Journal of Molecular and Cellular Cardiology 121 (2018) 69–80

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Cartilage oligomeric matrix protein is a novel notch ligand driving embryonic stem cell differentiation towards the smooth muscle lineage

Baihui Ma[a,d], Fang Yao[b], Nan Xie[a,d], Chenfeng Mao[a,d], Fei Liu[b], Ze Gong[a,d], Guizhen Zhao[a,d], Zhujiang Liu[a,d], Zeyu Cai[a,d], Fang Yu[a,d], Rongbo Dai[a,d], Zhongjiang Chen[a,d], Li Wang[b], Qingbo Xu[c,**], Wei Kong[a,d,*], Yi Fu[a,d,*]

[a] Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Peking University, Beijing, PR China
[b] State Key Laboratory of Cardiovascular Disease, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, PR China
[c] BHF Centre, School of Cardiovascular Medicine & Science, King's College London, London, United Kingdom
[d] Key Laboratory of Molecular Cardiovascular Science, Ministry of Education, Beijing, PR China

ARTICLE INFO

Keywords:
Embryonic stem cells
Vascular smooth muscle cells
COMP
Notch signaling

ABSTRACT

Cartilage oligomeric matrix protein (COMP), a protective component of vascular extracellular matrix (ECM), maintains the homeostasis of mature vascular smooth muscle cells (VSMCs). However, whether COMP modulates the differentiation of stem cells towards the smooth muscle lineage is still elusive. Firstly, purified mouse COMP directly induced mouse embryonic stem cell (ESC) differentiation into VSMCs both in vitro and in vivo, while the silencing of endogenous COMP markedly inhibited ESC-VSMC differentiation. RNA-Sequencing revealed that Notch signaling was significantly activated by COMP during ESC-VSMC differentiation, whereas the inhibition of Notch signaling attenuated COMP-directed ESC-VSMC differentiation. Furthermore, COMP deficiency inhibited Notch activation and VSMC differentiation in mice. Through silencing distinct Notch receptors, we identified that Notch1 mainly mediated COMP-initiated ESC-VSMC differentiation. Mechanistically, COMP N-terminus directly interacted with the EGF11-12 domain of Notch1 and activated Notch1 signaling, as evidenced by co-immunoprecipitation and mammalian two-hybrid assay. In conclusion, COMP served as a potential ligand of Notch1, thereby driving ESC-VSMC differentiation.

1. Introduction

Embryonic stem cells (ESCs) with the feature of pluripotency have the potential to differentiate into vascular smooth muscle cells (VSMCs), which express specific cell markers, such as α-smooth muscle actin (α-SMA), smooth muscle protein 22α (SM22α), calponin and smooth muscle myosin heavy chain (SM-MHC) and fine-tune vascular integrity and homeostasis [1,2]. Thus, ESCs serve as a promising source of VSMCs in vascular tissue engineering, angiogenesis, and vasculogenesis and play a pivotal role in vascular injury repair [3]. The downstream signals of cell surface receptors including integrins, Notch and growth factor receptors (e.g., TGFR, PDGFR) [4,5], as well as Nrf3-mediated transcriptional regulation [6], Nox4-derived ROS production [1] and HDAC- or miRNA-related epigenetic modulation [7], have been identified to mediate this differentiation process, although the mechanism of stem cell differentiation into the smooth muscle lineage is not fully understood.

The extracellular microenvironment, composed of a variety of extracellular matrix (ECM) components, ECM-bound growth factors, receptors and proteases, is a crucial regulator of the cell fate of ESCs [8–11]. In particular, ECM proteins, including collagens, elastins, proteoglycans and glycoproteins, fine-tune cell differentiation and behavior via protein-protein complex interaction. Moreover, the constitution and stiffness of the ECM coordinately direct cell division and differentiation. Through recent proteomic analysis, > 100 ECM proteins have been identified in human aortas and porcine stented coronary arteries, most of which are glycoproteins and proteoglycans with unknown functions [12,13]. Nevertheless, only a few ECM proteins have been suggested to be involved in smooth muscle lineage specification. For example, vascular wall resident collagen IV and hyaluronan have been reported to direct ESC differentiation into SMCs [14,15]. Exploring how ECM components mediate ESC differentiation towards the

