Bone Marrow-Derived Mesenchymal Stem Cells Up-Regulate Acetylcholine Receptor Delta Subunit through NRG/ErbB3-Mediated Mitogen-Activated Protein Kinase Pathway

Li Chen, M.D., Ph.D., Junjian Jiang, M.D., Ph.D., Jianguang Xu, M.D., Ph.D., Yudong Gu, M.D., Ph.D., and Lei Xu, M.D., Ph.D.

Abstract

To investigate the effect of bone marrow-derived mesenchymal stem cells (BMSCs) on the expression of acetylcholine receptor delta subunit (AChRδ), the murine skeletal muscle cell line Sol8 were grown in DMEM with 20% fetal bovine serum added with (conditional medium group) or without (control group) conditional medium of BMSC cells for 48 hours. RT-PCR and Western blot were performed to access the mRNA and protein levels of AChRδ in Sol8 cells, respectively. Western blot was used to detect total and phosphorylated protein levels of Ras, Raf-1, Mek1/2, and Erk1/2, respectively. NRG-1 antibody added in conditional medium of BMSCs, si-ErbB3, and four Ras/Raf/MEK/ERK pathway inhibitors (FTS, Sulindac, U0126, and PD98059) were using to investigate the effect of AChRδ levels. Our studies indicated that expression of AChRδ was significantly enhanced in the conditional medium group when compared with those in control group and phosphorylation of Ras, Raf, Erk1/2 in Sol8 cells was also increased. Although gene silencing for ErbB3 gene, adding of NRG-1 antibody in conditional medium of BMSCs or treatment of Ras/Raf/MEK/ERK pathway inhibitors can down-regulate expression of AChRδ and phosphorylation, which suggesting that the Ras/Raf/MEK/ERK pathway may be involved in BMSCs-induced expression of AChRδ. Clin Trans Sci 2012; Volume 5: 27–31

Keywords: BMSCs, AChRδ, Ras/Raf/MEK/ERK pathway, ErbB3, NRG-1

Introduction

Mesenchymal stem cells (MSCs), which reside within the stromal compartment of bone marrow (BM), were first identified in the pioneering studies of Friedenstein, who isolated bone-forming progenitor cells from rat marrow.[1] Human MSCs that have been isolated from BM, blood, and other adult tissues including adipose tissue have the potential to be useful candidates for therapy. In BM, MSCs serve two functions. One is the classically recognized function of providing a supportive microenvironment for hematopoiesis. The other is related to the development, stabilization, and maintenance of the sinusoidal network,[2] consistent with their subendothelial localization. In the bone/BM organ, the two functions are closely intertwined. Hematopoiesis requires establishment of a sinusoidal network, and hematopoietic stem cells localize to sinusoidal walls[3] in addition to endosteal surfaces.[4] Some striking examples of the therapeutic use of marrow-derived MSCs have been reported recently including cardiovascular repair, treatment of lung fibrosis, spinal cord injury, and bone and cartilage repair.[5] MSCs as an ideal target cell to gene therapy have shown a good prospect of application and extension.[6]

Acetylcholine receptors (AChRs) are among the proteins that become localized to this small patch of the muscle fiber membrane. Studies with transgenic mice have shown that AChR genes are transcribed at a higher rate in myofiber nuclei positioned near the synaptic site than in nuclei in nonsynaptic regions of the myofiber.[7] Research have showed Neuregulins (NRGs) functional receptors are ErbB tyrosine kinase receptors, known as transmembrane tyrosine kinase of epidermal growth factor receptor family members, including ErbB2/HER2/Neu, ErbB3/HERS, and ErbB4/HER4.[8,9]

In this study, we investigate the effect and molecular mechanisms of bone marrow-derived mesenchymal stem cells (BMSCs) on Sol8 cells and our results indicated that the Ras/Raf/MEK/ERK pathway may be involved in BMSCs-induced expression of AChRδ.

Materials and Methods

Chemicals and reagents

Mouse monoclonal anti-AchRδ antibody was purchased from Neo-Markers (Fremont, CA, USA). Anti-Ras, anti-Raf, anti-Mek1/2, anti-Erk1/2, and respective phosphospecific antibodies were purchased from Cell Signaling (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibodies to mouse and rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). All other chemicals were purchased from Sigma or Fisher Scientific (Pittsburgh, PA, USA).

