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GRP78 secreted by tumor cells stimulates differentiation of bone marrow mesenchymal stem cells to cancer-associated fibroblasts

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ABSTRACT

Cancer-associated fibroblasts (CAFs), one type of tumor-associated stromal cells, have been shown to provide a favorable environment for the malignant tumor progression. Extensive reports have demonstrated that mesenchymal stem cells (MSCs) can function as precursors for CAFs. However, the mechanisms by which tumor cells induce the transition of MSCs to CAFs have not been well established. GRP78, traditionally known as an endoplasmic reticulum (ER) chaperone, has been identified to overexpress in a variety of tumor entities and be involved in promoting survival and chemoresistance of tumor cells. Here, we interrogated the role of GRP78 in the generation of CAFs from MSCs. The results showed that GRP78 treatment induced expression of α-smooth muscle actin (α-SMA), a marker for CAFs, in human bone marrow mesenchymal stem cells (HBMSCs) as well as murine bone marrow mesenchymal stem cells (BMMSCs). This phenomenon was correlated with the stimulated phosphorylation of Smad2/3. Furthermore, the GRP78-induced α-SMA expression in HBMSCs was obviously attenuated by SB431542, a TGF-β type I receptor kinase inhibitor. Taken together, the present data suggested that tumor-derived secreted GRP78 elicited the differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) to CAFs through activating TGF-β/Smad signaling pathway, which may represent a novel mechanism for transition of BMSCs to CAFs and a hitherto unknown function of GRP78 in the tumor microenvironment.

1. Introduction

Solid tumors are composed of tumor parenchyma and stroma [1–3]. The tumor-associated stroma, encompassing a variety of stromal cells and their extracellular matrix [4–6], has been implicated in modulating the tumor progression through cell–cell, cell–ECM, cell-soluble factors interactions. Stromal activated fibroblasts, which are often termed as cancer-associated fibroblasts (CAFs), are the most prominent cell type within the tumor stroma of many cancers [4,7,8]. A wide range of evidence indicates that CAFs are potent to secrete increased levels of stromal cell-derived factor 1 (SDF-1), which is responsible for enhanced tumor growth and neoangiogenesis [3,9].

Mesenchymal stem cells (MSCs) are a population of multipotent progenitor cells that possess the ability to self-replicate *in vitro* and differentiate into diverse mesenchymal cells, including osteoblasts, chondrocytes, adipocytes, and myogenic lineages [10,11]. MSCs can be isolated from many types of adult and fetal tissues, like bone marrow, adipose tissue, umbilical cord blood, placenta and amniotic fluid [12]. Specially, MSCs exhibit marked tropism for inflamed or damaged tissues as well as tumor sites linked to wounds that never heal. When recruited to sites of tissue injury, MSCs give rise to connective tissue that facilitates wound healing [13–15]. As in wounds, MSCs incorporated into primary and metastatic tumors differentiate into CAFs, affecting tumor cell survival and angiogenesis [6,16–18]. While it is widely accepted that MSCs-derived CAFs contribute to tumor development, the molecular mechanism underlying transition of MSCs to CAFs is still poorly understood.

Glucose-regulated protein 78 (GRP78), also known as the immunoglobulin heavy chain binding protein (BiP), is a member

Abbreviations: CAFs, cancer-associated fibroblasts; MSCs, mesenchymal stem cells; ER, endoplasmic reticulum; α-SMA, α-smooth muscle actin; HBMSCs, human bone marrow mesenchymal stem cells; BMMSCs, murine bone marrow mesenchymal stem cells; SDF-1, stromal cell-derived factor 1; GRP78, glucose-regulated protein 78; BiP, immunoglobulin heavy chain binding protein; FBS, fetal bovine serum; IPTG, isopropyl-β-D-thiogalactoside; PBS, phosphate-buffered saline; ECL kit, enhanced chemiluminescence kit; HRP-tagged secondary antibodies, horseradish peroxidase-tagged secondary antibodies; cDNA, complementary DNA; SE, standard error; *p* values, probability values; TCM, tumor-conditioned medium; BMSCs, bone marrow-derived mesenchymal stem cells.

