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J Dent Res 93(9):898-903, 2014

ABSTRACT

The overexpression of the intracellular signaling molecule of the transforming growth factor- β family (TGF- β) Smad2 was found to induce apoptosis and inhibit the proliferation rate of oral epithelial cells. Therefore, the aim of this study was to investigate *in vivo* the effect of Smad2 overexpression on the proliferation rate of the junctional epithelium (JE). Smad2 overexpression was driven by the cytokeratin 14 promoter (K14-Smad2) in transgenic mice. The K14-Smad2 mice were compared with wild-type (WT) mice selected as the control group. Samples were stained with hematoxylin and eosin stains and analyzed by image analysis. Immunohistochemistry was conducted for proliferating cell nuclear antigen (PCNA) and c-Myc as markers of cell proliferation. The expression of cyclin-dependent kinase inhibitors (P15, P21, and P27) was determined by real-time polymerase chain-reaction (RT-PCR). The quantity of phosphorylated retinoblastoma (pRB) was determined with Western blots. The overexpression of Smad2 altered the area of the junctional epithelial cells in one-year-old K14-Smad2 mice. The area was $32,768 (\pm 3,473) \mu\text{m}^2$ for the WT and $24,937.25 (\pm 1,965) \mu\text{m}^2$ for the K14-Smad2 mice. There was a significant difference in the proliferation rates of the JE (PCNA-positive cells) between the WT and K14-Smad2 mice, 20.7% (± 1.1) and 2.1% (± 0.5), respectively. A significant difference in c-Myc expression occurred between experimental and control samples. The K14-Smad2 mice had a mean of 2.3% (± 0.6), and the WT mice had a mean of 20.1% (± 3.6). Smad2 overexpression up-regulated the mRNA expression of P15 by 2.3-fold and that of P27 by 5.5-fold in the K14-Smad2 mice. Finally, the pRB protein showed a 2.3 (± 0.5)-fold increase in K14-Smad2 mice when compared with WT mice. Smad2 overexpression inhibits the proliferation of JE cells by down-regulating c-Myc and up-regulating P15 and P27, which resulted in an increase in pRB, leading to cell-cycle arrest.

KEY WORDS: transforming growth factor- β , PCNA, c-Myc, cyclin-dependent kinase inhibitors, retinoblastoma, gingival biotype.

DOI: 10.1177/0022034514543016

Received May 2, 2014; Last revision June 18, 2014; Accepted June 19, 2014

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Smad2 Overexpression Reduces the Proliferation of the Junctional Epithelium

INTRODUCTION

The junctional epithelium (JE) is the part of the dento-gingival unit that is attached to the tooth surface. The JE develops from the reduced enamel epithelium, and, over time, it is replaced by the basal cells of the oral gingival epithelium (Salonen *et al.*, 1989). The JE extends coronally to the base of the oral sulcular epithelium and apically to the connective tissue attachment at the cemento-enamel junction and forms the lining of the interdental col (Gargiulo *et al.*, 1961). The JE is the first line of defense against periodontal disease; it provides an important barrier activity by coming into contact with the tooth surface *via* cells directly attached to the tooth (DAT cells) through hemidesmosomes (Listgarten, 1966). The JE cells play an active role in the synthesis of a variety of molecules that are part of the defense against bacterial invasion, such as the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), which directs and guides polymorphonuclear leukocytes (PMNs) through the junctional epithelium, and IL-8 cytokine, which has a chemotactic ability (Heymann *et al.*, 2005; Tonetti *et al.*, 2012). Therefore, the balance between cell death and mitotic activity is critical to maintain the number of JE cells, which affect the defense properties of the JE and, eventually, disease progression (Overman and Salonen, 1994; Bosshardt and Lang, 2005).