Cell culture

BMSC cells were maintained in Dulbecco's modified Eagle's medium (high glucose) with 10% fetal bovine serum and antibiotic-antimycotic (Sigma) at 37°C under 5% CO_2. The murine skeletal muscle cell line Sol8 were obtained from ATCC (Manassas, VA, USA) and grown in DMEM with 20% fetal bovine serum added with (conditional medium group) or without (control group) conditional medium of BMSC cells for 48 hours. 0, 5, 10, and 15 μg/mL NRG-1 antibody was added in conditional medium of BMSCs, respectively. Four Ras/Raf/MEK/ERK pathway inhibitors (FTS, Sulindac, U0126 and PD98059) were using to investigate the effect of AChRδ levels.

RT-PCR

Total RNA was extracted from Sol8 cells with Trizol reagent, according to the manufacturer's instruction. This was used as the template for cDNA synthesis. Reverse transcription was then carried out by M-MLV (Toyobo, Japan). After amplification, PCR products were electrophoresed in 2% agarose gel to confirm that PCR yielded a single product of the expected size. The GAPDH was used as an internal standard. The primer/probe sets were designed with Primer Express software (Applied Biosystems,

Department of Hand Surgery, Huashan Hospital, Fudan University, Shanghai 200040, China.
Correspondence: Lei Xu (xulei021@126.com)

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Foster City, CA, USA).[15] The primer sequences for the ErbB3 were as follows: forward, 5′-GCCTTTGTTTCCCATCAGAC-3′; reverse 5′-CCCGACTTCCCTTTGTGTAA-3′; and AChRδ forward 5′- CTTGTCTACCACTACGGCTTCG-3′, reverse 5′-GCGGTTCTCCTTGGCATC-3′. GAPDH as a restricted reference materials, forward 5′-CAGTAACCTATCTTACATGA-3′, reverse 5′-CCGAGAAAATTAAAGTCA-3′.

Western blot

The cells were lysed with lysis buffer (137 mm NaCl, 20 mm Tris-HCl [pH 7.5], 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 2mm EDTA [pH 8.0], 3 μg/mL aprotinin, 3 μg/mL leupeptin, 2 mm phenylmethylsulfonyl fluoride, 20 mm NaF, 10 mm sodium pyrophosphate, and 2 mm Na_3VO_4). Equal amounts of proteins were separated by SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) and incubated with a blocking buffer (5% nonfat milk in 20 mm Tris-HCl [pH 7.5], 137 mm NaCl, and 0.1% Tween 20) for 1 hour at room temperature. The membranes were incubated with primary antibodies overnight at 4°C. Washed three times (20 mm Tris-HCl [pH 7.5], 137 mm NaCl, and 0. 1% Tween 20], incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000 to 1:10,000 dilution) for 1 hour at room temperature, washed three times, and then detected with enhanced chemiluminescence.

Plasmid constructs and transfection

The si-ErbB3 vector (si-ErbB3-pSilencer4.1 with neomycin resistance), which produces specific siRNA (si-ErbB3) were used in the experiment. Vectors siRNA-random were used as the control for siRNA-ErbB3. Two sequences: (1) TCATTGTTAATTCAAGAGA, (2) ATTGTTACTTCAAACGGTT were used in the experiment for the hairpin construction of RNAi, and sequence[2] has a stronger silence effect. The day before transfection, about 1×10^6 Sol8 cells were seeded in the media onto a 60 mm dish and incubated for 24 hour. The next day, cells were transfected with Sofast gene transfection reagent kit (Sunma Corp., Xiamen, China) according to the manufacturer's instruction. The efficiency of ErbB3 RNAi in Sol8 cells was evaluated using RT-PCR and Western blot analysis. The transfected cells were collected for the following experiments after 24 hour of incubation.

Results

AChR gene is up-regulated after added with BMSCs conditional medium

Sol8 cell were grown in DMEM with 20% fetal bovine serum with a density of 4×10^5 cells per dish before added with (conditional medium group) or without (control group) conditional medium of BMSC cells were cultured in 35 mm dish for 48 hours. The mRNA (*Figure 1A*) and protein level (*Figure 1B*) of AChRδ was confirmed to obviously up-regulate when compared with those of the control group using RT-PCR and Western blot assay, respectively.

