EPITHELIAL AND MESENCHYMAL CELL BIOLOGY

Smad3* Suppresses Epithelial Cell Migration and Proliferation via the Clock Gene *Dec1*, Which Negatively Regulates the Expression of Clock Genes *Dec2* and *Per1

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Accepted for publication
January 2, 2019.

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Smad3 has circadian expression; however, whether *Smad3* affects the expression of clock genes is poorly understood. Here, we investigated the regulatory mechanisms between *Smad3* and the clock genes *Dec1*, *Dec2*, and *Per1*. In *Smad3* knockout mice, the amplitude of locomotor activity was decreased, and *Dec1* expression was decreased in the suprachiasmatic nucleus, liver, kidney, and tongue compared with control mice. Conversely, *Dec2* and *Per1* expression was increased compared with that of control mice. In *Smad3* knockout mice, immunohistochemical staining revealed that *Dec1* expression decreased, whereas *Dec2* and *Per1* expression increased in the endothelial cells of the kidney and liver. In NIH3T3 cells, *Smad3* overexpression increased *Dec1* expression, but decreased *Dec2* and *Per1* expression. In a wound-healing experiment that used *Smad3* knockout mice, *Dec1* expression decreased in the basal cells of squamous epithelium, promoting wound healing of the mucosa. Finally, the migration and proliferation of *Smad3* knockdown squamous carcinoma cells was suppressed by *Dec1* overexpression but was promoted by *Dec2* overexpression. *Dec1* overexpression decreased E-cadherin and proliferating cell nuclear antigen expression, whereas these expression levels were increased by *Dec2* overexpression. These results suggest *Smad3* is relevant to circadian rhythm and regulates cell migration and proliferation through *Dec1*, *Dec2*, and *Per1* expression. (*Am J Pathol* 2019; 189: 773–783; <https://doi.org/10.1016/j.ajpath.2019.01.006>)

Circadian rhythms are dominantly regulated by clock genes. Briefly, Clock/Bmal1 induces *Per1/2/3*, *Cry1/2*, and differentiated embryonic chondrocyte gene 1/2 (*Dec1/2*) transcription through E-box.^{1–3} The complex of Period (Per) and Cryptochrome (Cry) protein then represses their own transcription by inhibiting transcriptional activity of Clock/Bmal1. However, Dec suppresses their own transcription by competing with Clock/Bmal1 for the occupancy of the E-box in their own promoters.^{1–3} This negative feedback regulation by clock genes plays an important role in regulation of circadian rhythm. *Dec1* and *Dec2* are basic helix-loop-helix transcription factors, are ubiquitously expressed in whole tissues, and show circadian expression in the suprachiasmatic nucleus (SCN), peripheral tissues, mesenchymal stem cells, and tumor cells.^{3–9} *Dec1* and *Dec2* are induced by

Clock/Bmal1, hypoxia, serum shock, transforming growth factor (TGF)- β , and antitumor drugs, and they regulate circadian rhythm, sleep length, inflammation, lipid metabolism, adipogenic differentiation, the cell cycle, and tumor progression.^{5–10} *Per1* knockdown increased tumor proliferation and size in oral cancer cells, decreasing *Dec1* and *Dec2* expression.¹¹ It has also been reported that *Per1* and *Dec2* have tissue-specific interactions in circadian rhythm regulation.¹² Synergistic disruption of circadian rhythm in *Per1* and *Dec1* or *Dec2* double-mutant mice was observed in

Supported by JSPS KAKENHI grants 16K09624 (F.S.) and 15K08430 (Y.M.), and Nihon University Multidisciplinary Research Grant for 2018 (U.K.B.).

Disclosures: None declared.

comparison with that in single-mutant mice.¹³ It seems that Dec1 or Dec2 interacts with Per1 in not only the regulation of circadian rhythm but also in various other phenomena.