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of the HSP70 protein family, which has been well established as a endoplasmic reticulum (ER) chaperone and regulator of ER stress signaling [19,20]. As a multifunctional protein, GRP78 is implicated in proper protein folding and assembly, targeting misfolded protein for degradation, maintenance of Ca^{2+} homeostasis, and controlling the activation of the ER transmembrane sensor proteins [21]. Recent evidences show that in addition to its main location in ER, GRP78 is also found to exist in cell plasma membrane, cytoplasm, mitochondria, nucleus as well as cellular secretions of a variety of tumor cells [22,23]. The relocalization and overexpression of GRP78 in tumor cells is reported to be induced by ER stress from the microenvironment factors, such as glucose deprivation, hypoxia and acidosis [23]. While an array of studies has established that induced GRP78 contributes to tumor growth and confers drug resistance to cancer cells [24–26], little is elaborated about the role of GRP78 in tumor microenvironment. In this study, we aimed to investigate the possible role of secreted GRP78 in differentiation of BMSCs to CAFs.

2. Materials and methods

2.1. Cell isolation and culture

Murine bone marrow mesenchymal stem cells were isolated from femurs and tibias of 1-month-old Sprague–Dawley mice. Cells were seeded in culture dishes with complete medium constituted of IMDM (Gibco, Grand Island, NY, USA), 10% fetal bovine serum (Gibico), and penicillin/streptomycin (50 U/ml and 50 mg/ml, respectively; Gibco-Invitrogen, Carlsbad, USA). Cultures were incubated at 37 °C with 5% fully humidified CO_2. After 24 h, non-adherent cells were removed by washing with 1× PBS, and fresh medium was added every 3–4 days for a period of 4 weeks. When the culture was near 70–80% confluent, the monolayer cells were trypsinized and expanded in larger flasks. Cells were passaged twice prior to use.

Human bone marrow mesenchymal stem cells were purchased from Chinese Biowit Technologies and cultured in HBMSC-GM (Biowit, China). Human colon cancer DLD1 and SW480 cells were obtained from the Chinese Type Culture Collection and grown in RPMI-1640 (Gibico, Grand Island, NY, USA) containing 1 × penicillin/streptomycin and 10% fetal bovine serum (FBS, Gibico). DLD1 cells were stably transduced using a lentivirus expressing GRP78 protein.

2.2. Exposure of HBMSCs to tumor cell conditioned medium

SW480 and GRP78-overexpressed DLD1 were grown in RPMI-1640 + 10% FBS culture medium and when the cells were cultured to 50% confluency, they were switched to a serum free growth medium. After 24 h, the medium was harvested and centrifuged at 2000 rpm for 10 min. The supernatant was filtered using Millipore sterile 50 ml filtration system with 0.22 μm PVDF membrane. HBMSCs were conditioned with indicated concentration of tumor conditioned medium for 5 days.

2.3. Identification of GRP78 in supernatants of colon cancer cell lines

SW480 and DLD1 cells were cultured with serum-free RPMI-1640 for 24 h. Supernatants from the cultures were collected and centrifuged at 2000 rpm for 10 min to remove cell debris. And the clarified supernatant was concentrated by ultrafiltration through a 30-kDa MWCO hollow fiber membrane (Millipore) at 3800 rpm. 45 μg of total protein were used for Western blotting detection of GRP78.

2.4. Expression and purification of GST-GRP78

The recombinant pGEX-4T-2-GRP78 plasmid was transformed into host cells *Escherichia coli* DH5α. The expression of GRP78 protein was induced using 1.0 mM isopropyl-β-D-thiogalactoside (IPTG) at 30 °C for 4 h. The induced cells were collected at 11,000 rpm at 4 °C for 10 min and the soluble GST-tagged fusion protein was further purified using GST affinity column.

