Experimental Cell Research 347 (2016) 367–377

Contents lists available at ScienceDirect

Experimental Cell Research

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

Research Article

Roles of chondroitin sulfate proteoglycan 4 in fibrogenic/adipogenic differentiation in skeletal muscle tissues

Shiho Takeuchi [a], Shin-Ichi Nakano [a], Katsuyuki Nakamura [a], Atsufumi Ozoe [b], Peggie Chien [c], Hidehito Yoshihara [b,d], Fumihiko Hakuno [b], Takashi Matsuwaki [a], Yasushi Saeki [d], Shin-Ichiro Takahashi [b], Keitaro Yamanouchi [a,*], Masugi Nishihara [a]

[a] Department of Veterinary Physiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[b] Department of Animal Sciences and Applied Biological Chemistry, Graduate School of Agriculture and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[c] Department of Biology, The University of Rochester, Rochester, NY, USA
[d] Laboratory of Protein Metabolism, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan

ARTICLE INFO

Article history:
Received 23 April 2016
Received in revised form
11 August 2016
Accepted 27 August 2016
Available online 28 August 2016

Keywords:
Skeletal muscle
Mesenchymal progenitor cell
Cell surface marker
Chondroitin sulfate proteoglycan 4
Adipogenesis
Fibrosis

ABSTRACT

Intramuscular adipose tissue and fibrous tissue are observed in some skeletal muscle pathologies such as Duchenne muscular dystrophy and sarcopenia, and affect muscle strength and myogenesis. They originate from common fibrogenic/adipogenic cells in the skeletal muscle. Thus, elucidating the regulatory mechanisms underlying fibrogenic/adipogenic cell differentiation is an important step toward the mediation of these disorders. Previously, we established a highly adipogenic progenitor clone, 2G11, from rat skeletal muscle and showed that basic fibroblast growth factor (bFGF) is pro-adipogenic in these cells. Here, we demonstrated that 2G11 cells give rise to fibroblasts upon transforming growth factor (TGF)-β1 stimulation, indicating that they possess mesenchymal progenitor cells (MPC)-like characteristics. The previously reported MPC marker PDGFRα is expressed in other cell populations. Accordingly, we produced monoclonal antibodies that specifically bind to 2G11 cell surface antigens and identified chondroitin sulfate proteoglycan 4 (CSPG4) as a potential MPC marker. Based on an RNA interference analysis, we found that CSPG4 is involved in both the pro-adipogenic effect of bFGF and in TGF-β-induced alpha smooth muscle actin expression and stress fiber formation. By establishing an additional marker for MPC detection and characterizing its role in fibrogenic/adipogenic differentiation, these results will facilitate the development of effective treatments for skeletal muscle pathologies.

1. Introduction

Decreased muscle strength is observed in some skeletal muscle pathologies, such as Duchenne muscular dystrophy and age-related declines in muscle mass (sarcopenia) [1–3]. In Duchenne muscular dystrophy, muscle fiber degeneration and ectopic tissues, such as intramuscular adipose tissue (IMAT) and fibrous tissue are thought to cause muscle weakness [4,5]. IMAT is also observed in the skeletal muscle of elderly people and is associated with muscle function [1,6,7]. Although there is no direct evidence linking muscle fibrosis with age in humans, fibrous tissue is thought to be a cause of age-related declines in skeletal muscle function [7]. In addition to its impact on muscle strength, IMAT exerts a deleterious effect on myogenesis [8]. Therefore, it is important to elucidate the mechanisms of IMAT and fibrous tissue formation to facilitate the development of treatments to overcome muscle weakness.

Several groups have proposed that both IMAT and fibrous tissues are derived from mesenchymal progenitor cells (MPCs) in the

Abbreviations: ACTA2, α-actin 2; α-SMA, α-smooth muscle actin; bFGF, basic fibroblast growth factor; Col1a1, collagen type 1; CSPG4, chondroitin sulfate proteoglycan 4; CTGF, connective tissue growth factor; FAPs, fibro/adipogenic progenitors; FGFR, fibroblast growth factor receptor; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IMAT, intramuscular adipose tissue; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; mAb, monoclonal antibody; MPCs, mesenchymal progenitor cells; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PGs, proteoglycans; PM, proliferating medium; qPCR, quantitative RT-PCR; RT-PCR, Reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA; TGF-β, transforming growth factor-β

* Corresponding author.
E-mail address: akeita@mail.ecc.u-tokyo.ac.jp (K. Yamanouchi).

http://dx.doi.org/10.1016/j.yexcr.2016.08.023
0014-4827/

skeletal muscle of mice and humans [9–11]; these MPCs are also referred to as fibrogenic/adipogenic progenitors (FAPs) [9]. These cells express platelet-derived growth factor receptor α (PDGFRα) and typically reside in interstitial tissues, such as the endomysium, perimysium, and epimysium [9–12]. Some MPCs also express Tcf-4 (transcription factor 7-like 2, Tcf7L2) [13,14]. Type 1 pericytes (nestin$^-$/NG2$^+$ cells), which are associated with vessels, also possess adipogenicity and fibrogenicity and express PDGFRα [15,16]. However, some argue that MPCs lack NG2 expression and can be distinguished from type 1 pericytes by their location [10,14]. Although the relationship between MPCs and type 1 pericytes is controversial, they share phenotypic features, i.e., they both exhibit fibrogenicity and adipogenicity. Furthermore, in addition MPCs, PDGFRα is expressed in differentiated fibroblasts [11]. Thus, it is necessary to identify novel markers expressed in MPCs that can be used in place of PDGFRα to preferentially identify skeletal muscle MPCs that give rise to adipocytes and fibroblasts.