Smad3 has circadian expression in the gingiva, liver, heart, and mesenchymal stem cells.^{7,14} *Smad3* is induced by Clock/Bmal1, serum shock, and TGF- β and regulates cell proliferation, tumor progression, cardiac diseases, fibrosis, diabetes, and metabolic syndrome.^{7,15–18} The loss of *Smad3* in mice promotes re-epithelialization and the restoration of connective tissue, resulting in faster wound healing than in wild-type mice.^{19–23} This result correlates with the alternation of wound healing–associated genes, decreased production of collagen I and fibronectin, and increased expression of *p21*, *S100A4*, and *PCNA*.^{14,21,23–25} Several reports have shown that *Smad3* affects Dec1 and Dec2 expression.^{17,26,27} For example, activation of TGF- β signaling resets the circadian clock via Dec1.²⁷ TGF- β induced *Smad3* phosphorylation and then induced Dec2 expression through *Smad3*-binding elements of the *Dec2* promoter in pancreatic cancer cells.^{17,26} However, it is not yet clear how the loss of *Smad3* affects circadian rhythm and *Dec1*, *Dec2*, and *Per1* expression. Here, we investigated whether *Smad3*^{−/−} (*Smad3* knockout) affects *Dec1*, *Dec2*, and *Per1* expression in the SCN and peripheral tissues and how these genes interact in epithelial cell migration and proliferation.

Materials and Methods

Animal and Tissue Preparation

All animal experiments were performed according to protocols approved by the Animal Care and Use Committee of the Wakayama Medical University (approval number: 660). Six- to 8-week-old male and female *Smad3* (+/+) wild-type and (−/−) whole knockout mice with a C57BL/6 $\times$ 129SVEV background were generated as previously described.²⁸ Mice were housed, and tissues were prepared as previously described.^{6,14,29} Forty *Smad3*^{+/+} and 40 *Smad3*^{−/−} mice were used in this study. All mice were reared in 12:12 light/dark cycles (lights on at 8 AM, off at 8 PM). With the use of isoflurane anesthesia, mice were sacrificed by cervical dislocation. A dim red light was used to sacrifice at Zeitgeber time (ZT)12 and ZT18. Mouse brain dissection was performed at 0.5 mm thickness, using a mouse brain coronal matrix (BrainScience Idea, Osaka, Japan), and the SCN and prefrontal cortex from the mice were punched out onto a chilled dish with the use of a 0.5-mm diameter needle. The SCN and prefrontal cortex were quickly assessed for mRNA and protein purification.

Activity Recordings

Mouse locomotor activity was monitored by Supermex (Muromachi Kikai, Tokyo, Japan). Starting at 6 weeks of age, activity tests were conducted for 30 days. A sensor counts the movements of the mice by detecting the radiated

body heat. The data were recorded continuously in 1-minute bins by a data collection program (CompACT AMS; Muromachi Kikai). The free-running period of locomotor activity was calculated by periodogram analysis with the use of ClockLab software (Actimetrics, Wilmette, IL). Fast Fourier analysis was performed to determine the amplitude of the power spectral density of the circadian peak.

Wound-Healing Experiments of Mice

Mice were anesthetized by injection of ketamine (100 mg/kg of body weight) and xylazine (5 mg/kg of body weight) in sterile phosphate-buffered saline, and the wound was made by punching out a 3.0-mm hole with a Harris Uni-core puncher (BrainScience Idea) at the apex of the tongue. The mice were sacrificed at ZT6, and the tongue was prepared at 3 and 5 days after wounding. Tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (Wako, Osaka, Japan) and subjected to immunohistochemistry and hematoxylin and eosin staining.

Real-Time PCR

Three independent RNA samples were prepared from the mouse SCN, prefrontal cortex, and tongue. In addition, RNA was prepared from *Smad3* overexpressing NIH3T3 cells. Total RNA was isolated and first-strand cDNA was synthesized as previously described.^{6,14} Real-time PCR was performed with the SYBR Green Master Mix (Bio-Rad Laboratories, Inc., Hercules, CA). The amplification primer sequences were designed as follows: *Dec1* forward (F), 5'-ATCAGCCTCCTTTTGCCTTC-3', and reverse (R), 5'-AGCATTCTCCAGCATAGGCAG-3'; *Dec2* F, 5'-ATTGCTTTACAGAATGGGGAGCG-3', and R, 5'-AAAGCGCGGAGGTATTGCAAGAC-3'; *Per1* F, 5'-CCAGGATGTGGGTGTCTTCT-3', and R, 5'-GTCCTTGAGACCTGAACCTG-3'; and 18S rRNA F, 5'-GCGCCGCTAGAGGTGAAAT-3', and R, 5'-GAAAA-CATTCTTGGCAAATGCTT-3'. The data were normalized by using 18S rRNA.