NRG/ErbB activation involved in BMSC-mediated AChRδ regulation

Previous research has showed that nerve-derived and muscle-derived members of the NRG had been observed to stimulate myotube formation and induce AChR receptor expression.[10,11]

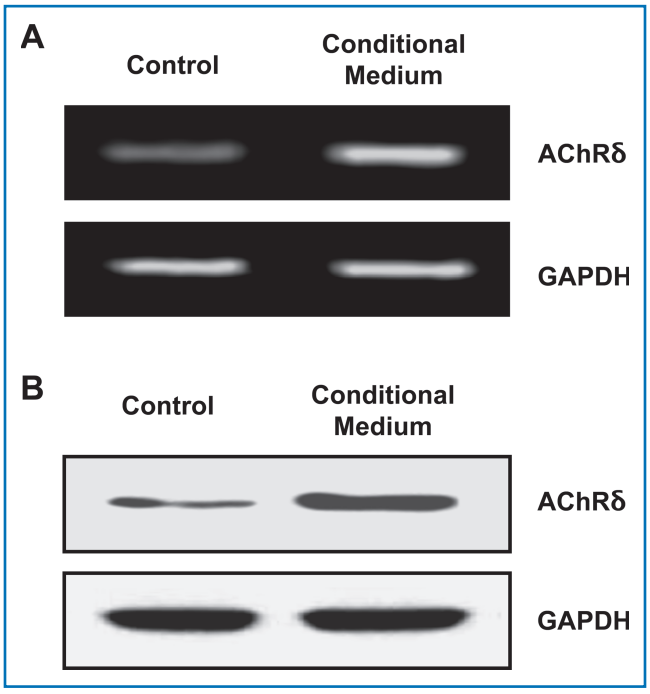

Figure 1. The level of AChRδ mRNA and protein in Sol8 cells is up-regulated when added with conditional medium of BMSCs. Sol8 cells added with (Conditional Medium group) or without (Control group) conditional medium of BMSC cells were cultured in 35 mm dish for 48 hours. The mRNA level of AChRδ was detected using RT-PCR (A). The protein level of AChRδ was detected using Western Blot (B). GAPDH is shown as an internal standard.

Generation of a muscle-specific ErbB2 deletion resulted in mice with impaired motor coordination and poor body condition, and contractile activity elicits NRG/ErbB activation.[4] So it promoted us to explore the signal transduction mechanisms involved.

First, the mRNA and protein levels of NRG-1 in BMSCs and Sol8 cells were detected by RT-PCR and Western Blot, respectively. Our results indicated that the mRNA and protein levels of NRG-1 in BMSCs were significantly more than those in Sol8 cells (*Figure 2A*). ELISA assay showed the protein levels of NRG-1 in BMSCs medium were also more than those in Sol8 cells (*Figure 2B*).

For further study the role of NRG-1 in BMSCs-mediated enhancement of AChRδ expression, Sol8 cells was cultured with the conditional medium of BMSC cells, which added with 0, 5, 10, and 15 μg/mL NRG-1 antibody to neutralize NRG-1 protein. The results showed that the mRNA (*Figure 2C*) and protein levels of AChRδ (*Figure 2D*) were obviously decreased after treatment with NRG-1 antibody when compared with those in control group and the AChRδ mRNA and protein levels decreased in a dose-dependent manner (*Figure 2C* and *D*), which suggested NRG-1 take part in the BMSCs-induced change of AChRδ expression.

NRGs are a family of growth and differentiation factors that are related to epidermal growth factor. The receptors for NRG are the ErbB family of tyrosine kinase transmembrane receptors, including the ErbB2/neu, ErbB3, and ErbB4 receptor. Through interaction with the ErbB receptors, HRG can promote the survival of epithelial cells and induce the expression of VEGF and stimulate angiogenesis. So we further investigated the effect of the receptors for NRG-1, ErbB3 on the change of AChRδ expression.

