Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Silencing of PMEL attenuates melanization via activating lysosomes and degradation of tyrosinase by lysosomes

Lijun Sun [a, 1], Lei Hu [b, 1], Ping Zhang [c], Huijin Li [d], Jingying Sun [a], Haifang Wang [a], Xin Xie [e, f, **], Jun Hu [a, *]

[a] Central Laboratory of Shaanxi Provincial People's Hospital, Xi'an, Shaanxi, China
[b] Department of Clinical Laboratory, Northwest Women and Children's Hospital, Xi'an, Shaanxi, China
[c] Department of Dermatology, The General Hospital of the Air Force, Beijing, China
[d] Institute of Basic and Translational Medicine, Xi'an Medical University, Shaanxi Key Laboratory of Ischemic Cardiovascular Disease, Xi'an, Shaanxi, China
[e] Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, College of Life Science, Northwest University, Xi'an, Shaanxi, China
[f] Department of Translational Medicine, Institute of Integrated Medical Information, Xi'an, Shaanxi, China

ARTICLE INFO

Article history:
Received 29 June 2018
Accepted 3 July 2018
Available online xxx

Keywords:
Pre-melanosomal protein
Melanogenesis
Tyrosinase
Melanosome
Lysosome

ABSTRACT

The functionally specialized melanosome is a membrane-enclosed lysosome-related organelle, which coexists with lysosomes in melanocytes. Pre-melanosomal protein (PMEL) initiates pre-melanosome morphogenesis and is the only cell-specific pigment protein required for the formation of fibrils on which melanin is deposited in melanosomes. But the effects of PMEL on melanin synthesis and lysosome activity remain unclear. In the study, PMEL was silenced in human epidermal melanocytes by siRNA transfection. Compared to the non-treated group, melanin content in the transfected cells was greatly reduced. Real-time qPCR, Western blotting and immunofluorescence analyses all showed that PMEL-siRNA transfection reduced protein level of tyrosinase, a key enzyme in melanogenesis, but it does not affect tyrosinase gene expression. Moreover, in the absence of PMEL, lysosomal activation was manifested by an increase in the number of lysosomes and activity of hydrolysis enzymes. The lysosome inhibitors restored tyrosinase expression after PMEL silencing, indicating that tyrosinase was degradated by lysosomes. The data collectively showed that silencing of PMEL suppressed melanization through activating lysosomes and degradation of tyrosinase by lysosomes. Our findings provide novel insight into the interaction between the melanosome and its related organelle, the lysosome, supplying a new idea for the pathogenesis and clinical treatment of pigmented diseases.

1. Introduction

Skin depigmentation is a generalized or local skin disorder resulting from melanocyte defects in the epidermis. Skin depigmentation caused by vitiligo alters patients' appearance, and complications, such as coincident diabetes mellitus, pernicious anaemia and cancers, are detrimental to their health [1]. Factors underlying the development of depigmenting skin disorders are not yet fully understood, but the proximate cause is defective melanin production.

Melanogenesis involves four morphological stages (stages I-IV) prior to melanosome maturation. The melanosome is a functionally specialized membrane-encased organelle, in which melanin is synthesized and stored [2]. Multiple enzymatic and structural proteins, known as melanosome-specific proteins, are involved in the melanosome maturation process, including tyrosinase (TYR), tyrosinase-related protein 1 (Tyrp1), tyrosinase-related protein 2 (Tyrp2), ocular albinism type 1 protein (OA1), pre-melanosomal protein (PMEL) and glycoprotein non-metastatic melanoma protein b (GPNMB), in which the melanosomal localization of the GPNMB is controversial [3–5]. Among the structural proteins, PMEL is one of the most important because it plays a crucial role in the

* Corresponding author. Central Laboratory of Shaanxi Provincial People's Hospital, No. 256, Youyi Road, Xi'an, Shaanxi, China.
** Corresponding author. Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, College of Life Science, Northwest University, Xi'an, Shaanxi, China.
E-mail addresses: xiexin@nwu.edu.cn (X. Xie), hujun116@gmail.com (J. Hu).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.bbrc.2018.07.012
0006-291X/

formation of intraluminal fibrils on which melanins are deposited in melanosomes [6]. The *PMEL* gene encodes a protein in the endoplasmic reticulum, which is delivered to the stage I melanosomes without being processed through the Golgi network; amino- and carboxy-terminal cleavage is necessary for fibrillogenesis [7]. Afterward, enzymatic components including tyrosinase and other enzymes are sorted and transported to participate in melanin synthesis [8]. In mice, mutation of the *PMEL* gene can cause melanocyte dysfunction, hair follicle loss and silver hair [9]. Studies have also shown that certain white mane traits in horse are closely related to the *PMEL* gene [10]. Therefore, PMEL is considered to be required for sorting of melanin enzymes to the melanosomes and for the ultimate production of pigment.

As a typical lysosome-related organelle, the melanosome coexists with lysosomes in melanocytes and shares several features with them. Both structures possess similar membrane proteins and specific proteins, they all have an acidic inner environment which mature melanosomes are much less acidic than lysosomes, and both contain lysosomal hydrolases and are relatively close to the endosome [11,12]. A single abnormal gene can affect several lysosomes and lysosome-related organelles, indicating that these organelles have closely related structures and functions [13].

In the current study, we report that silencing of PMEL suppressed melanization, which was manifested by reduced melanin content and level of tyrosinase. Moreover, in the absence of PMEL, the hydrolytic ability of lysosomes was enhanced and the number of lysosomes was increased. These changes appeared to be closely related to tyrosinase degradation by lysosomes.