Immunohistochemistry

Dec1, Dec2, and Per1 expression in mouse kidney, liver, and tongue tissues was evaluated by using serial deparaffinized sections. Six *Smad3*^{+/+} and six *Smad3*^{−/−} mice were used for immunohistochemistry. Five serial sections of 4 μ m per organ were prepared for staining. Immunohistochemistry was performed by using a Discovery Auto-Stainer with automated protocols (Ventana Medical Systems, Inc., Tucson, AZ; Roche, Mannheim, Germany) as previously described.¹⁴

Cell Culture and Treatment

NIH3T3 mouse fibroblasts were obtained from ATCC (Manassas, VA). CA9-22 human gingival cancer cells were

obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan). These cells were cultured in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum and 1% antibiotics. The construct expression vectors for human Dec1 and Dec2 were used for transfection.¹ Human Smad3 plasmid (11742) was purchased from Addgene (Cambridge, MA). Transient plasmid transfection and siRNA transfection were performed as previously described.³⁰ For Dec1 overexpressed-stable cells, empty (CA9-22^{vector}) and Dec1 (CA9-22^{Dec1}) plasmids were transfected in cells. After transfection, medium was changed, including 1000 µg/mL G418 (Roche) solution every 3 days until 3 weeks and selected stable cells. The Dec1 induction was confirmed in the stable cells by Western blot analyses. *Smad3* siRNA was synthesized by Qiagen (Hilden, Germany). The sense and antisense siRNA sequences were as follows: *Smad3* siRNA1, 5'-r (CAAGGGAUUUCCUAUGGAATT)-3' and 5'-r (UCCAUAGGAAAUCCCUUGAT)-3'; *Smad3* siRNA2, 5'-r (GAGAUUCGAAUGACGGUAATT)-3' and 5'-r (UUACCGUCAUUCGAAUCUCTT)-3', respectively.

Cell Migration Assay

CA9-22 cells were seeded in a 12-well plate and transfected with control or *Smad3* siRNA2. After 8 hours, the cells were transfected with empty, Dec1, or Dec2 plasmid, and then an artificial wound was carefully created at 0 hour by scratching the surface with the tip of a P-200 pipette. Pictures were taken by microscopic digital camera (Nikon DS-L3; Nikon Corporation, Tokyo, Japan) at 0 and 12 hours.

Cell Proliferation Assay

CA9-22 cells were seeded in 96-well plates. Transfection was performed as previously described.³⁰ After 10 hours of transfection, the cells were added to each well by using a cell proliferation kit (XTT based) (Biological Industries, Kibbutz Beit Haemek, Israel) and incubated at 37°C for an additional 2 and 14 hours. The absorbance at OD₄₈₀ and at OD₆₅₀ was measured with the use of a 96-well microplate reader (SH-9000; Hitachi, Tokyo, Japan).

Western Blot Analysis

Individual mouse liver and kidney were lyzed with the use of T-PER lysis buffer (ThermoFisher Scientific, Yokohama, Japan). Three independent SCN samples were pooled and then were lyzed with the use of T-PER lysis buffer. Cells were lyzed with M-PER lysis buffer (ThermoFisher Scientific, Yokohama, Japan). Lysates were run on SDS-polyacrylamide gels followed by Western blot analysis with the use of standard procedures. A WesternBright Sirius Kit (Advansta, CA) was used for antibody detection, and an AE-9300 Ez capture MG (ATTO, Tokyo, Japan) was used for image data capture. The intensity of the bands detected on the Western blot analysis

was quantified with ImageJ software version 1.47v (NIH, Bethesda, MD).

