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Platelet endothelial aggregation receptor-1 (PEAR1) is involved in C2C12 myoblast differentiation

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ABSTRACT

C2C12 murine myoblasts are a common model for studying muscle differentiation. Platelet endothelial aggregation receptor-1 (PEAR1), an epidermal growth factor repeat-containing transmembrane receptor, is known to participate in platelet contact-induced activation. In the present study, we demonstrated that PEAR1 is involved in the differentiation of C2C12 murine myoblasts. Western blotting and immunofluorescence staining were used to determine PEAR1 expression and localization during C2C12 cell differentiation. Subsequently, PEAR1 expression was activated and inhibited using clustered regularly interspaced short palindromic repeats-dCas9 technology to explore its effects on this process. PEAR1 expression was found to increase over the course of C2C12 cell differentiation. This protein was predominately localized on the membrane of these cells, where it clustered upon induction of differentiation. Expression of the myogenic markers Desmin, MYOG, and MYH2 revealed that PEAR1 positively regulated C2C12 cell differentiation. Moreover, induction of muscle injury by administration of bupivacaine to mice indicated that PEAR1 might play a role in muscle regeneration. In summary, our study confirmed the involvement of PEAR1 in C2C12 cell differentiation, contributing to our understanding of the molecular mechanisms underlying muscle development.

1. Introduction

Multiple epidermal growth factor (EGF)-like domain 10 (MEGF10) is known to function as a myogenic regulator of satellite cells in skeletal muscle [1,2]. Mutations in the *MEGF10* gene are implicated in various pathological conditions, including early onset myopathy, areflexia, respiratory distress, and dysphagia [3,4]. MEGF10 functions as a ligand of Notch and maintains myoblast number by regulating the Notch pathway during the differentiation of C2C12 murine myoblasts [5]. In humans and mice, MEGF10 has two paralogs, MEGF11 and MEGF12, the latter of which is also known as platelet endothelial aggregation receptor-1 (PEAR1) or JEDI-1. PEAR1 is a type-1 membrane protein with an extracellular EMI domain (characteristic of proteins of the EMILIN family) and is mainly expressed in platelets and endothelial cells [6,7]. Moreover, the extracellular domain of Jedi, which is similar to the corresponding domain of Jagged1, has been shown to regulate the differentiation of hematopoietic stem cells through the Notch signaling pathway [8]. However, a regulatory function for PEAR1 in skeletal muscle cell differentiation has not previously been demonstrated. In the present study, we investigated the role of PEAR1 in the differentiation of C2C12 murine myoblasts. In addition, an *in vivo* muscle injury experiment was performed using mice to confirm the involvement of PEAR1 in skeletal muscle regeneration. Establishing the impact of PEAR1 on muscle cell differentiation will provide insights into the molecular mechanisms underlying muscle development.

2. Materials and methods

2.1. Animals and housing

Male ICR mice were purchased from the Changchun Yisi Experimental Animal Technology Company (Jilin, China) and were housed in the Laboratory Animal Center of Northeast Agricultural University, Harbin, China. The mice were exposed to a 12:12-h light-dark cycle and had *ad libitum* access to normal rodent chow and water. Mice weighing 25 g were used for the muscle injury experiment. All experimental protocols were approved by the Animal Care Commission of Northeast Agricultural University and conducted in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978)

Abbreviations: EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; PBST, PBS containing 0.5% Triton X-100; BSA, bovine serum albumin; DAPI, 4′,6-diamino-2-phenylindole; TA, tibialis anterior; CRISPR, clustered regularly interspaced short palindromic repeats; CSA, cross-sectional area

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2.2. Cell culture and differentiation

The C2C12 cell (Sangon Biotech, Shanghai, China) culture medium comprised Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA), 10% fetal bovine serum (Gibco), 100 IU/mL penicillin, and 100 IU/mL streptomycin (Gibco). Subsequently, this medium was replaced with differentiation medium, comprising DMEM containing 2% horse serum (Gibco), 100 IU/mL penicillin, and 100 IU/mL streptomycin.

2.3. Plasmid construction

To test the effect of PEAR1 on C2C12 cell differentiation, four sgRNA sequences targeting the *Pear1* promoter (NCBI Gene ID: 73182) were designed, as follows: P1, GTGTCCCCCAGAGCCTAGTT; P2, CAC CGGGGCATAGATGACAAGTTTT; P3, CACCGCATATAGCCTAACTTCA CGA; and P4, CACCGTTTTGCCACGATAAAGTTT. These oligonucleotides were synthesized, annealed, and ligated into the BbsI site of the pSPgRNA expression vector, under control of the hU6 promoter (Addgene, Teddington, UK).

2.4. Western blotting

Proteins extracted from C2C12 cells were resolved by electrophoresis on a 10% SDS-polyacrylamide gel before being transferred to a PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membranes were incubated separately with primary antibodies against MYOG (diluted 1:200, sc-52903, Santa Cruz Biotechnology, Inc., Dallas, USA), PEAR1 (diluted 1:200, sc-292937, Santa Cruz Biotechnology, Inc.), MYH2 (diluted 1:200, sc-12117, Santa Cruz Biotechnology, Inc.), PAX7 (diluted 1:200, ab199010, Abcam, Inc., Cambridge, UK), and GAPDH (diluted 1:200, sc-66163, Santa Cruz Biotechnology, Inc.). Subsequently, the membrane was incubated with HRP-labeled goat anti-rabbit or rabbit anti-goat IgG secondary antibodies (diluted 1:2000, Bioss, Inc., Beijing, China). Proteins were visualized using a Super ECL Plus detection kit (Applygen Technologies Inc., Beijing, China) according to the manufacturer's instructions. Images of the membranes were acquired using a MiniChemi™ 500 Mini Chemiluminescent Imaging and Analysis System (Sage Creation Science, Beijing, China).

