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Original article

Smoothened activates breast cancer stem-like cell and promotes tumorigenesis and metastasis of breast cancer

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ABSTRACT

Smoothened (Smo) is a G protein-coupled receptor protein encoded by the *Smo* gene of the hedgehog signalling pathway, which is thought to play an important role in maintaining organ patterning, cell differentiation and self-renewal. The possible role of Smo in the process of tumorigenesis and metastasis of breast cancer still remains unclear. The present experiments were to investigate the effect of Smo on activating breast cancer stem-like $CD44^+CD24^-$ cells and the tumorigenesis and metastasis of breast cancer. By injected $CD44^+CD24^-$ cells (1×10^4) into the cleared fat pad of NOD/SCID mice, it was observed that $CD44^+CD24^-$ cells possess higher tumor-initiating capacity and metastasis properties than equal numbers of non-$CD44^+CD24^-$ cells. The mRNA and protein expressions of Smo in $CD44^+CD24^-$ cells were higher than those in non-$CD44^+CD24^-$ cells, indicating that Smo may play a role in maintaining breast cancer stem cell features. qRT–PCR results revealed that expressions of STAT3, Bcl-2 and cyclinD1 mRNA in MCF-7 cells were decreased after transfected by Smo siRNA. In addition, the expressions of MMP-2 and MMP-9 were downregulated in MCF-7 cells after Smo expression was inhibited. Smo inhibition may be a possible therapeutic target that potentially suppresses breast tumor formation and development.

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1. Introduction

Breast carcinoma is the leading female malignancy and the leading cause of death in women all over the world [1]. Although the early detection and treatments has significantly reduced the breast cancer mortality over the past decades, the incidence rate of breast cancer was increasing over the period in China. The breast cancer stem-like cells as $CD44^+CD24^{-/low}$ cells can initiate tumor formation when a few hundreds cells were injected into the mammary fat pad of NOD/SCID mice [2]. These cells possess the characteristics of cancer stem cells (CSCs), such as the potential of self-renewal and multilineage differentiation. The mechanisms whereby the neoplastic cells invade and destroy tissues are poorly understood, but emerging evidence suggests that these cells are responsible for breast cancer initiation, proliferation, drug resistance and relapses [3,4].

The Hedgehog (Hh) signalling pathway plays a crucial role in vertebrate embryogenesis by controlling cell fate, proliferation, and differentiation by sequentially activating downstream target genes. Hh signal transduction is initiated by the binding of the processed and lipid modified Hh ligand to its receptor Patched (Ptch), a 12-pass transmembrane protein. In the absence of the Hh protein, Ptch1 represses signal transduction by inhibiting the seven transmembrane domain containing protein, Smoothened (Smo) [5]. Upon Hh binding, the inhibitory function of Ptch1 on Smo is abolished, resulting in Smo activation and thereby sequential activating downstream target genes. The activation of Hh signalling has been identified in a variety of human tumors, including tumors of brain, lung, pancreatic, digestive tract, liver, ovarian and skin [6–12]. Moreover, many investigations have

Abbreviations: ALDH1A1, Aldehyde dehydrogenase isoform 1, member A1; NOD/SCID, Non-obese diabetes/severe combined immunodeficiency; Oct-4, Octamer-binding transcription factor 4; Bcl-2, B-cell lymphoma 2.

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showed that self-renewal and proliferation of cancer stem cells are associated with the activity of the Hh pathway [13–15].

It has been reported that the expression of constitutively activated Smo is related to the incidence of many kinds of carcinomas [16]. In human breast cancer, the expression of Smo is altered in clinical samples of human breast cancers, as well as in breast cancer cell lines [17–19], activated Smo leads to increased proliferation, altered differentiation, and ductal dysplasias, while cyclopamine, an antagonists of Smo, can inhibit breast cancer growth in vitro [20]. In addition, the metastatic tumors in some advanced basal cell carcinoma patients who were treated with Smo antagonist were inhibited [21], implicating that Hh signalling may play a role in facilitating the maintenance and movement of tumor cells. These results suggest that stimulation of Hh signalling in the mammary gland may be sufficient to induce breast cancer, but that additional molecular mechanisms are required to be explored.

In this study, we were to investigate the role of Smo in maintaining breast cancer stem-like cell and explore the potential molecular mechanism of Smo in breast cancer cell proliferation and invasion.

2. Materials and methods

2.1. Cell culture and tumor spheroid formation

The breast cancer MCF-7 cell line was purchased from KeyGEN Biotech (China), all cells were incubated in DMEM/F12 supplemented with 10% fetal bovine serum (Invitrogen, USA), penicillin (100 U/mL), streptomycin sulfate (100 μg/mL), and maintained at 37 °C in an atmosphere of 5% CO_2–95% air. For the tumorspheres forming, a single-cell suspension from trypsinization was cultured in ultra-low cluster 24-well plates (Corning, USA) with DMEM/F12 in the presence of 20 ng/mL EGF, 20 ng/mL bFGF and 2 mg/mL B-27 (Invitrogen, USA) without serum at a density of 4000 cells/well. After tumorspheres had formed, tumorspheres were collected and dissociated with Accutase (Sigma, USA) for 5 min to obtain single-cell suspensions. The single cells were replated on fresh ultra-low attachment plate to form secondary tumorspheres. The secondary tumorspheres were dissociated into single cell for $CD44^+CD24^-$ cell sorting.