Antibodies

The following commercial antibodies were purchased: Smad3 (dilution 1:1000; rabbit monoclonal; ab40854; Abcam, Cambridge, UK), Dec1 (dilution 1:5000; rabbit polyclonal; NB100-1800; Novus Biologicals, Centennial, CO), Dec2 (dilution 1:2000; mouse monoclonal; sc-373763; Santa Cruz Biotechnology Inc., Santa Cruz, CA), proliferating cell nuclear antigen (PCNA; dilution 1:10,000; rabbit polyclonal; sc-7907; Santa Cruz Biotechnology Inc.), Per1 (dilution 1:1000; rabbit polyclonal; TransGenic Inc., Hyogo, Japan), E-cadherin (dilution 1:20,000; mouse monoclonal; M106; TaKaRa Bio Inc., Otsu, Japan), and actin (dilution 1:10,000; mouse monoclonal; A5441; Sigma Chemical Co.).

Results

Smad3 Deficiency Decreases the Amplitude of Locomotor Activity

It was first examined whether *Smad3* deficiency affected circadian rhythm with the use of *Smad3*^{-/-} mice. *Smad3*^{+/+} and *Smad3*^{-/-} mice were fed under light/dark conditions for 14 days and constant dark conditions for 16 days. Locomotor activities were analyzed by Supermex. The amplitude of locomotor activity significantly decreased in *Smad3*^{-/-} mice compared with that in *Smad3*^{+/+} mice (Figure 1A). However, no significant differences were observed in the circadian period between *Smad3*^{+/+} and *Smad3*^{-/-} mice. *Dec1*, *Dec2*, and *Per1* mRNA expression was next examined at ZT6 and ZT12 in the SCN of *Smad3*^{+/+} and *Smad3*^{-/-} mice by real-time PCR, because time difference may affect clock gene expression. *Dec1* expression was higher, whereas *Dec2* and *Per1* expression was lower at both time points in *Smad3*^{+/+} mice than in *Smad3*^{-/-} mice (Figure 1, B and C), suggesting that *Smad3* up-regulates *Dec1* expression and down-regulates *Dec2* and *Per1* expression. In addition, Dec1, Dec2, and Per1 protein expression was analyzed at ZT6 and ZT12 in the SCN, and the result was similar at mRNA levels (Figure 1D). *Dec1*, *Dec2*, and *Per1* expression was found to be higher in the SCN of *Smad3*^{+/+} mice than in the prefrontal cortex (Supplemental Figure S1).

Diurnal Variation in the Expression of Dec1, Dec2, and Per1 in the Kidney

To examine Dec1, Dec2, and Per1 protein expression in *Smad3*^{+/+} and *Smad3*^{-/-} mice, Western blot analysis was performed. Compared with that in *Smad3*^{+/+} mice, Dec1 protein expression at ZT6 decreased in the liver and kidneys of *Smad3*^{-/-} mice, whereas Dec2 and Per1 protein expression increased (Figure 2A). Because the protein levels

Figure 1 Decreases in the amplitude of locomotor activity in *Smad3*^{-/-} mice. **A:** Representative locomotor activity records from *Smad3*^{+/+} and *Smad3*^{-/-} mice under 12:12 light/dark (LD; 14 days) and constant darkness (DD; 16 days) cycles. **Black squares** indicate the darkness. The records are double-plotted such that 48 hours are shown for each horizontal trace. The period and amplitude of *Smad3*^{+/+} and *Smad3*^{-/-} mice under DD are also shown. **B and C:** Relative *Dec1*, *Dec2*, and *Per1* mRNA expression in the suprachiasmatic nucleus (SCN) of *Smad3*^{+/+} and *Smad3*^{-/-} mice at Zeitgeber time (ZT)6 (**B**) and ZT12 (**C**) was analyzed by real-time PCR. The data were normalized to *18S* rRNA. **D:** Differentiated embryonic chondrocyte (Dec)1, Dec2, period (Per)1, and actin protein expression in the SCN of *Smad3*^{+/+} and *Smad3*^{-/-} mice at ZT6 and ZT12. Data are expressed as means $\pm$ SEM. $n = 3$ independent samples. * $P < 0.05$ (t -test).

of Dec2 was virtually undetectable in the liver of *Smad3*^{+/+}, the circadian expression of Dec2 and other clock genes was examined specifically in the kidneys of *Smad3*^{+/+} and *Smad3*^{-/-} mice. Circadian expression of Smad3 in *Smad3*^{+/+} mice was also examined. As expected, Dec1, Dec2, Per1, and Smad3 protein expression showed diurnal variation in *Smad3*^{+/+} mice (Figure 2B). *Smad3*^{-/-} mice also showed diurnal variation in the protein expression of these circadian

genes in the kidneys; however, the expression levels were altered. Specifically, Dec1 protein expression decreased from ZT0 to ZT18 in the kidneys of *Smad3*^{-/-} mice compared with that in *Smad3*^{+/+} mice. Interestingly, Dec2 protein expression greatly increased from ZT0 to ZT18 in the kidneys of *Smad3*^{-/-} mice compared with that in *Smad3*^{+/+} mice. Per1 protein expression was slightly increased in the kidneys of *Smad3*^{-/-} mice compared with *Smad3*^{+/+} mice.