2.5. Immunofluorescence staining

Cells on coverslips were fixed with methanol at −20 °C for 10 min, and subsequently washed with PBS containing 0.5% Triton X-100 (PBST). Immunofluorescence staining was performed following the manufacturer's instructions. Briefly, cells were incubated for 1 h with PBST containing 5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA), and for 60 min at 37 °C with primary antibody against Desmin (diluted 1:50, sc-14026, Santa Cruz Biotechnology, Inc.) or PEAR1 (diluted 1:50, sc-292937, Santa Cruz Biotechnology, Inc.). The cells were then rinsed three times with PBST and incubated with the corresponding FITC-conjugated secondary antibody (diluted 1:20, Bioss) for 60 min at 37 °C. Following a further three rinses with PBST, the cells were incubated with 4′,6-diamino-2-phenylindole (DAPI) for 2–4 min to visualize nuclei. Finally, the cells were again rinsed three times with PBST and exposed to an antifade mounting reagent (P0126, Beyotime Biotechnology, Shanghai, China) before observation.

2.6. In vivo muscle injury

Mice were anesthetized with ether prior to injection of 0.5% bupivacaine hydrochloride monohydrate (B5274-1G, Fluka, Milwaukee, WI, USA) in physiological saline (0.9% NaCl) along the length of the right tibialis anterior (TA) muscle. The mice were killed by cervical dislocation for collection of TA muscles on Day 0 (non-injected mice), and Days 1 (24 h after injury), 3, 5, 7, 14, and 21. Samples were taken from three animals at each time point, and each sample was then divided into two parts, one for western blotting and the other for immunohistochemistry.

2.7. Immunohistochemistry

TA muscles were fixed in Bouin's solution, embedded in paraffin wax, and cut into 5-μm sections. Endogenous peroxidase activity was eliminated with 3% hydrogen peroxide in distilled water for 10 min at room temperature. Antigen retrieval was performed at 97 °C for 10 min in citrate buffer, before blocking the sections with BSA to avoid non-specific binding. The sections were then blocked with 5% BSA at 37 °C for 2 h, mounted on slides, and incubated overnight at 4 °C with antibodies against PEAR1, MYOG, and PAX7 (diluted 1:50). The slides were subsequently incubated with a secondary antibody (diluted 1:200, Bioss) for 1 h at 37 °C. Immunoreactivity was detected with 0.05% 3,3′-diaminobenzidine (ZL1–9018, ZSGB-BIO, Beijing, China), and the slides were counterstained with hematoxylin and examined under an upright microscope (BX43, Olympus, Tokyo, Japan).

2.8. Statistical analysis

Myotube fusion rate was calculated by dividing the total number of nuclei in fused myotubes from five pictures by the total number of nuclei. Myofiber CSA was measured using cellSens software (Olympus Corp., Japan). Grayscale scanning of protein bands on western blotting was performed using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) and the resulting data were analyzed with GraphPad Prism (GraphPad Software, La Jolla, CA, USA). All data are expressed as means ± standard deviations. Statistical analyses were performed using SPSS software (IBM Corp., Armonk, NY, USA). *P* values less than 0.05 were considered to indicate statistical significance. The *t*-test was used for analysis of variance, and post ad-hoc test was used for the comparison of multiple groups.

3. Results

3.1. PEAR1 expression and localization during C2C12 cell differentiation

As a measure of the degree of C2C12 cell differentiation, MYOG and MYH2 expression was assessed using western blotting. Levels of these proteins increased during this process, peaking on Day 4 before subsequently decreasing (Fig. 1A, C, D). This finding therefore indicates that cells on Days 0, 1, and 4 are representative of the cell state prior to C2C12 differentiation, during early differentiation, and during late differentiation, respectively. PEAR1 expression demonstrated similar changes (Fig. 1A, B), increasing as C2C12 differentiation progressed, and peaking when the cells reached the late differentiation stage. In addition, visualization of the cellular localization of PEAR1 by immunofluorescence revealed this protein to be mainly expressed on the C2C12 cell membrane and to accumulate when these cells began to differentiate (from Day 1 to Day 4) (Fig. 1E).

3.2. PEAR1 upregulation and inhibition using clustered regularly interspaced short palindromic repeats (CRISPR)-dCas9

CRISPR technology was used to determine the effect of PEAR1 on C2C12 cell differentiation. pSPgRNA (empty), pSPgRNA-P1, pSPgRNA-P2, pSPgRNA-P3, and pSPgRNA-P4 vectors were separately co-transfected with a dCas9-VPR or dCas9 plasmid into C2C12 cells, which were collected 48 h later. PEAR1 expression increased significantly in cells co-transfected with dCas9-VPR and pSPgRNA-P1, pSPgRNA-P2, pSPgRNA-P3, or pSPgRNA-P4, being highest ($P < 0.01$) in the pSPgRNA-P2 group (Fig. 2A, B). Conversely, when co-transfected with dCas9, each of these plasmids resulted in decreased PEAR1 expression,