2.5. Western blot

After appropriate treatment, cells were scraped in ice-cold phosphate-buffered saline (PBS), spun down at 1000 rpm for 5 min, and incubated with lysis buffer (20 mM Tris–HCl, 1 mM EGTA, 1 mM EDTA, 10 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 30 mM sodium pyrophosphate, 25 mM β-glycerol phosphate, 1% Triton X-100, pH 7.4) for 30 min on ice. The cell lysates were then centrifuged at 13,000*g* at 4 °C to remove cell debris. 80 μg of the obtained supernatant proteins were subjected to a 12% polyacrylamide gel, and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). After blocking with 5% nonfat milk in TBST (10 mM Tris, 150 mM NaCl and 0.1% Tween 20) for 1 h at RT, the membrane was incubated with primary antibodies overnight at 4 °C. The labeled proteins of interest were visualized by exposing the blots to X-ray film in a dark room using an enhanced chemiluminescence (ECL) kit (Sigma), followed by incubation with horseradish peroxidase (HRP)-tagged secondary antibodies (Sigma) for 2 h at RT. Antibodies used for western were the following: a-SMA (1:1000 dilution; Biotime, China), β-catenin (1:1000 dilution; Abmart, Shanghai, China), Phospho-AKT (1:500 dilution; Bioworld, Nanjing, China), Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) (1:1000 dilution; Cell Signaling), Smad2/3 (1:500 dilution; Bioworld, Nanjing, China), GAPDH (1:5000 dilution; Abmart, Shanghai, China), secondary anti-mouse and anti-rabbit antibodies (1:1000 dilution; Invitrogen, Carlsbad, CA).

2.6. Quantitative RT-PCR

Total RNA was extracted from the cells using Trizol reagent (Takara, Shiga, Japan). 0.5 μg of RNA was used for the synthesis of complementary DNA (cDNA) with PrimeScript RT Master Mix (Takara). All the qRT-PCR samples were performed using SYBR Green PCR Master Mix (Takara) on an Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA). GAPDH served as an endogenous control. The relative expression of each targeted gene was normalized by subtracting the corresponding GAPDH threshold cycle (Ct) values using the ΔΔCt comparative method. α-SMA sense primer sequence: 5′-AGCGTGGCTATTCCTTCGT-3′. α-SMA antisense primer sequence: 5′-CCATCAGGCAACTCGTAACTC-3′.

2.7. 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. Tumor-derived GRP78 induce expression of a cancer-associated fibroblast marker in HBMSCs

Mishra et al. have documented that HBMSCs could assume a CAF-like phenotype and functional properties by prolonged

exposure to tumor-conditioned medium (TCM) [27]. To investigate whether TCM from colon cancer cells can also induce HBMSCs to express CAF markers, HBMSCs were either mock treated or exposed to TCM from human colon carcinoma SW480 cells. Then we assayed the differentiation ability of HBMSCs towards CAFs by the expression of α-SMA, the most reliable marker of CAFs. As showed in Fig. 1A, TCM increased the expression level of α-SMA in HBMSCs in a dose-dependently manner. In our previous studies (unpublished data), we have demonstrated that various colon cancer cell lines were able to release large amount of GRP78 into the tumor microenvironment. The similar results were identified with SW480 and DLD1 cells (Fig. 1B). To further determine whether GRP78 in tumor cell secretions also contributed to induced CAF differentiation of HBMSCs, HBMSCs were treated with TCM, which was from GRP78-overexpressed colon cancer cells DLD1 for 5 days. Compared to control group, TCM from GRP78-overexpressed DLD1 treatment resulted in upregulated α-SMA protein expression in HBMSCs (Fig. 1C).

