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ΔNp63/BMP-7-dependent expression of matrilin-2 is involved in keratinocyte migration in response to wounding

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ABSTRACT

p63 is expressed as multiple variants including TA and ΔN forms. Since *p63*-deficient mice displayed profound defects of stratified epithelia, p63 is an essential transcription factor required for epidermal morphogenesis. However, precise molecular mechanisms behind contribution of p63 to normal skin formation and healing skin wounds remained unclear. In this study, we demonstrated that ΔNp63/BMP-7 signaling pathway modulates wound healing process through the regulation of extracellular matrix protein matrilin-2. Knocking down of ΔNp63 in human keratinocyte HaCaT cells led to a significant reduction of *matrilin-2*. Intriguingly, BMP-7 which is one of ΔNp63-target gene products, induced *matrilin-2* and attenuated inhibitory effect of siRNA against ΔNp63 on *matrilin-2*. Furthermore, a remarkable cell migration in response to wounding took place in ΔNp63- or matrilin-2-knocked down cells. Taken together, our present findings indicate that ΔNp63/BMP-7 signaling pathway modulates wound healing process through the regulation of matrilin-2.

p63 is a *p53* family member which encodes a nuclear transcription factor with pro-apoptotic potential [1]. Like p53 and p73 [2,3], p63 is expressed as multiple variants including TA (transactivating) and ΔN (non-transactivating) forms arising from alternative splicing (α, β, and γ) and alternative promoter usage, respectively [1]. ΔNp63 which lacks NH_2-terminal transactivation domain exhibits a dominant-negative activity against TAp63, TAp73, and wild-type p53 [1]. In contrast to *p53*, *p63* and *p73* are rarely mutated in primary human tumors [4]. Initial genetic analysis demonstrated that *p63*-deficient mice fail to generate spontaneous tumors [5,6]. Intriguingly, mice lacking *p63* die soon after birth due to severe dehydration with significant defects in limb, craniofacial and skin development, suggesting that p63 plays an important role in the regulation of epidermal development and morphogenesis.

Recent genetic studies revealed that TA or ΔN isoform alone does not complement epidermal defects in *p63*-deficient mice, indicating that both isoforms are required for proper epidermal development [7]. TAp63 is the earliest p63 isoform expressed during epidermal development [8]. On the other hand, ΔNp63 is predominantly expressed in basal layer and reduced in suprabasal keratinocytes [1,9]. Sommer et al. described that mice overexpressing ΔNp63 display profound wound healing defects [10]. In contrast to the previous viewpoint, several lines of evidence indicate that ΔNp63 transactivates certain subset of genes [11,12]. Considering that ΔNp63 possesses a transcriptional potential, it is of interest to identify ΔNp63-target(s) implicated in the regulation of wound healing process.

Matrilin-2 is composed of two von Willebrand factor A-like domains, multiple epidermal growth factor-like modules as well as COOH-terminal coiled-coil domain [13] and belonging to extracellular matrix matrilin superfamily including matrilin-1, -2, -3 and -4 [14]. Matrilin-1 and -3 are expressed largely in cartilage, whereas matrilin-2 and -4 are detectable in a wide variety of extracellular matrixes [15]. Matrilin-2 is only deposited at lower part of basement membrane or upper part of dermis next to basement membrane in human skin [16]. Recently, it has been shown that matrilin-2 is induced in response to muscle or liver injury [17,18].

In this study, we found that ΔNp63/BMP-7 signaling pathway is implicated in the modulation of wound healing process through transcriptional regulation of *matrilin-2* in human keratinocyte cells.

Materials and methods

Cell culture. HaCaT and SAOS-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a water-saturated atmosphere of 95% air and 5% CO_2.