Figure 2 Diurnal variation in differentiated embryonic chondrocyte (Dec1, Dec2, and period (Per)1 expression in *Smad3*^{-/-} mouse livers and kidneys. **A:** Representative Western blot analyses of Dec1, Dec2, Per1, and actin at Zeitgeber time (ZT)6 in the liver and kidneys of *Smad3*^{+/+} and *Smad3*^{-/-} mice. **B:** Representative Western blot analyses of Dec1, Dec2, and Per1 circadian expression in the kidneys of *Smad3*^{+/+} and *Smad3*^{-/-} mice (top panel). Actin was used as a loading control. Quantitation of the intensities of bands in the data from Western blot analysis (bottom panels). The data were normalized to actin. Data are expressed as means ± SEM. *n* = 3 independent samples.

Smad3 Deficiency Down-Regulates Dec1 Expression, Up-Regulating Dec2 and Per1 Expression in Endothelial Cells of Mouse Kidney and Liver

To understand the localization and expression of Dec1, Dec2, and Per1, immunohistochemistry was performed with

the use of kidney and liver tissues of *Smad3*^{+/+} and *Smad3*^{-/-} mice. Strong positive staining for Dec1 was detected in endothelial cells in the kidney and liver and in renal tubular epithelial cells of *Smad3*^{+/+} mice, whereas no significant immunostaining was observed in those of *Smad3*^{-/-} mice (Figure 3A). In contrast, strong positive staining for Dec2 and Per1 was detected in endothelial cells in the kidneys and liver of *Smad3*^{-/-} mice, but not in those of *Smad3*^{+/+} mice (Figure 3, B and C). These results suggested that Smad3 regulated Dec1 expression and negatively regulated Dec2 and Per1 expression.

Smad3 Overexpression Increases Dec1 Expression, Decreasing Dec2 and Per1 Expression

Next, it was examined whether overexpression by Smad3 plasmid affected Dec1, Dec2, and Per1 expression with the use of NIH3T3 cells. Smad3 overexpression increased Dec1 protein expression in NIH3T3 cells compared with control treated cells (Figure 4A). Endogenous protein expression of Dec2 and Per1 was low in NIH3T3 cells. Therefore, real-time PCR was performed to assess the mRNA levels. Smad3 overexpression increased *Dec1* mRNA expression but decreased *Dec2* and *Per1* mRNA expression (Figure 4B). These results were in good agreement with immunohistochemistry in mouse tissues.

Wound Healing Is Promoted by the Alternation of Clock Gene Expression in *Smad3*^{-/-} Mice

It was further examined how Dec1, Dec2, and Per1 expression was affected by wound healing in *Smad3*^{+/+} and *Smad3*^{-/-} mice. On day 3 and day 5 after wounding, re-epithelialization of the tongue was accelerated in *Smad3*^{-/-} mice compared with *Smad3*^{+/+} mice, whereas the degree of inflammation decreased in *Smad3*^{-/-} mice (Supplemental Figure S2). On day 3 ZT6 after wounding, positive staining for Dec1 was dominantly observed in basal cells of the adjacent squamous epithelium of *Smad3*^{+/+} mice, whereas positively stained cells were significantly decreased in the wound lesions (Figure 5A). By contrast, the intensity of immunostaining for Dec1 was weak in both the adjacent squamous epithelium and wound lesions of *Smad3*^{-/-} mice. However, positive staining for PCNA, Dec2, and Per1 was weak in basal cells of the adjacent squamous epithelium of *Smad3*^{+/+} mice, whereas a number of cells positive for PCNA, Dec2, and Per1 expression were observed in *Smad3*^{-/-} mice (Figure 5A and Supplemental Figure S3). Positive staining between Dec1 and PCNA had a significant correlation. In addition, *Dec1* mRNA expression was decreased in *Smad3*^{-/-} mice compared with that in *Smad3*^{+/+} mice (Figure 5B). On the fifth day after wounding, a similar immunostaining pattern for Dec1, Dec2, and Per1 was observed in *Smad3*^{+/+} and *Smad3*^{-/-} mice (Supplemental Figure S4). From the above data, it was hypothesized that Smad3 suppressed basal cell