3.2. GRP78 protein treatment stimulates differentiation of BMMSCs to CAFs in vitro

To further test our hypothesis that GRP78 in tumor cell secretions play roles in the generation of CAFs from bone marrow-derived mesenchymal stem cells (BMSCs), we firstly constructed recombinant plasmid pGEX-4T-2-GRP78 and obtained the fused GST-GRP78 protein in *E. coli* system (Fig. 2A). Then, BMMSCs were treated with various concentrations of purified GST-GRP78 fusion protein. Western blotting analysis showed that addition of 1 μM GST-GRP78 protein to BMMSCs cultured in 5% fetal bovine serum medium resulted in markedly promoted α-SMA expression on day 9, compared with control GST protein-treated cells (Fig. 2B and C). In agreement with the Western blotting results, α-SMA mRNA expression of BMMSCs in response to the conditions mentioned above, was dramatically elevated (Fig. 2D). Collectively, these results indicate that BMMSCs treated with GST-GRP78 fusion protein undergo CAF differentiation *in vitro*.

3.3. GRP78 protein treatment induces differentiation of HBMSCs to CAFs in vitro

In order to confirm the activation of BMMSCs by GST-GRP78 fusion protein exposure was not species specificity, we also examined the effects of GST-GRP78 on HBMSCs. As expected, the induced expression of α-SMA protein in HBMSCs occurred on day 3 and it was maximally increased on day 6 (Fig. 3A). Results of quantitative RT-PCR analysis of α-SMA were identical with that from Western blots (Fig. 3B). Taken together, all these results clearly imply that tumor-derived secreted GRP78 can function in communication between tumor cells and circulating bone marrow-derived mesenchymal cells, and switch BMSCs to cancer-associated fibroblasts in the neoplastic tumor microenvironment.

3.4. GRP78 trigger differentiation of HBMSCs to CAFs through TGF-β/Smad pathway

To clarify the specific molecular mechanism responsible for GRP78-induced transition of BMSCs to CAFs, we evaluated herein the activation of pathways, which have been reported to be involved in cell differentiation processes. We performed Western blot analysis against β-catenin, the phosphorylated form of AKT and SMAD2/3, indicators of Wnt, PI3K/AKT and Smad pathway activation, respectively. The results showed in Fig. 4A that the phosphorylated form of SMAD2/3 was prominently increased in HBMSCs following GST-GRP78 induction, in contrast to no obvious activation of the other two pathways. Moreover, HBMSCs exposed to TCM from GRP78-overexpressed DLD1 showed the same pattern as presented in HBMSCs treated with GST-GRP78 fusion protein (Fig. 4B). These data suggested that GRP78 protein specifically activates Smad2/3 in HBMSCs.

Smad-dependent pathway has been known as a TGF-β receptor-mediated downstream signaling pathway. A growing body of findings also reported that TGF-β from tumor cells are important regulators involved in the differentiation of stromal cell types to myofibroblasts, as evidenced by increased α-SMA expression. In this regards, we then analyzed the expression of α-SMA in HBMSCs after exposure to GST-GRP78 along with SB431542, a TGF-β type I receptor inhibitor. The results showed that when TGF-β receptor-mediated signaling was blocked, GRP78-induced α-SMA protein expression, as well as its expression at transcriptional level, was strongly reversed (Fig. 4C and D). In summary, it demonstrated that GRP78 mediated BMSCs–CAFs differentiation through the activation of TGF-β/Smad signaling pathway.

