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Microtubule disassembly prevents palatal fusion and alters regulation of the E-cadherin/catenin complex

YUKIKO KITASE and CHARLES F. SHULER*

Department of Oral Biological and Medical Sciences, Faculty of Dentistry, University of British Columbia, Vancouver BC, Canada

ABSTRACT During palatal fusion, the midline epithelial seam (MES) degrades to achieve mesenchymal confluence. Epithelial mesenchymal transition (EMT) is one mechanism which is active in MES degradation. TGF-β induces EMT in medial edge epithelium (MEE) by down-regulation of an epithelial marker, E-cadherin. Microtubule disassembly impaired palatal fusion leading to a multilayered MES in the mid-region. In this study, we investigated the effect of microtubule disruption on the regulation of the E-cadherin/catenin adhesion complex. Nocodazole (NDZ) enhanced the accumulation of the adhesion complex at cell-cell contacts in MEE, while loss of the adhesion complex was observed in the control. NDZ caused aberrant regulation of the E-cadherin transcriptional repressors (Snail and Zeb) and the activator (c-MYC) through inhibition of the TGF-β/SMAD2 signaling pathway, which led to a failure in EMT. These results suggest that the microtubule cytoskeleton plays an important role in mediating TGF-β/SMAD2 signals to control *E-cadherin* gene expression in MEE during palatal fusion.

KEY WORDS: *microtubule, palatal fusion, EMT, E-cadherin/catenin adhesion complex, TGF-β/SMAD2*

Introduction

Formation of the secondary palate is a complex and critical event involving tightly regulated palatal shelf outgrowth, re-orientation, adhesion and fusion. Disturbance at any step during palatal development may result in cleft palate, a common birth defect in humans (Ferguson, 1988). The opposing palatal shelves approach each other in the midline after re-orientation from a vertical position. The medial edge epithelia (MEE) adhere and form, via intercalation, a single layer called the medial epithelial seam (MES). Degradation of the MES proceeds to achieve the confluence of palatal mesenchyme and complete the process of palatal fusion (Ferguson, 1988).

Epithelial mesenchymal transition (EMT) plays a crucial role in MES degradation (Greene and Pisano, 2010, Griffith and Hay, 1992, Jalali *et al.*, 2012, Jin and Ding, 2006, Shuler *et al.*, 1992, Yu W, 2009). It is characterized by loss of epithelial cell morphology and acquisition of mesenchymal properties. To maintain epithelial integrity a calcium-dependent homophilic adhesion molecule, E-cadherin, exerts adhesive function by binding to the cytoplasmic α- and β-catenin proteins, which serve to link the cadherin to the actin cytoskeleton (van Roy and Berx, 2008). E-cadherin transcription is tightly regulated through the promoter that contains a CAAT box, a GC rich region and an E-box. The

transcription factor c-MYC transactivates E-cadherin through its direct interaction with the E-box or as a co-activator of AP-2 transcription factor, which interacts with the GC-rich region (Batsche *et al.*, 1998, Liu *et al.*, 2009). TGF-β negatively regulates c-MYC (Pietenpol *et al.*, 1990). Transcriptional repression of E-cadherin is a key molecular event in EMT (Peinado *et al.*, 2004). Down regulation of E-cadherin by transcriptional repressors such as the zinc finger factor Snail (Snai1 and Snai2), two-handed zinc finger factor Zeb (Zeb1 and Zeb2) is an essential mechanism by which TGF-β elicits EMT (Dave *et al.*, 2011, Jalali *et al.*, 2012, Pirozzi *et al.*, 2011, Shirakihara *et al.*, 2007). The signaling pathway is well known to induce EMT in MEE during palatal fusion (Kaartinen *et al.*, 1997, Sun *et al.*, 1998).

We have shown that microtubule disruption by nocodazole (NDZ), a widely used microtubule de-stabilizing agent, inhibited palatal fusion accompanied with the development of a multilayered MES in the mid-palatal region (Kitase and Shuler, 2012). In this study, we investigated the underlying mechanism by which NDZ exposure resulted in the accumulation of cell-cell adhesion proteins at intercellular junctions in MEE. These findings provided evidence that microtubule disruption impaired the mechanism

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*****Address correspondence to:** Charles F. Shuler. 2194 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada. Tel: +1-604-822-5773. Fax: +1-604-822-4532. e-mail: cshuler@dentistry.ubc.ca

for EMT. In this study we investigated the role of microtubules on the transcriptional regulation of the adhesion proteins. Our results provided evidence that the microtubule cytoskeleton plays a crucial role in EMT through regulation of TGF-β/SMAD2 signaling during palatal fusion and inhibiting the repression of E-cadherin.