* Corresponding authors at: Department of Physiology and Pathophysiology, Peking University Health Science Center, 38 Xueyuan Rd, Haidian District, Beijing 100191, PR China.
** Correspondence to: Q. Xu, BHF Centre, School of Cardiovascular Medicine & Science, King's College London, 125 Coldharbour Lane, London SE5 9NU, United Kingdom.
E-mail addresses: qingbo.xu@kcl.ac.uk (Q. Xu), kongw@bjmu.edu.cn (W. Kong), yi.fu@bjmu.edu.cn (Y. Fu).

https://doi.org/10.1016/j.yjmcc.2018.07.002
Received 13 April 2018; Received in revised form 6 June 2018; Accepted 3 July 2018
Available online 04 July 2018
0022-2828/

smooth muscle lineage would facilitate the development of new regenerative therapies for repairing injured VSMCs in vascular diseases.

Cartilage oligomeric matrix protein (COMP), a 524 kDa pentameric noncollagenous glycoprotein, is a matricellular protein that is abundant in the cardiovascular system. Our recent studies have shown that COMP plays protective roles in maintaining mature VSMC homeostasis by interacting with various binding proteins (e.g., integrin α7 and BMP-2) [16,17]. COMP maintains the contractile phenotype of VSMCs and suppresses atherosclerotic neointima formation and VSMC calcification [16–18]. Here, we unexpectedly discovered that COMP may serve as a novel ligand of Notch1, thereby directly driving ESC differentiation towards the smooth muscle lineage.

2. Materials and methods

2.1. Materials

Recombinant platelet-derived growth factor-BB (PDGF-BB) (220-BB) was obtained from R&D (Flanders, NJ, USA), and transforming growth factor-β (TGF-β) (AF-100) was obtained from PeproTech Group Inc. (Chicago, IL, USA). Antibodies against COMP (ab28400), Notch1 (ab65297), SM α-actin (α-SMA) (ab119952), calponin (ab46794), and SM22α (ab10135) are from Abcam plc (Cambridge, UK). DAPT (orb65891) was purchased from Biorbyt (Cambridge, UK). Antibody against cleaved Notch1 (Val1744) was obtained from Cell Signaling Technology (Boston, MA, USA). Gelatin was obtained from Merck Millipore (Darmstadt, Germany). Heparin-agarose beads (H0402) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Matrigel™ Basement Membrane Matrix (356234) was bought from BD Biosciences (San Diego, CA, USA).

2.2. Cell culture and ESC-VSMC differentiation

Mouse ESCs (ES-D3 cell line, CRL-1934) were obtained from ATCC Inc. (Manassas, VA, USA). ESCs were seeded at density of 2×10^5 cells per 25 cm^2 on mitomycin C (10 μg/mL)-treated confluent mouse embryonic fibroblast (MEF) cell feeder in 0.1% gelatin (Millipore, Boston, MA, USA)-coated flasks and cultured in ESC medium including Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Carlsbad, FL, USA), 10% EmbryoMax fetal bovine serum (FBS) (Millipore, Boston, MA, USA), 10 ng/mL of leukemia inhibitory factor (LIF) (Millipore, Boston, MA, USA), 0.1 mM of 2-mercaptoethanol (2-ME) (Life Technologies, Carlsbad, FL, USA), 100 U/mL of penicillin (Life Technologies, Carlsbad, FL, USA), 100 μg/mL of streptomycin (Life technologies, Carlsbad, FL, USA) and 2 mM of glutamine (Life Technologies, Carlsbad, FL, USA). They were split at a ratio of 1:6 every other day and seeded at density of 2×10^5 cells per 25 cm^2. For ESC-VSMC differentiation, cell culture dishes were pre-coated with 0.5 mL of Tris-buffered saline containing various concentrations of gelatin, mouse collagen IV or mouse COMP per cm^2 area at room temperature overnight. Undifferentiated ESCs (2×10^5 cells per 60 mm dish) were seeded on mouse collagen IV (10 μg/mL)- or purified-COMP-coated dishes in differentiation medium (DM) containing α-minimal essential medium (Life Technologies, Carlsbad, FL, USA) supplemented with 10% FBS, 0.05 mM of 2-ME, 100 U/mL of penicillin, 100 μg/mL of streptomycin and 2 mM of glutamine. DM was refreshed every day after the second day of differentiation. The cells were cultured in DM for 3–7 days, after which they were harvested and further analyzed. 293A cells and COS-7 cells from ATCC were maintained and passed in high-glucose DMEM supplemented with 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin in an incubator with a 5% CO_2/water-saturated atmosphere at 37 °C.