Transforming growth factor- β (TGF- β) is a potent cytokine that is involved in both development and disease (Chang *et al.*, 2013). TGF- β signaling occurs through the binding of TGF- β to the type II receptor that in turn phosphorylates the TGF- β type I receptor, activating the intracellular kinase. The activation of type I results in the phosphorylation of the intracellular transcription molecule Smad2. Smad2 and Smad4 bind together and enter the nucleus to start the transcription of TGF- β -dependent genes that mediate multiple processes, including apoptosis, cell proliferation, and the secretion of inflammatory cytokines (Wyllie, 2010). To study the effect of Smad2 overexpression on the JE cells, we used a mouse model (K14-Smad2) that overexpresses Smad2 specifically in epithelium, using a cytokeratin14 promoter (K14) (Ito *et al.*, 2001). The transgenic mouse model presents an advantage, since it induces an overexpression of Smad2 controlled by the K14 promoter, specifically, JE cells, which makes the model suitable for the investigation of the role of Smad2 overexpression in JE cells (Fujita *et al.*, 2012).

Our laboratory recently published data showing an increased apoptotic index of the JE cells in Smad2 overexpression mice. Smad2 overexpression induced apoptosis of JE cells through the down-regulation of the anti-apoptotic molecule Bcl2 (Fujita *et al.*, 2012). The JE cells ordinarily have a high proliferation rate, which could overcome the increase in apoptosis, maintaining the homeostasis of the JE (Watanabe *et al.*, 2004). Previous studies have shown that TGF- β inhibits the proliferation rate of cells through the up-regulation of the cyclin-dependent kinase inhibitor and through the repression of the

c-Myc. c-Myc is a transcription protein that represses cyclin-dependent kinase inhibitors (P15 and P21), leading to an inhibition of the cell cycle from the G1 to the S phase (Seoane, 2006). The hypothesis of this study is that Smad2 overexpression will reduce the proliferation rate of JE cells through the up-regulation of the cyclin-dependent kinase inhibitors and the down-regulation of c-Myc. The availability of K14-Smad2 transgenic mice permits the effect of Smad2 overexpression on JE cells to be examined *in vivo*; thus, the aim of the current study was to investigate the effect of Smad2 overexpression on the proliferation rate of the JE cells *in vivo*.

MATERIALS & METHODS

Animals and Genotyping

K14-Smad2 mice that induce Smad2 overexpression through a K14 promoter were selected to represent the model of this study. Dr. Yang Chai, University of Southern California Center for Craniofacial Molecular Biology, generously provided these mice. All methods were within the guidelines and approved by the Animal Care Committee of The University of British Columbia. The genotype of the mice was detected by means of a primer set that detected the K14-Smad2 transgene through the cytokeratin14 promoter region. Mice overexpressing K14-Smad2 were analyzed at 3 and 12 mos of age and were selected as the test group and compared with age-matched wild-type controls. Forty mice were selected for this study. Sample size was divided as shown in Fig. 1. The sample size was based on power analysis (G*power software), which was conducted for all methods, which gave power of 0.8 for each.

Histology and Immunohistochemistry

Decalcification and Paraffin Embedding of the Samples

Hemisections of mice maxillae were dissected under the microscope, and the samples were decalcified with ethylenediaminetetraacetic acid (EDTA) for 4 to 6 wks at room temperature. The decalcified samples were embedded in paraffin blocks and stored at -50°C . We obtained 7- μm sections for subsequent analysis.

Hematoxylin and Eosin

Slides were stained with Harris's hematoxylin for 2.5 min, then were transferred to running tap water for 20 min. Thereafter, the

Figure 1. Smad2 overexpression altered the junctional epithelium (JE) surface area. (A) Hematoxylin and Eosin (H&E) image showing the JE of 3-month-old wild-type (WT) mice. (B) H&E image of K14-Smad2 JE surface area at 3-month time-point. (C) H&E image showing the JE of 12-month-old WT mice. (D) H&E image showing the JE of 12-month-old K14-Smad2 mice. (E) Quantification of the JE surface area of the 3-month and one-year samples. * $p < .005$.

slides were immersed in eosin for 40 sec, dehydrated, and cleared through a series of ethanol and xylene, respectively. Finally, slides were coverslipped with mounting media and viewed under light microscopy (Wazen *et al.*, 2009).