Results

Microtubule disassembly inhibits palatal fusion leading to a multi-layered MES formation in the mid-region

We observed induction of multi-layered MES formation in the palate mid-region when *in vitro* palate shelf organ cultures were treated with a microtubule-disrupting agent, NDZ. The MES disappeared to achieve complete mesenchymal confluence in control organ cultures while NDZ treated cultures had a persistence of a multilayered MES (Fig.1).

Microtubule disassembly results in the accumulation of E-cadherin/catenin adhesion complexes at cell-cell junctions in MEE

We examined whether MEE treated with NDZ maintained intact E-cadherin/catenin cell adhesion complexes at the intercellular junction. In control organ cultures, down-regulation of E-cadherin and the associated catenins such as p120 catenin, β-catenin and α-catenin occurred during palatal fusion. The intensity of p120 catenin and α-catenin signals is approximately 3- and 10-fold weaker than for E-cadherin in all cultures (Fig. 2Aa,b). The adhesion molecule proteins became concentrated at cell-cell junctions in MEE treated

Control   **NDZ**

Fig. 1. Microtubule disassembly inhibits palatal fusion resulting in multi-layered MES formation in the mid-region. *Hematoxylin staining. Representative sections shown for control* **(A,C)** *and 30 µM NDZ* **(B,D)** *were taken from 72 h organ cultured palatal tissue.* **(C,D)** *High magnification images from the rectangular area shown in* **(A,B)** *respectively. Scale bar, 100 µm.*

with NDZ over long-term culture periods (Fig. 2Bc-e). Palatal organ cultures from TGF-β3 null mice exhibited failure of degradation of E-cadherin/catenin adhesion complex in control and developed multi-layered MES and accumulation of the adhesion complex under long-term treatment of NDZ (Fig. 2Ac and Bd).

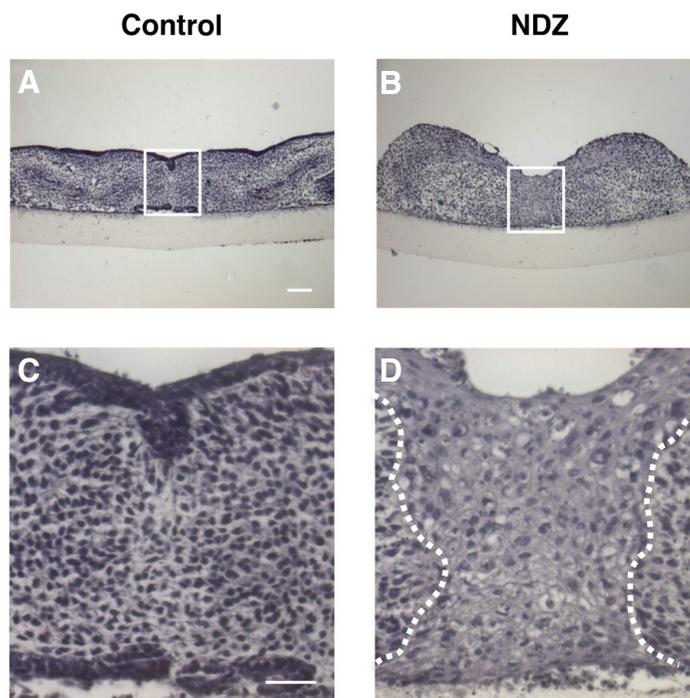### Microtubule disassembly alters the expression of E-cadherin/catenin adhesion molecule genes

We investigated the effect of NDZ on the expression of adhesion molecule genes by quantitative real-time PCR to understand the underlying mechanism by which the accumulation of the adhesion molecule proteins occurred at cell-cell junctions (Fig. 3). E-cadherin mRNA was increased more than 2-, 4- and 5-fold at 24, 48 and 72 h respectively. p120 catenin mRNA was also increased more than 2-fold at 72 hours while β-catenin mRNA was inhibited approximately 2-fold at all time points measured. There were no significant changes in α-catenin mRNA expression.