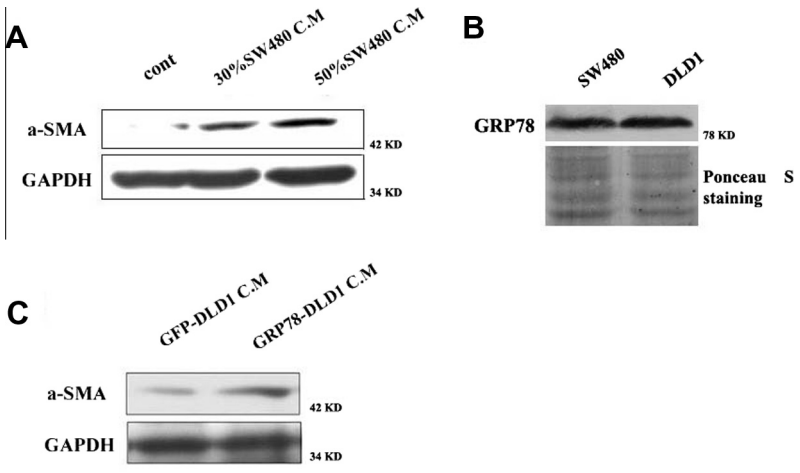

Fig. 1. Tumor-derived GRP78 stimulates the expression of a-SMA in HBMSCs. (A) Increased expression of α-SMA was observed in HBMSCs upon prolonged exposure to conditioned medium from human colon cancer cell line SW480. HBMSCs were treated with indicated concentrations of C. M from SW480 for 10 days. The expression level of α-SMA was determined by Western-blot. (B) Detection of GRP78 in secretions of human colon cancer cell lines SW480 and DLD1. (C) HBMSCs had upregulations of α-SMA expression following treatment with C. M from GRP78 over-expressed human colon cancer cell line DLD1 for periods up to 7 days.

