody was used for primary antibody detection. After three PBS washes, the slides were mounted in gelvatol for the confocal immunofluorescence analysis.

2.5. Western blotting and quantitative RT-PCR

Western blotting and quantitative RT-PCR assays were performed as previously described [17]. Antibodies used for western were as follows: α-SMA (1:1000; Biotime, China), β-catenin (1:1000; Abmart, Shanghai, China), NF-κB (1:1000; Bioworld, Nanjing, China), Phospho-AKT (1:500; Bioworld, Nanjing, China), Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) (1:1000; Cell Signaling), Smad2/3 (1:500; Bioworld, Nanjing, China), GAPDH (1:5000; Abmart, Shanghai, China), secondary anti-mouse and anti-rabbit antibodies (1:1000; Invitrogen, Carlsbad, CA). The primers for qPCR used in this study were depicted in Supplementary Table 2.

2.6. Statistical analysis

Data are presented as mean ± standard error (SE). Student's *t*-test was used to analyze differences between two groups. We considered probability (P) values <0.05 as significant.

3. Results

3.1. Human colon cancer cells induce α-SMA expression in BMSCs through direct cell–cell contacts

To investigate whether colon cancer cells can trigger differentiation of BMSCs into TAFs through direct cell–cell contacts, we first established direct co-cultures by placing colon cancer cells in the same well with human bone marrow mesenchymal cells (HBMSCs) on primaria slides for 3 days. Two colon cancer cell lines labeled with lentivirus expressing green fluorescent protein (GFP), GFP-DLD1 and GFP-SW480, were selected for our co-culture assay. Then, expression of α-SMA, the most reliable marker of TAFs in HBMSCs was examined by immunofluorescence staining. As shown in Fig. 1, clear expression of α-SMA was observed in HBMSCs adjacent to colon cancer cells, indicating that direct cell–cell contacts between colon cancer cells and HBMSCs can promote the differentiation of HBMSCs into TAFs.

To further support the notion above, the parallel experiments were performed with murine bone marrow-derived mesenchymal stem cells (BMMSCs). Consistent with observations from HBMSCs, BMMSCs in co-culture were also found to display strongly positive α-SMA staining at sites of cell contact with human colon cancer cells (Supplementary Fig. 1).

3.2. Notch and Smad-dependent signaling pathways are activated in the co-culture system

The Notch signaling pathway is known as an evolutionarily ancient cell–cell interaction mechanism [18]. As a primary candidate, Notch signaling molecules were examined to investigate whether signaling through Notch receptors could control the induced differentiation of HBMSCs to TAFs by direct contacts with colon cancer cells. We first co-cultured HBMSCs with GFP-DLD1 and GFP-SW480 respectively. After culturing for 3 days, the ratio of the two cell types in co-cultures was determined by flow cytometry. Next, corresponding colon cancer cells and HBMSCs cultured separately at the same time, were mixed to the final cell ratio as in the co-culture system and used for control groups. Quantitative RT-PCR analysis showed that both the direct co-cultured groups resulted in dramatically increased mRNA expression of the downstream Notch effector Hes1 (Fig. 2A).

Extensive studies indicate that α-SMA gene transcription is regulated by the interplay between a variety of signal transduction pathways [19,20]. To address this we performed Western blot