Microtubule disassembly inhibits TGF-β/SMAD2 signaling resulting in down-regulation of EMT-related transcription factors

Both E-cadherin and β-catenin are downstream target genes regulated by TGF-β signaling, which is also essential to mediate EMT signals (Peinado *et al.*, 2004, Pirozzi *et al.*, 2011). Failure in transcriptional down-regulation of E-cadherin and up-regulation in β-catenin following microtubule disassembly led us to examine whether NDZ effects TGF-β signaling. TGF-β signaling during palatal fusion was evaluated by detecting phospho-SMAD2. Immunofluorescence analysis revealed strong phospho-SMAD2 signals in most MEE after adhesion of the opposing palatal shelves, while few signals were detected in MEE before adhesion in control (Fig. 4Aa, b). By contrast, NDZ blocked activation of TGF-β signaling. The NDZ exposed MEE have almost no phospho-SMAD2 signals at all the time points examined (Fig. 4Ac-e). We performed quantitative real-time PCR to examine the expression level of EMT-related transcription factors regulated by TGF-β. All of the transcription factors exhibited an approximately 2-fold decrease at all the time points measured (Fig. 4B).

### Microtubule disassembly failed to down regulate c-MYC associated with ID2 induction

The balance between negative and positive regulatory pathways controlling transcription affects total E-cadherin mRNA level. To explore the effect of NDZ on the inductive regulatory pathway, we performed immunofluorescence analysis to investigate the expression level of c-MYC. The strong signals were observed in MEE before adhesion in control and its expression was inhibited after adhesion, which is inversely correlated with the expression level of phospho-SMAD2 during palatal fusion (Fig. 5Aa,b). By contrast, NDZ maintained c-MYC expression in MEE even at 48 h (Fig. 5Ac-d). c-MYC is well known to induce a bHLH transcriptional factor ID2 in association with the MAX transcription factor (Lasorella *et al.