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Expression plasmid for BMP-7 and matrilin-2 luciferase reporters. Human *BMP-7* cDNA was amplified by PCR-based strategy. Sense and antisense primers used are as follows: 5′-CT**GGATCC**ACCGCCATGCACGTGCGCTCACTG-3′ (sense) and 5′-AA**GCGGCCGC**CTGTGGCAGCCACAGGCCCG-3′ (antisense). BamHI and NotI sites were shown in boldface type. PCR products were digested with BamHI and NotI and inserted into identical sites of pcDNA3-FLAG (Invitrogen) to give BMP-7-FLAG. The indicated luciferase reporters driven by *matrilin-2* promoter were generated using following primer sets: *MATN2*-promoter-1 (−289/+39), 5′-AT**ACGCGT**A AAGCTGCCTGTGTTCCG-3′ (sense) and 5′-AT**CTCGAG**TTGCTCCAAGTCCCATCC-3′ (antisense); *MATN2*-promoter-2 (−289/+205), 5′-AT**ACGCG**TAAAGCTGCCTGTGT TCCG-3′ (sense) and 5′-AT**CTCGAG**CTGCAGCCGCCGGCGCCT-3′ (antisense). MluI and XhoI sites were shown in boldface type. PCR products were then inserted into MluI and XhoI sites of pGL3-basic plasmid (Promega) upstream of *luciferase* gene. These constructs were verified by DNA sequencing (Applied Biosystems).

Transfection. Cells were transfected with the indicated expression plasmids using LipofectAMINE 2000 transfection reagent (Invitrogen) following the manufacturer's recommendations.

Short interference RNA. siRNA targeting human ΔNp63 was designed (5′-CAAUGCCCAGACUCAAUUU-3′) and purchased from Sigma. siRNA against human Smad4 (D-003902-05), human matrilin-2 (J-011329-09 and J-011329-11), luciferase (Luciferase GL2 Duplex) and control siRNA (siCONTROL) were purchased from Dharmacon. HaCaT cells were transfected with 25 nM of the indicated siRNAs using LipofectAMINE RNAiMAX (Invitrogen).

Luciferase reporter assay. HaCaT cells were transfected with 10 ng of pRL-TK *Renilla* luciferase cDNA (Promega) and 100 ng of matrilin-2 luciferase reporter or pGL3-basic plasmid and incubated with serum-free medium. Twenty-four hours after incubation, cells were treated with or without 100 ng/ml of human BMP-7. At the indicated time points after treatment, luciferase activities were determined by Dual-Luciferase Assay System (Promega) according to the manufacturer's recommendations.

Immunoblotting. Equal amounts of cell lysates were separated by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred onto Immobilon-P Transfer Membrane (Millipore). Membranes were incubated with monoclonal anti-FLAG (M2, Sigma), monoclonal anti-Smad4 (B-8, Santa Cruz Biotechnology), monoclonal anti-p63 (4A4, NeoMarkers) or with polyclonal anti-actin (20–33, Sigma) followed by incubation with HRP-conjugated goat anti-mouse or with anti-rabbit IgG (Santa Cruz Biotechnology). Bound antibodies were detected by ECL system (Amersham Biosciences).

Reverse transcription PCR analysis. Total RNA was isolated from the indicated cells using the ISOGEN reagent (Nippon gene) according to the manufacturer's instructions and treated with RNase-free DNase I. Five micrograms of total RNA were used to synthesize first-strand cDNA using random primers and SuperScript II reverse transcriptase (Invitrogen). The list of primer sets used will be provided upon request.

Indirect Immunofluorescence staining. HaCaT cells were fixed in ice-cold acetone/methanol at room temperature for 1 min and then washed with ice-cold PBS. Cells were incubated with 4% normal goat serum (NGS) in PBS for 1 h at room temperature and then probed with monoclonal anti-p63 antibody followed by subsequent incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen). After extensive washing with PBS, cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI).

In vitro wound healing. Subconfluent HaCaT cells were switched to serum-free medium and incubated for 24 h. Monolayer cultures of HaCaT cells were scratched by pipette tip and then incubated with 2% serum. Where indicated, HaCaT cells were exposed to mitomycin C (10 μg/ml) for 2 h [19].

Cell migration assays. Boyden chamber cell migration assay was performed using transwell chambers with 8-μm pore size membranes (Becton–Dickinson). HaCat cells were transfected with the indicated siRNAs. Twenty-four hours after transfection, medium was changed to fresh serum-free medium and subsequently incubated for 24 h. Cells were resuspended in serum-free medium and added to upper chamber at 1×10^5 cells/well and incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. Cells were stained with Calcein AM (BD Bioscieneces) for 20 min at room temperature followed by fixation in 3.7% formaldehyde for 10 min at room temperature. The migrated cells on lower surface of membrane were examined under a fluorescence microscopy.