Immunohistochemistry

EDTA antigen retrieval was done, then the sections were blocked with 2% goat serum blocking solution for 30 min at room temperature. A 2- μL quantity of primary antibody against E-cadherin (mouse polyclonal; Cell Signaling Inc., Danvers, MA, USA), PCNA (mouse polyclonal; Cell Signaling Inc.), and c-Myc (rabbit polyclonal; Abcam Inc., Cambridge, MA, USA) were incubated with the sections for 2 hrs and then rinsed with PBS three times for 5 min each. Secondary antibody goat anti-mouse (Alexa Fluor 488 IgG; Invitrogen, Inc., Carlsbad, CA, USA) and goat anti-rabbit (Alexa Fluor 568 IgG; Invitrogen, Inc.) were incubated with the slides for 1 hr at room temperature, followed by PBS rinse three times for 4 min each. Finally,

Table.

Primer	Forward	Reverse
P15	5'-CCCTGCCACCCTTACCAGA-3'	5'-CAGATACCTCGCAATGTCACG-3'
P21	5'-CCTGGTGATGTCCGACCTG-3'	5'-CCATGAGCGCATCGCAATC-3'
P27	5'-TCAAACGTCAGAGTGTCTAACG-3'	5'-CCGGGCCGAAGAGATTCTG-3'
GAPDH	5'-GGTCCTCAGTGTAGCCCAAG-3'	5'-AATGTGTCCGTCGTGGATCT-3'

Figure 2. Smad2 overexpression reduced the proliferation rate of JE cells. **(A)** Representative image of PCNA-positive cells (green) and DAPI (blue) from WT mice at the 3-month time-point. **(B)** PCNA-positive cells and DAPI from K14-Smad2 mice at the 3-month time-point. **(C)** PCNA window of WT mice at 3 mos, showing multiple positive cells. **(D)** PCNA window of K14-Smad2 mice showing limited positive cells. The dotted line represents the JE. Original magnification (x600). **(E)** Magnification x1200 of WT mice showing multiple co-localizations between PCNA and DAPI. **(F)** Magnification x1200 of K14-Smad2 mice showing limited co-localizations between PCNA and DAPI. **(G)** Positive control small intestine tissue showing multiple PCNA-positive cells. **(H)** Negative control JE cells showing no PCNA signal. **(I)** Quantification of the PCNA-positive cells represented by PCNA + DAPI-positive cells. ** $p < .001$. The white bar represents 100 μ m.

slides were coverslipped with mounting media, which included 4',6-Diamidino-2-Phenylindole (DAPI) for the identification of cell nuclei. Nikon Laser Scanning Confocal microscopy (C1) was used to examine the slides (Watanabe *et al.*, 2004). Liver and small intestine tissues were used as positive control samples for PCNA and c-Myc proteins, respectively. Negative control samples were obtained by omission of the primary antibody.

Real-time PCR

The samples were collected from the buccal and palatal attached gingiva of the first and second molar teeth of the K14-Smad2 and wild-type mice. Tissues were homogenized by mortar and pestle, and the RNA was purified with RNeasy Mini Kit (Qiagen, Alameda, CA, USA). The total RNA was measured, and equal amounts of the RNA were used for cDNA synthesis with an Iscript Kit (Bio-Rad, Hercules, CA, USA). Nucleotide sequences for the PCR primers were obtained from the National Center for Biomedical Information as shown in the Table.

cDNA samples were added to a PCR amplification mixture containing forward and reverse primers and SsoFast EvaGreen Supermix PCR master mixture (Bio-Rad). Samples were then subjected to a denaturation reaction for 5 min at 94°C, followed by annealing and DNA synthesis for 60 sec each at 60°C. The data were normalized against GAPDH and calculated by the CT method (Hart *et al.*, 2004).