*, 2000). Failure of c-MYC down-regulation by TGF-β/SMAD2 due to microtubule disassembly may secondarily lead to ID2 induction. We therefore examined the expression level of the protein, which also acts as a negative regulator of TGF-β-induced EMT through negatively modulating the transcription of Zeb (Shirakihara *et al.*, 2007). In control, most MEE showed strong ID2 signals before adhesion, but weak signals were detected in some MEE after adhesion, which indicates down-regulation by TGF-β during palatal
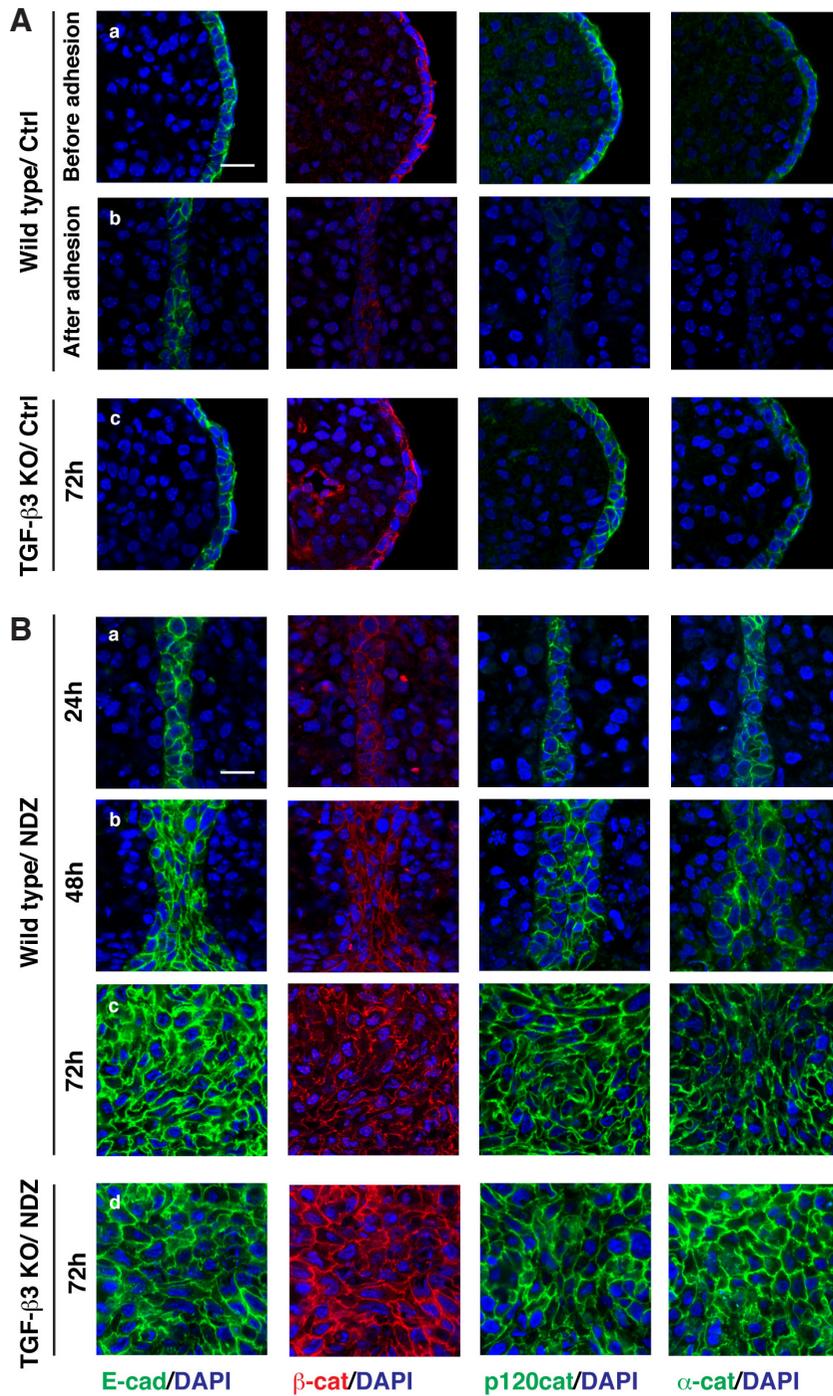
Fig. 2 (left). Microtubule disassembly results in the accumulation of E-cadherin/catenin adhesion complexes at cell-cell junctions in MEE. *Organ cultured were performed with* **(B)** *or without 30 µM NDZ* **(A)***. Fluorescent images of E-cadherin (green), β-catenin (red), p120 catenin (green), α-catenin (green) and DAPI (blue). Representative sections shown for wild type/ control (a: before adhesion, b: after adhesion), TGF-β3KO/ control (c: 72h), wild type/ 30 µM NDZ (c: 24 h, d: 48 h, e: 72 h) and TGF-β3KO/ 30 µM NDZ (d: 72h) were taken from palatal tissue. Scale bar, 20 µm.*
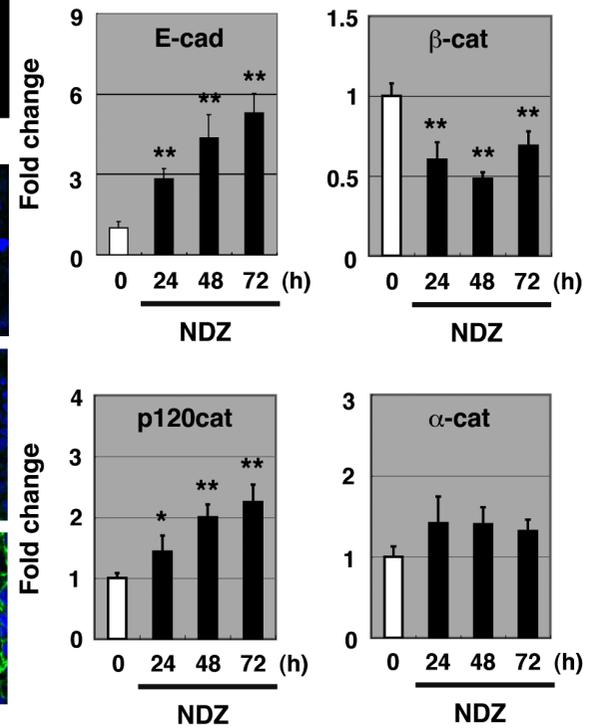
Fig. 3 (above). Microtubule disassembly alters expression of E-cadherin/catenin adhesion molecule genes. *Quantitative real-time PCR analysis of gene expression encoding E-cadherin, β-catenin, p120 catenin and α-catenin in palatal tissue treated with 30 µM NDZ for the indicated period. GAPDH was used as reference for normalization. *P<0.05 and **P<0.01 compared to control.*