2.3. Recombinant COMP expression in 293A cells and COMP purification

The plasmid pcDNA3.1 encoding cDNA of full-length mouse COMP was constructed and transfected into 293A cells using of Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). Stably transfected cells were selected with 750 mg/L G418. For COMP purification, stably transfected 293A cells were incubated in serum-free Opti-MEM (GIBCO, Rockford, CA, USA) with G418 (250 mg/L) for 48 h. The conditioned medium was incubated with heparin-agarose beads (Sigma-Aldrich, St. Louis, MO, USA) equilibrated with Tris-buffered saline containing 2 mM of $CaCl_2$ for binding overnight at 4 °C with gentle agitation. After five-time washes, COMP was eluted with 0.75 M NaCl (buffered in 10 mM Tris, pH 7.5) containing 2 mM of $CaCl_2$. The purified mouse COMP protein was verified by western blot analysis, while the purity of COMP was > 98% as identified in SDS-PAGE followed by Coomassie Brilliant Blue G250 staining (Fig. S1). [17,19,20]

2.4. Western blotting

Cells (1×10^6 cells per sample) subjected to different treatments or aortic tissue dissected from 8-week old mice were lysed in RIPA buffer and prepared for extraction of whole-cell protein samples. Then, equal amounts of total protein (50–100 μg) per sample were resolved using 8% or 10% SDS-PAGE and transferred onto nitrocellulose membranes. The blots were subsequently incubated with primary antibodies (SM22α, α-SMA, calponin, GAPDH, COMP, cleaved Notch1 and Notch1; 1:1000) and IRDye-conjugated secondary antibodies (1:10000, Rockland Inc., Gilbertsville, PA, USA). The fluorescence signal was detected with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA), and the band density was analyzed using NIH Image J software.

2.5. Quantitative PCR analysis

Total RNA was extracted from 5×10^5 cells per sample by using Trizol reagent (Life Technologies, Carlsbad, CA, USA), and equal amounts (2 μg) were reverse transcribed to cDNA by using 5× All-In-One RT MasterMix (TransGen Biotech, Beijing, China). Quantitative PCR amplification was performed using a QuantStudio 3 Real-Time PCR System (Stratagene Corp., La Jolla, CA, USA). SYBR Green 2× PCR mix (TransGen Biotech, Beijing, China) was used according to the manufacturer's instructions. The data were normalized to the internal control 18S. The primer sequences for the target genes are listed in Table S1.

2.6. Immunofluorescent staining

Cells or tissue slides were first fixed by using 4% paraformaldehyde for 10 min, then incubated with rabbit anti-calponin or goat anti-SM22α antibody overnight at 4 °C. Subsequently, Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:400) (Life Technologies, Carlsbad, FL, USA) or Alexa Fluor 555-conjugated donkey anti-goat IgG (1:400) (Life Technologies, Carlsbad, FL, USA) antibody was applied, and the samples were incubated for 1 h. Nuclei were stained with Hoechst 33342 for 1 min. As a negative control, rabbit or goat IgG was applied instead of the primary antibodies. The fluorescence signal was visualized by confocal laser scanning microscopy (Leica Microsystems, Wetzlar, Germany).

2.7. Cell contraction assay

A Cell Contraction Assay kit (CBA-021) was purchased from Cell Biolabs Inc. (San Diego, CA, USA). The contractile ability of the cells was evaluated according to the manufacturer's instructions. Cells were harvested and resuspended in culture medium at density of 2×10^6 cells/mL. A collagen lattice was prepared by mixing cell suspension and ice-cold collagen gel solution in a volume ratio of 1:4. Then, 0.5 mL of the cell-collagen mixture per well was added to a 24-well plate and incubated for 1 h at 37 °C. After collagen polymerization, 1.0 mL of culture medium was added on top of each collagen gel lattice. Twenty-