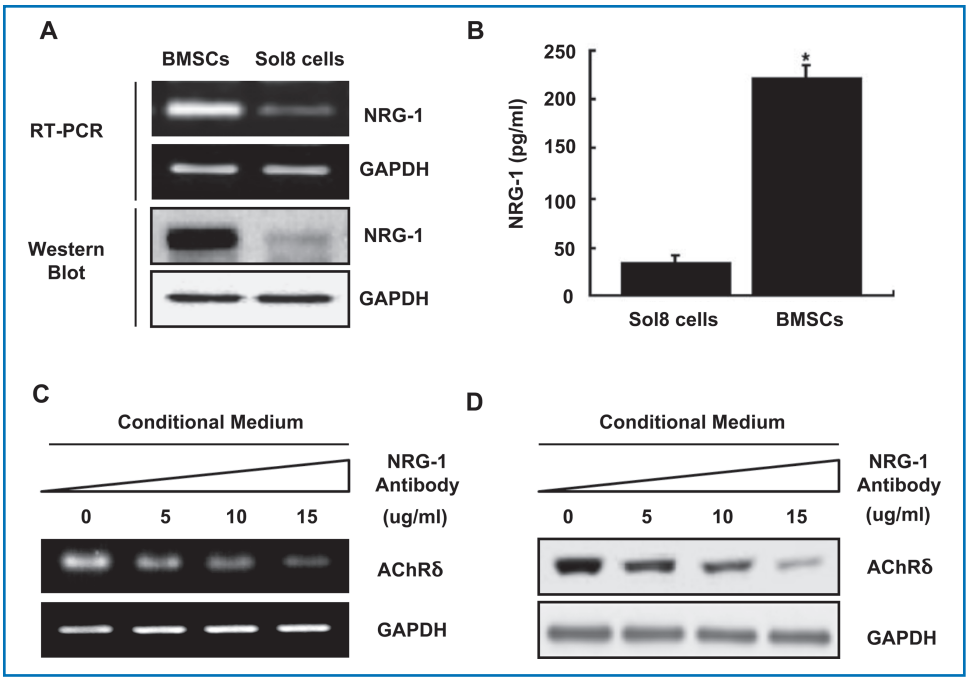

Figure 2. The level of AChRδ mRNA and protein in Sol8 cells is down-regulated after neutralized with NRG-1 antibody in conditional medium of BMSCs. The mRNA and protein levels of NRG-1 in BMSCs and Sol8 cells were detected by RT-PCR and Western Blot, respectively (A). The levels of NRG-1 in conditional medium of BMSC and Sol8 cells were detected by ELISA, respectively (B). The mRNA levels of AChRδ in Sol8 cells were detected by RT-PCR after neutralized with 0, 5, 10, and 15 μg/mL NRG-1 antibodies in conditional medium of BMSCs, respectively (C). The protein levels of AChRδ in Sol8 cells were detected by Western Blot after neutralized with 0, 5, 10, and 15 μg/mL NRG-1 antibodies in conditional medium of BMSCs, respectively (D).

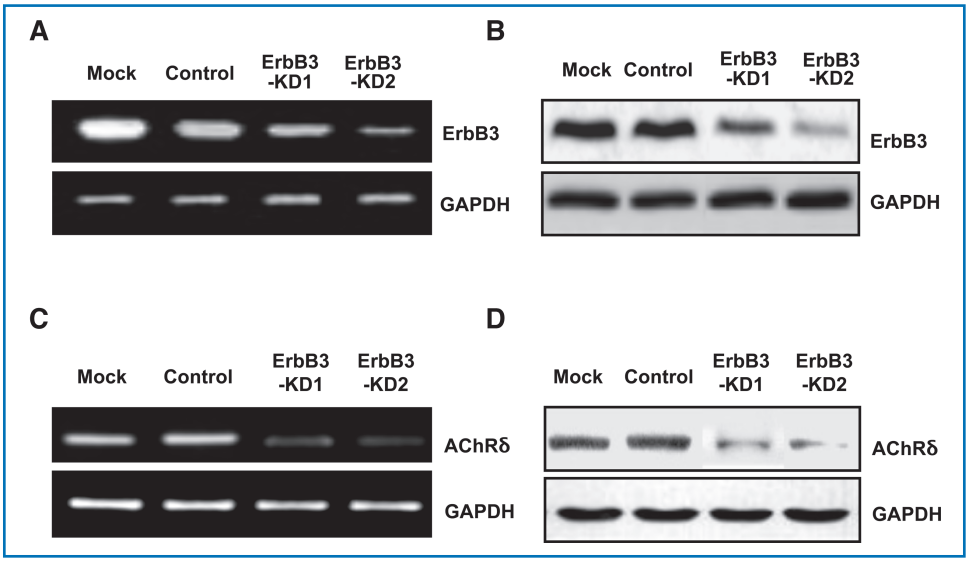

Figure 3. The level of AChRδ mRNA and protein in Sol8 cells is down-regulated after ErbB3 RNAi. The efficiency of ErbB3 RNAi in Sol8 cells was evaluated using RT-PCR (A) and Western blot analysis (B). The mRNA levels of AChRδ in Sol8 cells were detected by RT-PCR after ErbB3 RNAi (C). The protein levels of AChRδ in Sol8 cells were detected by Western Blot after ErbB3 RNAi (D).