fusion (Fig. 5A). NDZ increased expression of ID2 with maximum induction at 72 h (Fig. 5B).
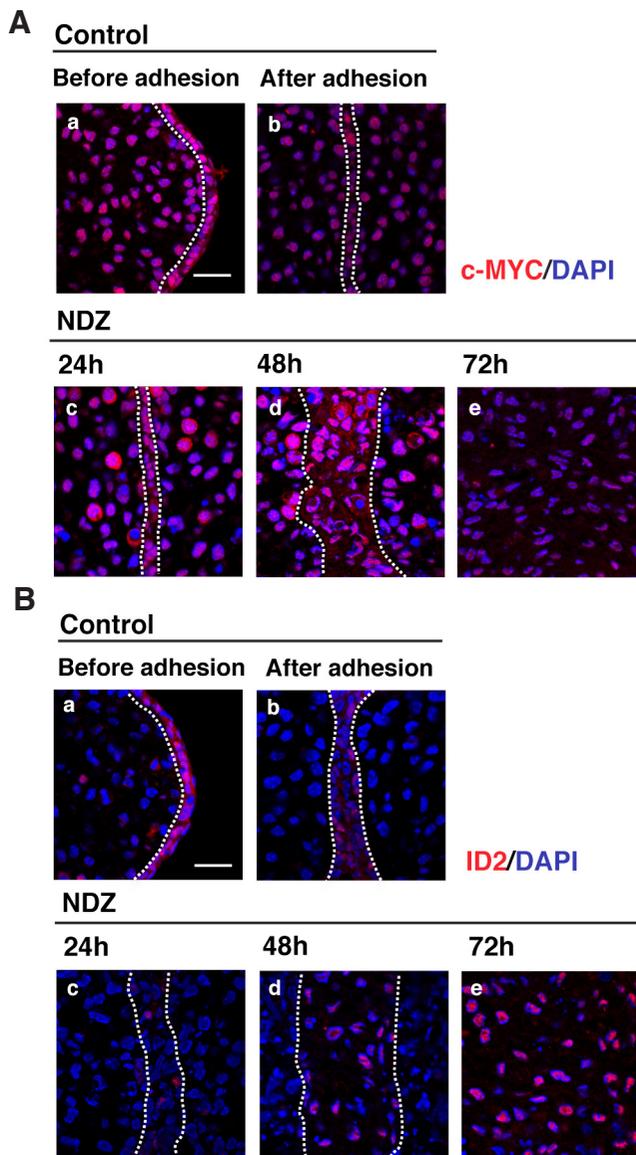
Discussion

E-cadherin and its associated catenin adhesion complex are down-regulated in MEE destined to undergo EMT to accomplish mesenchymal confluence (Schmid, 2002). E-cadherin plays crucial roles in maintaining epithelial cell polarity and integrity. We observed earlier onset of degradation of p120 catenin and α-catenin than of E-cadherin when palatal fusion was examined in control samples.