Fibrogenic/adipogenic differentiation is regulated by several growth factors; for example, transforming growth factor (TGF)-β induces the expression of several fibroblast markers (*Col1a1*, *Col3a1*, *CTGF*, and *Acta2)* [11,16,17]. We previously reported that exposure to basic fibroblast growth factor (bFGF) activates the adipogenic program of skeletal muscle adipogenic cells [18,19]. These growth factors act on cells via direct binding to their corresponding receptors, but their accessibility to receptors is regulated by several proteoglycans (PGs) [20,21]. For example, an extracellular domain of chondroitin sulfate proteoglycan 4 (CSPG4) binds to platelet-derived growth factor (PDGF)-AA and bFGF [22]; furthermore, the bFGF captured by CSPG4 is thought to interact with FGF receptor (FGFR) 1 and 3 in cells derived from leiomyosarcoma-derived cells [21]. Therefore, it is plausible that the PGs expressed in MPCs regulate differentiation via the modulation of growth factor accessibility.

To better understand the characteristics of fibrogenic/adipogenic cells, we previously established a highly adipogenic progenitor clone, 2G11, from rat skeletal muscle [23] and showed that bFGF is pro-adipogenic in these cells [18]. Considering that mouse/human FAPs have fibrogenic as well as adipogenic potential, 2G11 cells might represent the rat counterparts and retain fibrogenic potential. However, it is not clear whether 2G11 cells are able to differentiate to fibroblasts.

In the present study, we examined whether 2G11 cells give rise to fibroblasts. Subsequently, to identify a novel marker for fibrogenic/adipogenic cells other than PDGFRα, we produced monoclonal antibodies (mAbs) that bind to cell surface antigens of 2G11 cells. The role of a candidate antigen, CSPG4, in adipogenic and fibrogenic differentiation was then examined.

2. Materials and methods

2.1. Animals

BALB/c mice (female, 6 weeks old) were purchased from Charles River Japan (Yokohama, Japan). Female Wistar-Imamichi rats (6 weeks old) were purchased from the Institute for Animal Reproduction (Ibaraki, Japan). They were housed in a room at 23 °C with a light/dark cycle of 12 h light and 12 h darkness (lights on at 0800 h). Food and water were provided ad libitum. All animal experiments performed in this study were in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tokyo and were approved by the Institutional Animal Care and Use Committee of the University of Tokyo.

2.2. Cells

The rat highly adipogenic progenitor cell clone 2G11 [23], was cultured in Dulbecco's modified Eagle medium (Gibco, Life Technologies, Palo Alto, CA, USA) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin (proliferating medium; PM), and 10 ng/ml bFGF (cat. no. 233-FB; R&D Systems, Minneapolis, MN, USA) on poly-L-lysine and fibronectin-coated multi-well culture plates and culture dishes. The rat skeletal muscle L6 cell line (American Tissue Culture Collection, Manassas, VA, USA) was cultured in PM.

Procedures for the isolation of progenitor cells from skeletal muscles were described previously [14]. In brief, rats were euthanized by inhalation of carbon dioxide gas and their hind limbs and back muscles were trimmed from associated fat and connective tissue. The muscles were hand-minced with scissors and digested for 1 h at 37 °C with 1.25 mg/ml protease (from *Streptomyces griseus*, type XIV; Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS). Cells were separated from the muscle fiber fragments and tissue debris by differential centrifugation and plated on poly-L-lysine and fibronectin-coated dishes in PM.

2.3. Cloning of rat skeletal MPCs

Procedures for isolating progenitor cells are described above. Typically, cells from 6 mg of starting tissue were resuspended in 30 ml of PM with 10 ng/ml bFGF, and 0.1 ml of cell suspension was plated per well of a 96-well plate for a final cell density of between 0.5–1/well. After 24 h, the plates were washed and replaced with PM. Only the wells that contained a single cell were checked, and the culture was continued. When the density reached pre-confluency, cells were trypsinized and seeded on larger plates. The clones were then used for the clonal assay.

2.4. Induction of fibrogenic and adipogenic differentiation

To induce fibrogenic differentiation, the cells were cultured in PM containing 10 ng/ml TGF-β1 (R&D Systems; cat. no. 240-B) for 1 day. The medium was then replaced with PM and the cells were cultured for another 2 days.

To induce adipogenic differentiation, cells were cultured in adipogenic differentiation medium (PM containing insulin (1 μg/ml), dexamethasone (0.1 μg/ml), isobutylmethylxanthine (27.8 μg/ml), and troglitazone (10 μmol/l); kindly gifted by Daiichi-Sankyo Co. Ltd, Tokyo, Japan) for 2 days. The medium was then replaced with PM containing insulin and troglitazone and the cells were further cultured for another 2 days. At 1 day prior to the induction of adipogenic differentiation, cells were cultured in PM containing 10 ng/ml bFGF for 1 day.

2.5. Chondroitinase treatment

To remove chondroitin sulfate chains, the cells were cultured in PM containing 0.1 U/ml chondroitinase ABC (chABC, cat. no. C36667, Sigma) for 24 h.

2.6. Establishment of monoclonal antibodies

mAbs were established according to a standard procedure. Briefly, 2G11 cells were detached from the culture dish by treatment with 10 mM EDTA in PBS. Then, 1×10^7 cells were injected intraperitoneally into BALB/c mice six times at weekly intervals. After determining the increased serum antibody titer against 2G11 cells by flow cytometry, mice were euthanized by cervical dislocation and their spleens were trimmed from associated connective tissue. To isolate splenocytes, the spleens were cut and