Western Blots

The attached gingiva from the first and second maxillary molars of K14-Smad2 transgenic mice and wild-type mice were collected under the microscope. The tissue samples were homogenized in 500- μ L lysis buffer NP-40 and 10 μ L of the proteinase inhibitor cocktail on ice. The samples were placed in dry ice for 20 min, after which the tubes were centrifuged at 12,000 rpm for 15 min at -4°C. The protein concentrations were

determined through the bovine serum assay standard, and equal protein samples were then subjected to 5% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) buffer and electrophoresis for 45 min at 160V at room temperature. The samples were transferred from the SDS-PAGE gels to a membrane (transblot pure nitrocellulose; BioRad) at 60V for 2 hrs. The membranes were then washed with PBS for 10 min on

a shaker. Blocking solution Odyssey (LI-COR, Lincoln, NE, USA) was applied for 1 hr at room temperature. The primary antibody for phosphorylated retinoblastoma (rabbit polyclonal; Abcam) was applied at a concentration of 0.2 $\mu\text{g}/\text{mL}$ for 1 hr. The membrane was washed in PBS five times for 5 min each. The samples were incubated with a fluorescent secondary anti-rabbit IgG antibody for 1 hr. The membrane was then washed in PBS five times for 5 min each. Finally, the membrane was scanned, and the band intensities were quantified by the Image tool program (Lohinai *et al.*, 2001).

Statistical Analysis

The data were interpreted by one-way ANOVA and Student's *t* test.

RESULTS

Smad2 Overexpression Altered the Surface Area of the JE Cells

The JE surface area was measured by Image tool at 3 and 12 mos of age for both K14-Smad2 and wild-type mice. As shown in Fig. 1, the mean JE surface area of the K14-Smad2 remained almost unchanged, from 23,036.5 ($\pm 3,754$) μm^2 at 3 mos to 24,937.25 ($\pm 1,965$) μm^2 at the 12-mo time-point. In contrast, the mean surface area of the wild-type mice was 21,728 ($\pm 4,724$) μm^2 at 3 mos and increased significantly to 32,768 ($\pm 3,473$) μm^2 at the 12-mo time-point (Fig. 1).

Smad2 Overexpression Reduces the Proliferation Rate of JE Cells

PCNA-positive cells were selected as representative of proliferating cells. The positive cells (PCNA+DAPI) were counted and divided by the total number (DAPI) of JE cells to identify the percentage of proliferating cells. The 3-month-old K14-Smad2 mice had a significantly lower proliferation rate than their wild-type counterparts: The mean proliferation rate of the JE cells was 20.79% in the wild-type mice and 1.31% in the K14-Smad2 mice (Fig. 2).

Increased Smad2 Up-regulates c-Myc

The mean c-Myc-positive cells in the JE were counted, and the percentage of c-Myc-positive cells was calculated. Increased Smad2 reduced the number of c-Myc-positive cells in the JE in K14-Smad2 experimental mice (2.3%) compared with that in the wild-type controls (20.1%) (Fig. 3).

Figure 3. Smad2 overexpression inhibits c-Myc. (A) Representative image of c-Myc (red) expression in the JE with E-cadherin (green) from WT mice at the 3-month time-point. (B) c-Myc-positive cells and E-cadherin from K14-Smad2 mice at the 3-month time-point. (C) c-Myc window showing positive cells of WT mice. (D) c-Myc window showing no positive cells of K14-Smad2 mice. The dotted line represents the JE. Original magnification ($\times 600$). (E) Magnification $\times 1,200$ of WT mice showing multiple co-localizations between c-Myc and DAPI. (F) Magnification $\times 1,200$ of K14-Smad2 mice showing limited co-localizations between c-Myc and DAPI. (G) Positive control liver tissue showing multiple c-Myc-positive cells. (H) Negative control of JE cells showing no c-Myc signal. (I) Quantification % of c-Myc-positive cells. $**p < .001$. The white bar represents 100 μm .