The stability of E-cadherin at cell junctions is dependent on p120 catenin and α-catenin. p120 catenin stabilizes the adhesion complex at cell-cell junctions via masking the di-leucine motif and tyrosine residues of the juxtamembrane domain of E-cadherin to prevent the clathrin-mediated endocytosis and Hakai-dependent ubiquitination of E-cadherin (Ishiyama *et al.*, 2010). Knockdown of p120 catenin increases the level of E-cadherin endocytosis (Miyashita and Ozawa, 2007). α-catenin links E-cadherin through β-catenin to the actin cytoskeleton to provide stable cell adhesion (Rimm *et al.*, 1995). Loss of α-catenin reduced E-cadherin cell-cell adhesion and the restoration of α-catenin rescued E-cadherin
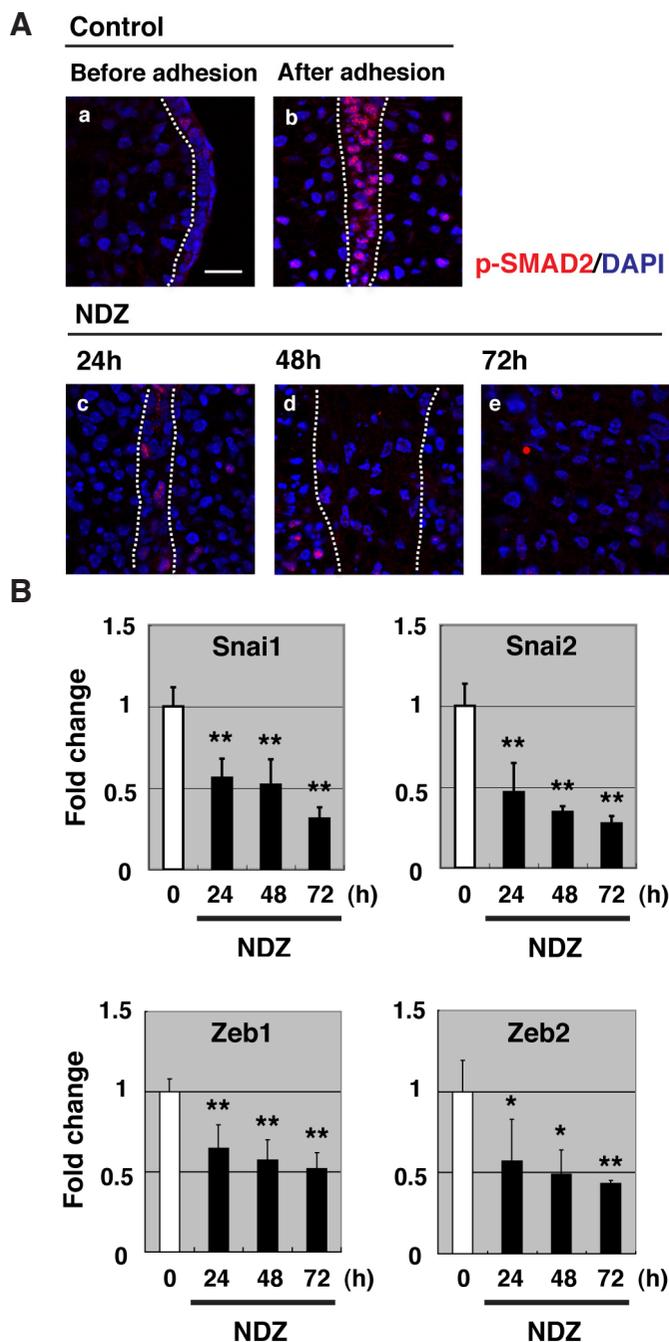
Fig. 4. Microtubule disassembly inhibits TGF-β/SMAD2 signaling resulting in the down-regulation of EMT-related transcription factors. **(A)** *Fluorescent images of phospho-SMAD2 (red) and DAPI (blue). Representative sections shown for control (a: before adhesion, b: after adhesion) and 30 μM NDZ (c: 24 h, d: 48 h, e: 72 h) were taken from palatal tissue. Scale bar, 20 μm.* **(B)** *Quantitative real-time PCR analysis of gene expression encoding Snai1, Snai2, Zeb1 and Zeb2 in palatal tissue treated with 30 μM NDZ for the indicated period. GAPDH was used as reference for normalization.* **P<0.05 and **P<0.01 compared to control.*

Fig. 5. Microtubule disassembly inhibited down-regulation of c-MYC associated with ID2 induction. **(A)** *Fluorescent images of c-MYC (red) and DAPI (blue). Representative sections shown for control (a: before adhesion, b: after adhesion) and 30 μM NDZ (c: 24 h, d: 48 h, e: 72 h) were taken from palatal tissue.* **(B)** *Fluorescent images of ID2 (red) and DAPI (blue). Representative sections shown for control (a: before adhesion, b: after adhesion) and 30 μM NDZ (c: 24 h, d: 48 h, e: 72 h) were taken from palatal tissue. Scale bar, 20 μm.*

cell-cell adhesion (Inge *et al.*, 2008, Shimoyama *et al.*, 1992). The antecedent degradation of both proteins indicates the acceleration of E-cadherin endocytosis and loss of stability of the adhesion complexes in control. In contrast, prolonged treatment with NDZ

induced aberrant accumulation of the adhesion molecule proteins at cell-cell junctions in the MEE over the course of time, which was associated with the altered gene expression. Up-regulation of p120 catenin and E-cadherin transcription by NDZ may provide the means to increase the stability of the adhesion complex at intercellular junctions in the MEE.