To investigate the role of ErbB3 in Sol8 cells we transfected UCH37 siRNA in Sol8 cells that express ErbB3 in a relatively high level. The transfection efficiencies of ErbB3-KD1 and ErbB3-KD2 transfectants were initially evaluated using RT-PCR and Western blot analysis. As shown in *Figure 3A*, the ErbB3 mRNA levels in both ErbB3-KD1 and ErbB3-KD2 siRNA transfectants displayed a significant reduction when compared with their negative controls. Western blot analysis showed the responsive changes of ErbB3 protein levels in corresponding transfectants (*Figure 3B*). In addition, ErbB3-KD2 showed a stronger silence effect when compared with ErbB3-KD1. As shown in *Figure 3C*, the level of AChRδ mRNA in Sol8 cells is down-regulated after ErbB3 RNAi. Western blot analysis showed the responsive changes of AChRδ protein levels after ErbB3 RNAi (*Figure 3D*). Moreover, the stronger silence in ErbB3-KD2 group induced a more decrease of AChRδ mRNA and protein levels in Sol8 cells when compared with those in ErbB3-KD1 group, respectively.

Constitutively active Ras→Raf→MAPK may be involved in BMSCs-induced expression of AChRδ

Because AChRδ gene have been defined as target gene of the Ras/MAPK signaling pathway, we next explored whether the Ras/MAPK signaling pathway was involved in BMSCs-induced expression of AChRδ. Notably, the conditional medium of BMSCs induced Ras, Raf, Mek1/2, and Erk1/2 phosphorylation obviously when compared with those in the control group (*Figure 4*). Using 0, 5, 25, and 50 μM FTS (a Ras inhibitor), 0, 1, 10, and 100 μg/mL Sulindac (an inhibitor of Ras activation of Raf-1), 0, 5, 10, and 15 μM U0126 (a highly selective inhibitor of MEK 1 and MEK 2) or 0, 10, 20 and 40 μg/mL PD98059 (an Erk1/2 inhibitor) to offset phosphorylation of Ras/MAPK signaling pathway, the expression of AChRδ is obviously down-regulated (Figure 5). So the typically Ras→Raf→MAPK signal transduction pathway play an important role BMSCs-induced expression of AChRδ.

Discussion

To the best of our knowledge, this is the first study to report AChRs were up-regulated by conditional medium of BMSCs in Sol8 cells. Our results indicate that NEG-1 mainly was produced, and then stimulation results in activation of a Ras/Raf/MAP kinase signaling cascade.

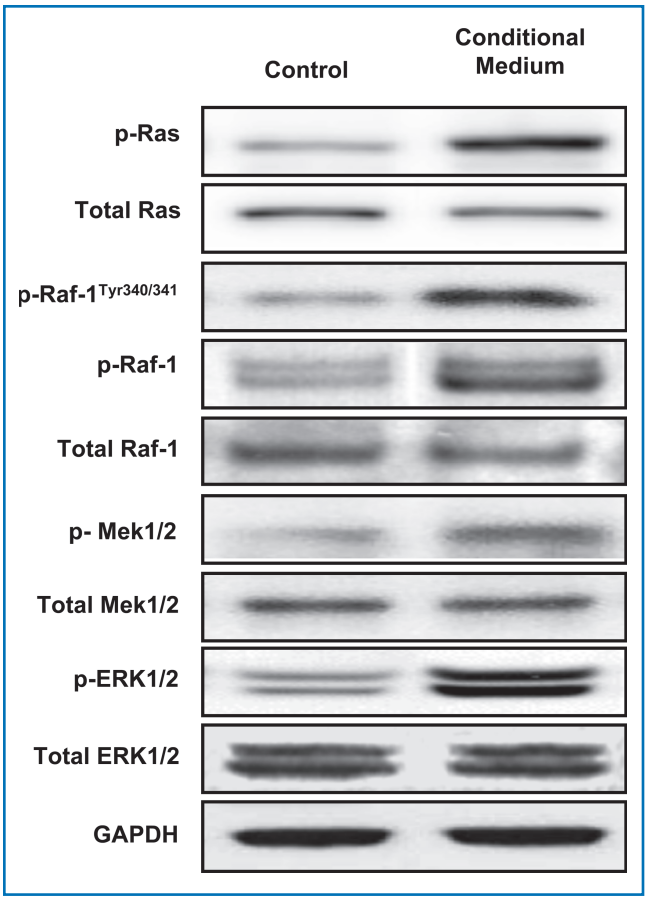

Figure 4. Effect of BMSCs conditional medium on the Ras/Raf/MEK/ERK pathway of Sol8 cells. Western blotting analysis was used to detect the total and phosphorylated form of Ras, Raf-1,Mek1/2, and ERK1/2, respectively. GAPDH is shown as an internal standard.