Increased Smad2 Increased both P15 and pRB to Inhibit JE Cell Proliferation

Smad2 increased the expression of the mRNA of the cyclin-dependent kinase inhibitor p15 to a 5.56-fold increase when compared with that in wild-type mice. P21 gene expression did not show any statistically significant difference between K14-Smad2 and wild-type mice. Smad2 overexpression increased (2.3-fold) the pRB protein in the JE cells of K14-Smad2 mice when compared with that in wild-type mice (Fig. 4).

DISCUSSION

The junctional epithelium has an important protective role providing the barrier ability and the secretory of defense molecules (Listgarten, 1966; Overman and Salonen, 1994; Bosshardt and Lang, 2005; Heymann *et al.*, 2005; Tonetti *et al.*, 2012). In our laboratory, we previously published data showing that Smad2 overexpression causes an increased apoptotic index in the JE cells through the inhibition of Bcl2 (Fujita *et al.*, 2012). The present study expanded on those original findings to determine if increased levels of Smad2 also altered the JE proliferation,

Figure 4. Smad2 overexpression up-regulates P15 and P27 and at the same time increases the protein level of pRB. (A) Western blots of K14-Smad2 and WT mice representing pRB and GAPDH. (B) Quantification of the pRB Western blot results. ** $p < .001$. (C) Real-time PCR expression of P15, P21, and P27. ** $p < .001$.

and thus the combined effects of increased apoptosis and decreased proliferation would have a negative impact on JE homeostasis. In the current study, the JE of K14-Smad2 mice had a reduced surface area when compared with that of wild-type mice at 12 mos, which indicates that the JE does not have the same size/integrity as the wild-type control tissue. The change in JE area at 12 mos can be explained by a combination of increased apoptosis and a reduced proliferation rate that decreases the replacement of the dead cells. A limitation of the current methodology for the collection of JE tissue samples for the Western blots and the real-time PCR was a potential confounding variable. The JE cells were isolated by doing modified Widman incisions, which included oral and JE epithelium cells for both test and control groups. To understand the underlying mechanism of the role of Smad2 overexpression on the reduction of the proliferation rate, we examined molecules that control the proliferation rate, such as c-Myc, cyclin-dependent kinase inhibitors (P15, P21, and P27), and pRB (Warner *et al.*, 1999). It was reported *in vitro* that Smad2 overexpression induces P21 and P15, cyclin-dependent kinase inhibitors that inhibit cell-cycle progression from the G1 to the S phase, thus preventing proliferation (Shimoe *et al.*, 2013). Our findings had the same outcomes as the previous *in vitro* study, since increased levels of Smad2 resulted in an up-regulation of the expression of both P15 and P27. Some differences in specific cyclin-dependent kinase inhibitors can be explained by the specific epithelial cells and tissues examined. The *in vitro* study used oral epithelial cells, and in our study, we specifically investigated the JE epithelial cells. It has been reported that TGF- β down-regulates transcription factors involved in proliferation such as c-Myc (Warner *et al.*, 1999). Previous studies have shown that c-Myc was a repressor of P27 (Amendola *et al.*, 2009) and P15 (Staller *et al.*, 2001) and that down-regulation of c-Myc would up-regulate these cyclin-dependent kinase inhibitors—again, results supported by the current *in vivo* analysis of increased intracellular signaling in the TGF- β pathway. Studies have shown that the activation of cyclin-dependent kinase inhibitors would lead to

increased levels of pRB, consistent with the findings in the present study. The increased levels of pRB in turn release E2F, a transcriptional factor that causes cell-cycle arrest (Ravitz and Wenner, 1997).

From the results presented above, we can draw a conclusion based on a working model that explains the role of Smad2 overexpression in the reduced proliferation rate of JE cells. Smad2 overexpression reduces the surface area of JE cells by inhibiting c-Myc, which in turn up-regulates the cyclin-dependent kinase inhibitors (P15 and P27), leading to excess phosphorylation of the retinoblastoma protein.

ACKNOWLEDGMENTS

The research was conducted within the University of British Columbia. Dr. Alotaibi received the Joseph Tonzetich Fellowship award. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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