A **B** **C**

Figure 3 Immunohistochemical detection of differentiated embryonic chondrocyte (Dec)1, Dec2, and period (Per)1 in the kidneys and liver of *Smad3*^{+/+} and *Smad3*^{-/-} mice. **A–C:** Representative images for immunohistochemistry of Dec1 (**A**), Dec2 (**B**), and Per1 (**C**) at Zeitgeber time (ZT)6 in the kidneys and liver of *Smad3*^{+/+} and *Smad3*^{-/-} mice (**left panel**). Positive staining for Dec1, Dec2, and Per1 in endothelial cells of *Smad3*^{+/+} and *Smad3*^{-/-} mouse kidneys and liver was counted in nine random microscopic fields (**right panel**). In addition, positive staining for Dec1 in 10 renal tubules was counted as above. Data are expressed as means $\pm$ SEM. $n = 3$ independent samples. $^{**}P < 0.01$ (t -test). Original magnification, $\times 400$ (**A–C**).

Figure 4 Smad3 overexpression increases differentiated embryonic chondrocyte (Dec)1 expression but decreases Dec2 and period (Per)1 expression. **A:** Representative Western blot analyses of Smad3, Dec1, and actin expression in NIH3T3 cells in the presence (+) or absence (–) of Smad3 plasmid (**left panel**). Quantitation of the intensities of bands in the data from the Western blot analysis (**right panels**). **B:** Relative Dec1, Dec2, and Per1 mRNA expression in the presence or absence of Smad3 plasmid from NIH3T3 cells as measured by real-time PCR. The data were normalized to 18S rRNA. Data are expressed as means $\pm$ SEM. $n = 3$ independent samples. * $P < 0.05$ and ** $P < 0.01$ (t -test).

proliferation in re-epithelialization during wound healing via Dec1, which in turn down-regulated Dec2 and Per1 expression.

Smad3 Regulates Cell Migration and Proliferation via Dec1 and Dec2

To explore this hypothesis, it was examined whether *Smad3* knockdown with or without Dec1 or Dec2 overexpression affected the migration and proliferation of human oral squamous cancer CA9-22 cells. Both siRNA1 and siRNA2 for *Smad3* knockdown greatly decreased endogenous Smad3 expression in CA9-22 cells (**Figure 6A**). *Smad3* siRNA2 had effective knockdown compared with *Smad3* siRNA1. Dec1 overexpression greatly increased exogenous Dec1 expression, but it decreased endogenous Dec2, Per1, E-cadherin, and PCNA expression in CA9-22 cells (**Figure 6A**). However, Dec2 overexpression increased both E-cadherin and PCNA expression, whereas it had little effect on Dec1 and Per1 (**Figure 6A**). After a wound was created at 12 hours, *Smad3* knockdown by siRNA2 greatly induced cell migration compared with control siRNA (**Figure 6B**). However, Dec1 overexpression with or without *Smad3* siRNA2 greatly suppressed cell migration. In addition, Dec1 overexpression with or without *Smad3* siRNA2 significantly decreased cell proliferation (**Figure 6C**). Migration and cell proliferation assays were also performed with the use of Dec1 overexpressed-stable cells. The results were compatible with transient transfection experiments (**Supplemental Figure S5**). Conversely, Dec2 overexpression with or without *Smad3* siRNA2 significantly induced cell migration and proliferation. These results suggested that cell migration and proliferation were promoted by *Smad3* deficiency through Dec1 down-regulation and Dec2 up-regulation.