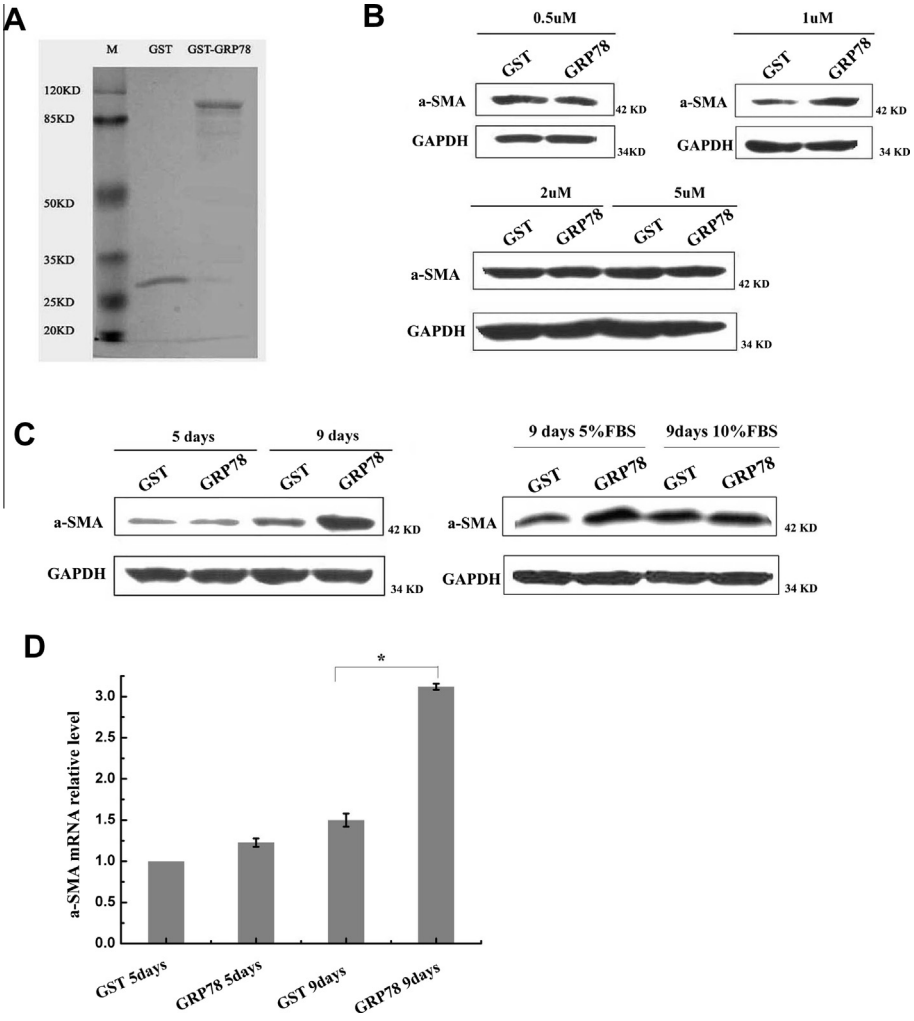

Fig. 2. Murine bone marrow mesenchymal stem cells (BMMSCs) have increased expression of α-SMA under the influence of GST-GRP78. (A) SDS–PAGE analysis on the purified GST and GST-GRP78 recombinant protein. Lane M: protein molecular weight marker. (B) BMMSCs were incubated with 0.5, 1, 2 or 5 μM of GST-GRP78 fusion protein, respectively. As shown by Western blot, the GRP78-induced expression of α-SMA was detected in BMMSCs exposed to 1 μM of GST-GRP78. (C) Western blotting analysis of the α-SMA protein level in BMMSCs cultured in different concentrations of FBS supplemented medium with 1 μM of GST-GRP78 at different time points. (D) α-SMA expression in BMMSCs was elevated at transcriptional level on day 9 after treatment with 5% FBS-medium containing 1 uM GST-GRP78 protein. $^{*}p < 0.05$ versus control group.

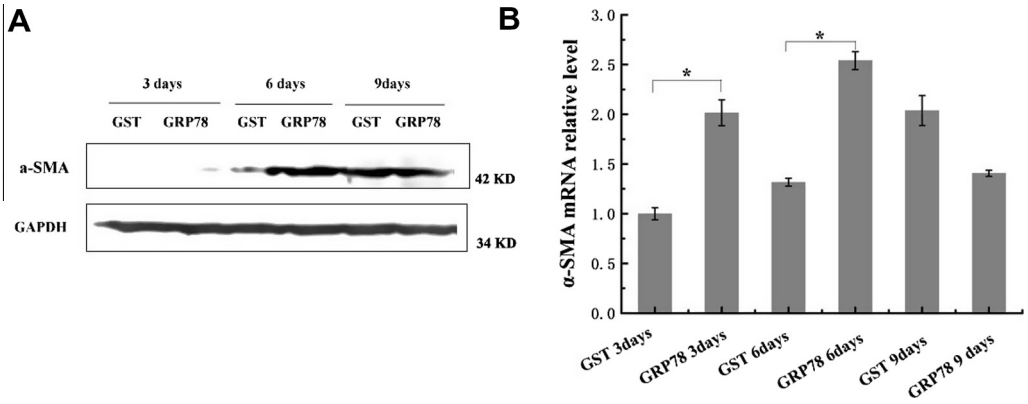

Fig. 3. Human bone marrow mesenchymal stem cells (HBMSCs) treated with GST-GRP78 fusion protein exhibited higher expression of α-SMA. (A) Western blotting analysis of α-SMA expression in HBMSCs by treatment with GST-GRP78 (1 μM) for different time periods. (B) Quantitative analysis of the a-SMA mRNA expression in HBMSCs exposed to GST-GRP78 (1 μM) for different time periods. $^{*}p < 0.05$ versus control group.