2. Materials and methods

2.1. Antibodies and reagents

Rabbit anti-GPNMB monoclonal antibody (Ab), rabbit anti-PMEL monoclonal Ab, mouse anti-cathepsin L(CTSL) monoclonal Ab, rabbit anti-β-actin polyclonal Ab, horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) and HRP-conjugated goat anti-mouse IgG were purchased from Abcam (Cambridge, MA). Mouse monoclonal Ab to tyrosinase was purchased from Abgent (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG were purchased from Invitrogen (Grand Island, NY).

2.2. Cell culture

The immortalized human melanocyte cell line PIG1 was cultured in 254 medium supplemented with 5% foetal calf serum (FCS) and human melanocyte growth supplement (S-002-5) at 37 °C in a humidified atmosphere with 5% CO_2. The 254 medium, FCS and S-002-5 were all purchased from Gibco BRL (Gaithersburg, MD).

2.3. Transfection of siRNAs

For targeted knockdown of PMEL, a mixture of three pairs of PMEL-siRNA were designed according to a human PMEL gene transcript (NCBI GenBank accession number, NM_001200054) and synthesized by Invitrogen (Gaithersburg, MD). Their nucleotide sequences were PMEL-homo-829:5′-GGACUUUGGAGACAGUAGUTT-3′(sense) and 5′-ACUACUGUCUCCAAAGUCCTT-3′ (antisense), PMEL-homo-1251:5′-CACCUGCAGAGGUAUCAAUTT-3′ (sense) and 5′-AUUGAUACCUCUGCAGGUGTT-3′ (antisense), PMEL-homo- 1921:5′-GCGCAGACUUAUGAAGCAATT-3′ (sense) and 5′- UUGCUUCAUAAGUCUGCGCTT-3′ (antisense), respectively. A pair of NC-siRNA with nucleotide sequences of 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACACGUUCGCAGAATT-3′ (antisense) was used as negative control. For transfection, 5×10^4 melanocytes were seeded in each of the 24-well micro-plates, grown for 1 day to reach 30%–50% confluence and incubated with 15 pmol of siRNAs which were a mixture of three different PMEL-siRNAs and 1.5 μl of Lipofectamine™ RNAi-MAX (Invitrogen, Carlsbad, CA) in 50 μl of serum-free medium 254 at 37 °C with 5% CO_2.

2.4. Cell viability assay

The cell viability assay was performed using a Cell Titer-Blue H Cell Viability Assay Kit (Promega, Madison, WI) according to the manufacturer's instructions.

2.5. RNA extraction and real-time qPCR

Total RNA was extracted using the Total RNA kit (TIANGEN, Beijing, China). First-strand cDNA was synthesized using the PrimeScript™ RT kit (TaKaRa, Dalian, China), according to the manufacturer's instructions. Real-time qPCR amplifications were performed on the iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with SYBR® *Premix Ex Taq*™ II kit (Takara, Dalian, China). The following components were added in a 25-μl reaction system: 25 ng of cDNA and 0.4 mM specific primers for each gene synthesized by Sangon Biotech (Shanghai, China). The primers for each gene were as follows: PMEL (F: 5′-CCC CAG GAA AC TGA CGA TGC-3′, R: 5′-AGC CAC AGG AGG TGA GAG GAA T-3′), GPNMB (F: 5′-AAG TGA AAG ATG TGT ACG TGG TAA CAG-3′, R: 5′-TCG GAT GAA TTT CGA TCG TTC T -3′), TYR (F: 5′-GGC CTC AAT TTC CCT TCA CA-3′, R: 5′-CAG AGC ACT GGC AGG TCC TAT-3′), CTSL (F: 5′-TGT TGC TAA TGA CAC CGG CT-3′, R: 5′-CTC CGG TCT TTG GCC ATC TT-3′), β-actin (F: 5′-CTG GAA CGG TGA AGG TGA CA-3′, R: 5′-AAG GGA CTT CCT GTA ACA ATG CA-3′).

The PCR conditions consisted of an initial 3 min denaturation at 95 °C, 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. All reactions were performed in triplicate, and results were represented as relative mRNA expression. The change in transcript abundance of all tested genes was calculated using the $2^{-\Delta\Delta Ct}$ method. All gene mRNA amounts were normalized to a β-actin control.

2.6. Western blotting

Total protein of the epidermal melanocytes was extracted using RIPA lysis buffer (Beyotime, Nantong, China) and measured using a BCA protein assay kit (Beyotime). The collected proteins were electrophoresed on a 12% polyacrylamide gel and then transferred onto a nitrocellulose membrane (Pharmacia, Piscataway, NJ). After blocking with 10% defatted milk in phosphate-buffered saline (PBS) at 4 °C overnight, the target proteins were incubated with the primary Ab diluted 1:1000–1:200. The HRP-conjugated IgG was used as the secondary Ab. The blots were detected using ECL reagents (Boehringer Mannheim, Mannheim, Germany) and photographed (Kodak, Rochester, NY). All experiments were performed in triplicate, and the levels of each protein relative to that of β-actin were analysed.

2.7. Immunofluorescence microscopy

A total of 3×10^5 epidermal melanocytes were cultured on glass cover slips overnight and then fixed with methanol at 22 °C for 1 h. Coverslips were incubated with primary Ab (mouse anti-TYR monoclonal Ab and mouse anti-CTSL monoclonal Ab) at 4 °C. FITC-conjugated goat anti-mouse IgG was used as the secondary Ab. The cells were then incubated with 1 mg/ml fluorescent dye

Please cite this article in press as: L. Sun, et al., Silencing of PMEL attenuates melanization via activating lysosomes and degradation of tyrosinase by lysosomes, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.07.012