Discussion

The decreases in the circadian amplitude imply the disruption of activity patterns and sleep quality. In addition, these decreases are associated with metabolic syndrome, depression, and cardiac diseases.^{31,32} Because *Smad3*^{−/−} mice experienced great decreases in the amplitude of locomotor activity, it was hypothesized that it may depend on the abnormality of clock genes through *Smad3* deficiency. As expected, alternation of *Dec1*, *Dec2*, and *Per1* expression was observed in the SCN, liver, kidneys, and tongue in *Smad3*^{+/+} and *Smad3*^{−/−} mice. In addition, *Bmal1* expression decreases in the heart of *Smad3*^{−/−} mice compared with that in control mice.¹⁴ This finding implies that *Smad3* deficiency certainly alters the expression of clock genes. Meanwhile, *Smad3*^{−/−} mice did not show significant change during the circadian period. Dec1, Dec2, and Per1 had circadian expression in the SCN and kidneys of *Smad3*^{−/−} mice, so the circadian period may have a minimal effect. Smad3 may compensate for other Smads, such as Smad2. In other studies, *Dec1* or *Per1* single-knockout mice had only a slight change in circadian rhythm, whereas *Dec1/Dec2* or *Per1/Per2* double-knockout mice had a marked change.^{33,34} Similarly, Smad3 forms a heterodimer with Smad2 and is bound to the DNA of target genes.³⁵ Smad2, therefore, may compensate for *Smad3* deficiency. *Smad3*^{−/−} mice have a circadian rhythm in central and peripheral tissues, although the amplitude of the locomotor activity and the expression of Dec1 and Dec2 are dampened. Note that *Dec1* and *Dec2* double mutant mice reduce the amplitude of sleep–wake cycle.³³ Therefore, the dampened amplitude of activity rhythm in *Smad3*^{−/−} mice may be induced by the altered expressions of both *Dec1* and *Dec2* observed in the SCN.

Figure 5 Differentiated embryonic chondrocyte (Dec1) expression decreased in basal cells of the squamous epithelium of the tongue of *Smad3*^{-/-} mice. **A:** Representative images of immunohistochemistry for Dec1 and proliferating cell nuclear antigen (PCNA) at Zeitgeber time (ZT)6 in the tongue of *Smad3*^{+/+} and *Smad3*^{-/-} mice 3 days after punch biopsy (**top panels**). Positive nuclear staining for Dec1 and PCNA in the squamous epithelium was counted in nine random microscopic fields (**bottom panels**). Immunostaining data of Dec2 and Per1 were included in the [Supplemental Figure S3](#). **B:** Relative *Dec1* mRNA expression in the adjacent (Adj) and wound lesions (Wou) of *Smad3*^{+/+} and *Smad3*^{-/-} mouse tongue at ZT6 was analyzed by real-time PCR. The data were normalized to 18S rRNA. The average numbers are shown (**A**). Data are expressed as means $\pm$ SEM. $n = 3$ independent samples. ** $P < 0.01$ [multiple comparison—Dunnett test (straight)]; $^{\dagger}P < 0.05$ (Bonferroni test); $^{\ddagger}P < 0.05$ (Dunnett test). Original magnification, $\times 400$.

There is accumulating evidence that Dec1, Dec2, and Per1 play important roles not only in circadian rhythm but also in apoptosis, differentiation, and disease progression.^{1,4,31,36–41} In other words, Dec1, Dec2, and Per1 exert their effects under stress. Several reports have shown that Dec1 overexpression affects the expression of clock genes *in vitro*.^{42,43} Nevertheless, the differential expression of Dec1, Dec2, and Per1 *in vivo* has been unclear. It has been proved that *Smad3* knockout accelerates wound healing.^{20–23} As a stress, wounding was performed by punch biopsy, and it was

investigated whether *Smad3* deficiency affects Dec1, Dec2, and Per1 expression during wound healing. The tongue is quickly repaired after wounding, and punch biopsy of tongue causes comparatively little damage to the mouse compared with liver and kidney. Wound healing was therefore performed on the tongue. By wounding the tongue of *Smad3* knockout mice, Dec1 expression decreased in the basal cells of the squamous epithelium, whereas Dec2 and Per1 expression increased. Although it is unclear how Dec2 and Per1 expression increased, a clear negative relationship was