BMSC isolated from BM, periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle, and deciduous teeth, have two defining characteristics of a stem cell including multipotency and self-renewal. Stem cell therapy involves the transplantation of autologous or allogeneic stem cells into patients, either through local delivery or systemic infusion.[12–15] Someone has showed that murine MSCs, injected into the quadriceps muscle of mdx mice, expressed dystrophin in association with the muscle fiber sarcolemma, and pointed towards a potential therapy for muscular dystrophy.[16] But Kuroiwa shows that, in a murine model of allogeneic BM transplantation, treatment with rhHGF strongly reduces the incidence of GVHD.[17] Though Serakinci used adult mesenchymal stem cells (hMSC) transduced with the telomerase hTERT gene to find that the adult hMSCs can be targets for neoplastic transformation.[5] But in the future, there may be a way to resolve this problem.

AChRs is likely to be one nerve-supplied signal that induces expression of AChR genes at the developing neuromuscular junction.[6] Many researchs have showed AChRs can be up-regulated by MAPK and PI3K.[8,9,18] And in this study, conditional medium of BMSCs was used to stimulate Sol8 cells and found that the AChRδ mRNA and protein was up-regulated. Further study indicated that NEG-1 was produced and a Ras/Raf/MAP kinase signaling cascade was activated meanwhile. Finally lead to transcriptional activation of Erk1/2 and induction of AChRδ genes.

Knockdown ErbB3 and neutralization with NRG-1 antibody decreased this effect, which indicated AChRδ up-regulated through NRG/ErbB partly at least. This study may provide an opportunity to help cure some musculoskeletal disorders.

Competing Interests

All authors have no competing interest to declare.

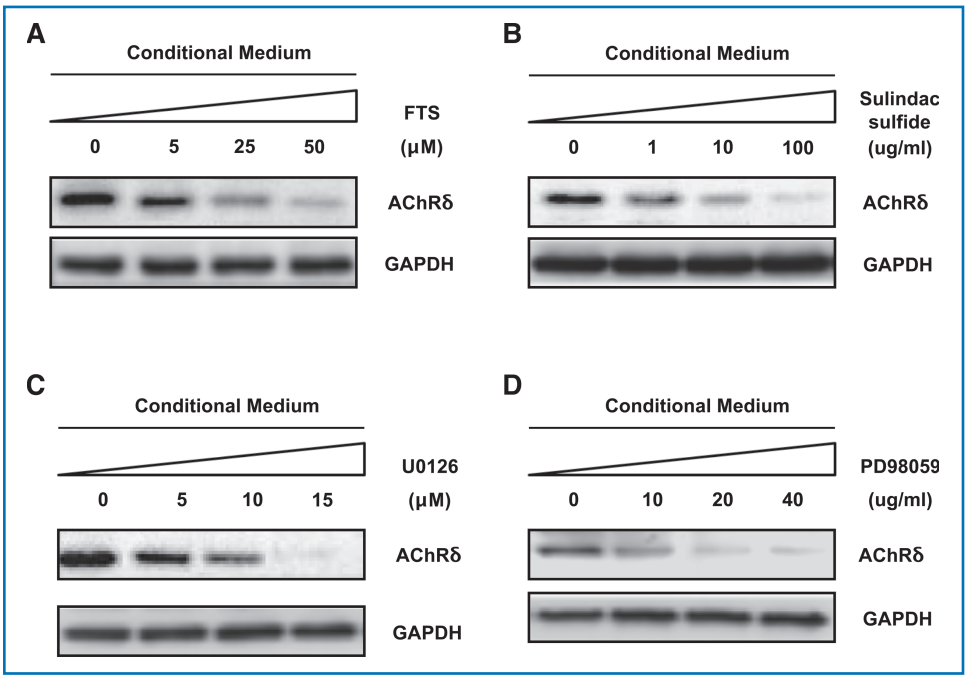

Figure 5. Effect of the Ras/Raf/MEK/ERK pathway inhibitor on AChRδ protein in Sol8 cells. Western blotting analysis was used to detect AChRδ protein in Sol8 cells after treated by FTS (A), Sulindac sulfide (B), U0126 (C), and PD98059 (D), respectively. GAPDH is shown as an internal standard.

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