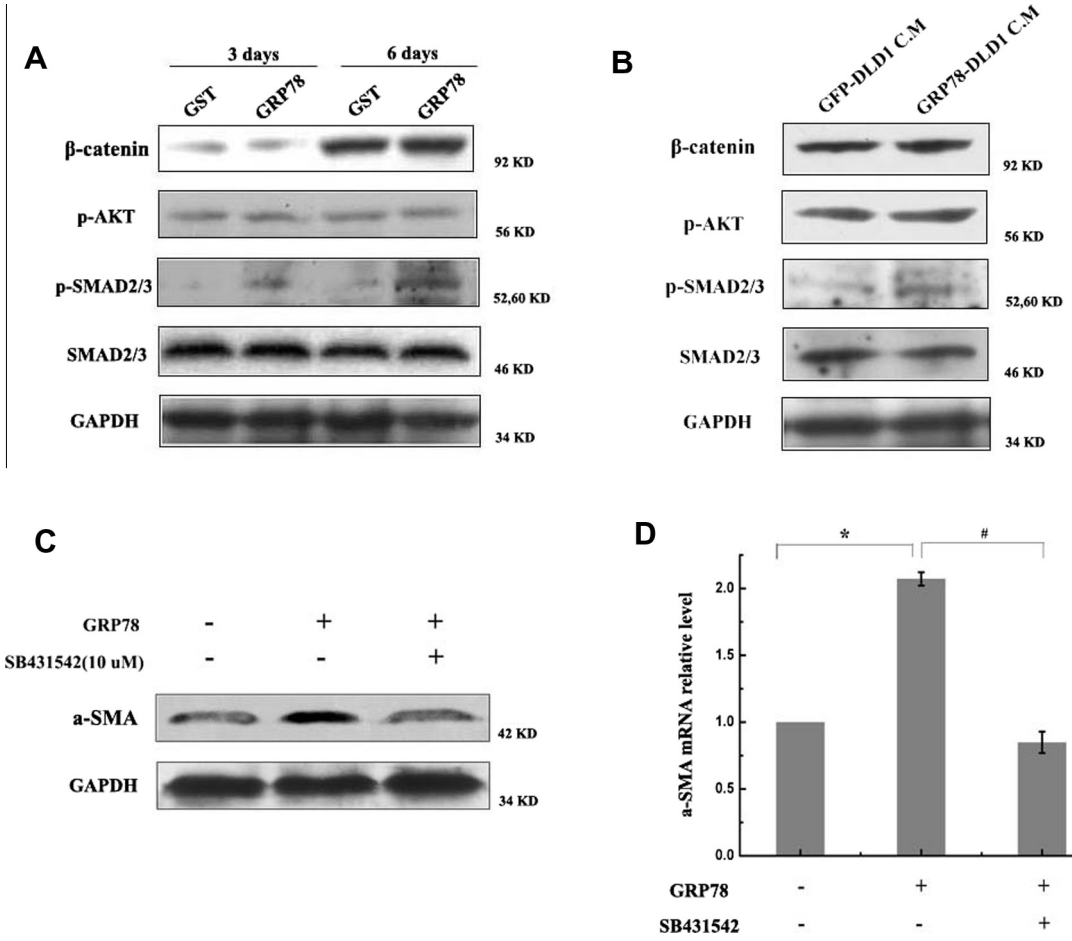

Fig. 4. GRP78-induced α-SMA expression in HBMSCs is dependent on TGF-β/Smad signaling pathway. (A) HBMSCs were treated with GST-GRP78 fusion protein (1 μM). The levels of β-catenin, p-AKT and p-Smad2/3 were analyzed by Western blot. (B) HBMSCs were treated with C. M from GRP78-over expressed DLD1. The levels of β-catenin, p-AKT and p-Smad2/3 were measured by Western blot. (C) HBMSCs were exposed to GST-GRP78 fusion protein in the presence of 10 μM SB431542 for 3 days. The expression of α-SMA protein was assessed by Western blot. (D) Quantitative analysis of the α-SMA mRNA expression in HBMSCs incubated with GST-GRP78 in combination with 10 μM SB431542. $^{*}p < 0.05$ versus control group. $^{\#}p < 0.05$ versus GRP78 group.