A **B** **C**

Figure 6 Differentiated embryonic chondrocyte (Dec1) overexpression suppressed whereas Dec2 overexpression induced the migration and proliferation of oral squamous carcinoma cells. **A:** Representative Western blot analyses of Smad3 and actin by *Smad3* siRNA1 or *Smad3* siRNA2 in CA9-22 cells. The samples were prepared from two independent wells (**left panel**). Representative Western blot analyses of Dec1, Dec2, period (Per)1, E-cadherin, proliferating cell nuclear antigen (PCNA), and actin protein expression in Dec1- or Dec2-overexpressing CA9-22 cells (**middle and right panels**). **B:** Representative images for the cell migration assay (**top panels**). CA9-22 cells were transfected with control or *Smad3* siRNA2. After 8 hours, the cells were transfected with control, Dec1, or Dec2 plasmid, and then a wound was made with the use of a pipette tip. Photographs of the wound area were taken at 0 and 12 hours. The three different unrepared areas in each well were measured by scale and quantified (**bottom panel**). The average unrepared areas at 12 hours are shown. **C:** Cell proliferation assay. CA9-22 cells were treated with (+) or without (-) *Smad3* siRNA2 and Dec1 or Dec2 plasmid. The absorbance (OD₄₈₀/OD₆₅₀) at 0, 12, and 24 hours is shown. Data are expressed as means $\pm$ SEM. $n = 3$ independent samples (**B**). $*P < 0.05$ (Dunnett test). Original magnification, $\times 100$.

found between Dec1 and Dec2 and Per1. *In vitro* experiments revealed that Dec1 overexpression suppressed cell migration and proliferation, whereas Dec2 overexpression had the opposite effect on the cells. This finding implies that the

suppression of cell migration and proliferation by Smad3 may occur via Dec1. Moreover, these results demonstrated that Dec1 and Dec2 regulate cell migration and proliferation via E-cadherin and PCNA. In particular, Dec1 had significant

correlation with PCNA in wound-healing experiments. In addition, Dec1 overexpression decreased PCNA expression *in vitro*. Dec1 function in the regulatory mechanisms of cell proliferation is not well understood. Notably, Dec1 suppressed proliferation, inhibiting PCNA.

The interaction between Smad3 and Dec2 and Per1 is likely as follows: First, Smad3 increases the Dec1 expression, although it decreases the Dec2 and Per1 expression. Second, although Dec1 overexpression decreases the Dec2 and Per1 expression, Dec2 overexpression has little effect on the Dec1 and Per1 expression. Third, Dec1 is induced early by stresses, whereas Dec2 is induced later.^{3,4,7,32} Collectively, these results suggest that Smad3 negatively regulates Dec2 and Per1 expressions via Dec1. Furthermore, in wound-healing experiments that used *Smad3*^{+/+} and *Smad3*^{-/-} mice, it was revealed that Dec1 expression was decreased in the wound lesions, even in *Smad3*^{+/+} mice. Tumor necrosis factor- α , which is a suppressor of TGF- β , also suppresses Smad3 and Dec1 expression in NIH3T3 cells.¹⁴ It is possible that tumor necrosis factor- α production in wound lesions may influence Smad3 and Dec1 expression. Intriguingly, a recent study has demonstrated that the actin cytoskeleton of fibroblasts has rhythmic expression, influencing wound-healing efficacy.⁴⁴ Smad3 expression may be involved in the mechanism by which tongue wounds incurred during daytime heal faster than nighttime wounds. Although it was found that the alternation in the healing processes observed under the *Smad3*-deficient condition is associated with Dec1 and Dec2 function, further studies are needed to elucidate the precise mechanisms of wound healing. These issues include the roles of Smad3 and clock genes in squamous epithelium and fibroblasts.

In summary, *Smad3* deficiency alters *Dec1*, *Dec2*, and *Per1* expression in circadian central and peripheral tissues. We propose that Smad3 is a new regulator of clock genes that are involved in circadian rhythm, wound healing, and cell proliferation and migration. Investigation of the interaction between Smad3 and clock genes may shed light on chronotherapy alternatives for wound healing and cancer.

Acknowledgments

F.S., T.O., and H.T.L. performed experiments; A.K. and U.K.B. analyzed data; F.S. prepared the draft and made figures; A.K. and Y.M. edited the manuscript.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2019.01.006>.

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