Results

Expression of p63 at wound edge of human keratinocyte HaCaT cells

Since it has been unclear how p63 could regulate epidermal homeostasis, we examined expression levels of p63 in response to wounding. To this end, mechanical scratch was introduced on surface of confluent HaCaT cells. Cells were then transferred into fresh medium containing 2% FBS. At the indicated time periods after addition of FBS, cells were stained with anti-p63 antibody. As shown in Fig. 1A, p63 was undetectable in wound border 2 h after addition of FBS, whereas p63 levels returned to those in non-wounded cells 24 h after wounding.

Transcriptional regulation of matrinin-2 by ΔNp63

As described previously [17], matrilin-2 was transiently down-regulated in early phase of muscle injury and then increased in its late phase. Under our experimental conditions, p63 was undetectable in wound border in response to wounding. To address whether ΔNp63 could regulate *matrilin-2*, HaCaT cells were transfected with siRNA against luciferase (siLuc) or with ΔNp63 (siΔNp63). At the indicated time points after transfection, total RNA was subjected to RT-PCR. As shown in Fig. 1B, knockdown of ΔNp63 resulted in a significant reduction of *matrilin-2*, suggesting that *matrilin-2* is one of direct or indirect downstream targets of ΔNp63.

BMP-7 induces matrilin-2

During search of human *matrilinin-2*-promoter region, we found seven putative Smad-binding sites [20] (Fig. 2A). Since *BMP-7* is one of direct targets of p53 family including ΔNp63 [21], we examined whether BMP-7 could regulate *matrilin-2*. Consistent with recent observations [21], enforced expression of ΔNp63α resulted in a remarkable up-regulation of *BMP-7* but not of *BMP-2* and *BMP-4* (Fig. 2B). Similarly, knockdown of ΔNp63 reduced *BMP-7* but not *BMP-2* and *BMP-4* (Fig. 2C).

BMP-7 treatment induced *matrilin-2* in a time-dependent manner (Fig. 2D). BMP-7 had marginal effects on ΔNp63, whereas $p21^{WAF1}$ was significantly up-regulated in response to BMP-7 [22,23]. Our present findings suggest that BMP-7 induces *matrilin-2* through several Smad-binding sites.

BMP-7/Smad4 signaling pathway contributes to expression of matrilin-2

To further confirm a possible involvement of BMP-7 signaling pathway in the regulation of *matrilin-2*, knockdown of Smad4 was performed. As shown in Fig. 2E, siRNA against Smad4 efficiently reduced Smad4 in HaCaT cells. HaCaT cells were then transfected with siLuc or with siSmad4 and treated with BMP-7 or left untreated. Twenty-four hours after treatment, total RNA was analyzed for *matrilin-2* and $p21^{WAF1}$ by RT-PCR. As shown in Fig. 2F, BMP-7-mediated up-regulation of *matrilin-2* and $p21^{WAF1}$ was inhibited in Smad4-knockdown cells. As expected, knockdown of ΔNp63 led to a reduction of *matrilin-2* and *BMP-7*, whereas addition of BMP-7 partially attenuated inhibitory effects of siΔNp63 on *matrilin-2* (Fig. 2G).

Regulation of matrilin-2-promoter activity through ΔNp63/BMP-7 pathway

To examine effect of ΔNp63 and BMP-7 on promoter activity of *matrilin-2*, we generated luciferase reporters termed *MATN2*-promoter-1 (−289/+39) and *MATN2*-promoter-2 (−289/+205) (Fig. 3A). Twenty-four hours after starvation, HaCaT cells were transfected with *MATN2*-promoter-2 (−289/+205) and pRL-TK. Four hours after transfection, cells were treated with BMP-7 or left untreated. Consistent with our present results, luciferase activity driven by *MATN2*-promoter-2 (−289/+205) was significantly enhanced in response to BMP-7 as compared with those in untreated cells (Fig. 3B). Knockdown of ΔNp63 led to a significant reduction of luciferase activity driven by *MATN2*-promoter-2 (−289/+205) (Fig. 3C). Luciferase activity driven by *MATN2*-promoter-1 (−29/+39) was also decreased in ΔNp63-knocked down cells but to a