2.2. Flow cytometry sorting

Dissociated cells were counted and resuspended in PBS at 1×10^6 cell/100 μL. Then, cells were incubated with PE anti-human CD44 antibody or FITC anti-human CD24 antibody (Biolegend, USA) at 4 °C for 30 min on ice in dark. The respective isotype control antibodies were used at the same concentrations according to the manufacturer's instructions. After washing twice with PBS, samples were resuspended in 500 μL PBS and analyzed on a flow cytometer (FACSAria II, USA). Isolated $CD44^+CD24^-$ cells or non-$CD44^+CD24^-$ cells were collected for mouse injection or assessing genes expression.

2.3. Animal experiments

The $CD44^+CD24^-$ cells or non-$CD44^+CD24^-$ cells were mixed with a collagen gel in a 1:1 volume, and 5–6-week-old NOD/SCID mice ($n = 4$/group) were randomly assigned to receive either $CD44^+CD24^-$ cells, non-$CD44^+CD24^-$ cells or an equal volume of collagen gel as control. The $CD44^+CD24^-$ cells or non-$CD44^+CD24^-$ cells (1×10^4) were respectively injected into the cleared fat pad of NOD/SCID mice, without cultivation in vitro. All mice were examined every other day for morbidity and for tumor growth (measured using a digital caliper). When the largest axis of the tumors in the $CD44^+CD24^-$ mice reached 1 cm, all mice were necropsied to determine possible gross metastases, and major organs removed and stored in 4% paraformaldehyde before processing for histopathology. All experiments using mice were approved by the Institutional Animal Care and Use Committee at Dalian Medical University. All efforts were made to minimize animal suffering to reduce the number of animals used.

2.4. RNA extraction and real-time reverse transcriptase-PCR

Total RNA was isolated using Trizol (Invitrogen, USA) according to the manufacturer's specification. Purified RNA was oligo(dT) primed and cDNA was synthesized at 42 °C with PrimeScript™ RT reagent Kit (TaKaRa, JPN). For mRNA analysis, real-time PCR was performed using stratagene Mx 3005P (Agilent Technologies, USA). Expression levels of Smo, Oct-4, ALDH1A1, STAT3, Bcl-2, cyclinD1, MMP-2 and MMP-9 were analyzed. For PCR amplification, the following primers were used: human Smo (forward 5′-CTTTGTCATCGTGTACTACGCC, reverse 5′-CGAGAGAGGCTGGTAGGTG), human Oct-4 (forward 5′-GGAGATATAGCAAAGCAGAAACC, reverse 5′-CTCAAAATCCTCTCGTTATGC), human ALDH1A1 (forward 5′-CCATAACAATCTCCTCTGCTCTG, reverse 5′-TCTCCCAGTTCTCTTCCATTTC), human STAT3 (forward 5′-CTACAGTGACAGCTTCCCAATG, reverse 5′-TTGGCTTCTCAAGATACCTACT), human Bcl-2 (forward 5′-ATGTATGTGGAGAGCATCAAC, reverse 5′-AGAGACAGCCAGGAGAAATCAAAC), human cyclinD1 (forward 5′-CGGAGGAGAACAAACAGATCAT, reverse 5′-AGGCGGTAGTAGGACAGGAAAT), human MMP-2 (forward 5′-AACTACGATGATGACCGCAAG, reverse 5′-GACAGACGGAAGTTCTTGGTG), human MMP-9 (forward 5′-GAACTTTGACAGCGACAAGAAGT, reverse 5′-AGTGAAGCGGTACATAGGGTACA), human GAPDH (forward 5′-GAAGGTGAAGGTCGGAGTCA, reverse 5′-AATGAAGGGGTCATTGATGG). Triplicate assays were performed with RNA samples isolated from at least 3 independent experiments. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.5. Transfection of Smo-specific shRNA

Smo-specific siRNA (target sequence: GCTACAAGAACTACCGATACC) was purchased from Shanghai Genepharma Co., Ltd (China). Transfection in MCF-7 cells was performed using Lipofectamine2000 (Invitrogen, USA) following the manufacturer's instructions [the ratio of plasmid (μg) to lipid (μL) was 1:3]. After 48 h, the transfected cells were collected for verification of Smo knockdown and analysis of knockdown effects.

2.6. Western blotting

Smo protein in breast cancer cells was detected as described previously [16]. For western blot analysis, whole cell lysates were mixed with direct lysis buffer and separated in a 10% SDS-PAGE. The protein was then transferred onto polyvinylidenedifluoride membranes, blots were incubated with anti-Smo (ab72130, Abcam, USA) diluted 1:200 at 4 °C overnight. Membranes were incubated with a secondary antibody, HRP-labelled goat anti-mouse/rabbit antibody (Santa Cruz, USA) diluted 1:2000. The bands were visualized using the enhanced chemiluminescence (ECL) system (Santa Cruz, USA).

2.7. Statistical analyses

The data were statistically analyzed using the SPSS software version 13.0. The data are presented as means ± SEM. The differences between the mean values of two groups were evaluated using the Student's *t*-test (unpaired comparison). For comparison of more than three groups, we used one-way analysis of variance (ANOVA) test followed by Tukey's multiple comparison. *P* values of < 0.05 were considered statistically significant.

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