4. Discussion

The significant role of reactive tumor stroma in tumorigenesis and tumor progression is increasingly appreciated. Accumulating evidences support the view that cancer-associated fibroblasts are essential components among diverse tumor stromal cells and play pivotal roles in the progression and malignancy of tumor cells [28–30]. To date, a serious of relevant studies have explored the origins of cancer-associated fibroblasts, including local tissue fibroblasts, endothelial cells, transdifferentiating epithelial cells or bone marrow-derived progenitor cells from the circulation [2,7,31,32]. While extensive observations have shed light on the origin and functions of CAFs, the possible mechanisms underlying transdifferentiation of BMSCs to cancer-associated fibroblasts remain poorly elucidated. An important recent study proved that under long-term exposure to tumor conditioned medium, HBMSCs exerted CAFs differentiation, characterized by higher expression of CAFs-like proteins [2]. In agreement with previously published data, the present result here showed that exposure of colon cancer cells-conditioned medium also induced expression of α-SMA, the most reliable hallmarker of CAFs, in HBMSCs by 10 days. Besides, we also demonstrated that 30% or 50% conditioned medium from colon cancer cell lines was sufficient to induce differentiation of HBMSCs to CAFs. Whereas full TCM may augment the differentiation process, the result was not addressed in our study owing to inadaptation of HMSCs to a long incubation with TCM *in vitro*. From our data, we can conclude that BMSCs are also an attractive candidate for CAFs in tumor stroma of colorectal carcinoma, and some soluble factors secreted by colon cancer cells positively modulate the differentiation of BMSCs to CAFs.

While there are several reports that have identified key tumor-derived factors contributing to the formation of active tumor stroma [32–36], such information is still relatively scanty in terms of a better understanding of the interplay between bone marrow derived cell and the tumor cells within the tumor microenvironment. In this study, we mainly focus on GRP78, a resident endoplasmic reticulum molecular chaperone protein, which is also reported to be highly induced in tumor cells [22,23]. Multifarious stress conditions in the tumor microenvironment also promote relocalization of GRP78 from ER to cytosol, mitochondria, nucleus, cell surface as well as cell secretions of tumor cells. Cell-surface GRP78 has emerged as a regulator for tumor survival and a prime target for anti-cancer therapy [37–39]. With regard to GRP78 in tumor secretions, its role in a multitude of biological processes is only described in a limited number of literatures. In a study concerning the antiangiogenic effect of bortezomib, it was proposed that bortezomib increased secretion of GRP78 by tumor cells, which subsequently bound to cell-surface receptors and activated the ERK and AKT pathways in endothelial cells, thereby resulting in resistance of endothelial cells to bortezomib [40]. However, we have identified a novel biological function of secreted GRP78 in the present study. Our findings showed that conditioned medium from

GRP78-overexpressed colon cancer cell line DLD1, triggered the derivation of CAFs from BMSCs. We further verified the participation of GRP78 in promoting BMSCs–CAFs transition by GST-GRP78 fusion protein treated BMSCs. GST-GRP78 was observed to cause induction of α-SMA expression in both murine and human bone marrow mesenchymal stem cells, implying the possibility that tumor-derived secreted GRP78 is implicated in generation of CAFs in the tumor stroma from bone marrow mesenchymal stem cells.

Additionally, our experiments discovered that GST-GRP78 recombinant protein or TCM from GRP78-overexpressed DLD1 enhanced α-SMA expression in BMSCs accompanied by increased Smad2/3 phosphorylation. Furthermore, the GRP78-induced α-SMA expression was suppressed by pharmacological inhibition of TGF-β receptor with SB431542, a TGF-β type I receptor kinase inhibitor. Taken together, our work provides evidences that tumor-derived secreted GRP78 have the ability to stimulate the transition of BMSCs to CAFs, and the differentiation process is associated with activation of TGF-β/Smad signaling pathway. Our findings implicate a new mechanism by which tumor cells educate the BMSCs to become part of the tumor microenvironment, and thereby contribute to tumor progression. These observations, although preliminary, will be important in developing improved stroma-targeted therapies for cancer.

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