The microtubule cytoskeleton plays a critical role in intracellular vesicle transport. Disassembly of microtubules by NDZ has been shown to influence endocytosed and endocytic vesicle transports to various endosomal compartments (Hamm-Alvarez and Sheetz,

A

B
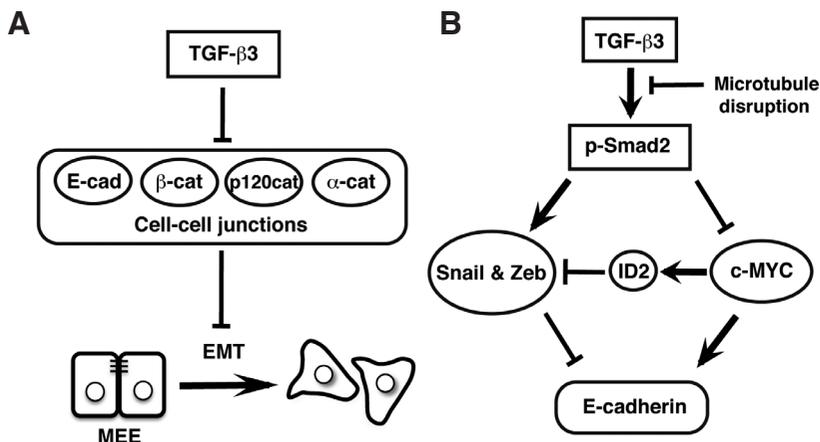
Fig. 6. A proposed model for the underlying mechanism of altered regulation of E-cadherin/catenin adhesion complex by long-term treatment of NDZ. (A) *Cell-cell adhesion complex, E-cadherin and catenins in MEE are to degrade leading to EMT during palatal fusion. TGF-β negatively regulates the E-cadherin/catenin adhesion complex, which maintains epithelial phenotype, at transcriptional and post-transcriptional levels.* **(B)** *The role of microtubule cytoskeleton in the regulation of E-cadherin expression in MEE. Microtubule disruption by NDZ prevents phosphorylation of Smad2 induced by TGF-β during palatal fusion and thereby causes aberrant regulation of E-cadherin transcriptional repressors; Snail and Zeb and an activator; c-MYC leading to accumulation of E-cadherin at cell-cell junction, which resulted in accumulation of E-cadherin at intercellular junctions and failure of EMT.*

tightly regulated positively and negatively by the transcription factors. We therefore speculated that microtubule disruption affects the positive regulatory pathways that result in the induction of E-cadherin mRNA. c-MYC is one of the down-stream transcription factors regulated by TGF-β and reported to be an E-cadherin transcriptional activator (Batsche *et al.*, 1998, Liu *et al.*, 2009, Pietenpol *et al.*, 1990). We have shown that strong c-MYC signals were maintained even at 48 h in the MEE treated with NDZ, which was associated with its down-stream target ID2 induction. It suggests that the positive regulatory signals of c-MYC may control E-cadherin mRNA expression actively and that ID2 induced by c-MYC may affect the E-cadherin negative regulatory pathway via modulation of Zeb expression.

This study investigated the underlying mechanism by which microtubule disruption induced the aberrant accumulation of E-cadherin/catenin adhesion complexes at intercellular junctions in MEE. We evaluated the effect of NDZ on the transcriptional regulation of E-cadherin controlled by TGF-β/SMAD2. Our results provided evidence that microtubule disruption caused aberrant expression of both negative (Snail and Zeb) and positive (c-MYC) E-cadherin transcriptional regulators by blocking the TGF-β/SMAD2 signaling pathway, which resulted in a failure in EMT. The working molecular model we have developed is depicted in Fig. 6. The microtubule cytoskeleton plays an important role to mediate TGF-β/SMAD2 signals to control E-cadherin expression in MEE during palatal fusion. However, we can not exclude the possibility that long-term treatment of NDZ causes the accumulation of E-cadherin/catenin complex independently of TGF-β/SMAD2 signaling pathway since this experimental system does not allow us to conduct rescue experiments, which are dependent on intact microtubule cytoskeleton to activate the signaling pathway, and to provide direct evidence of involvement of TGF-β signaling pathway.

Materials and Methods

Palate organ culture

All animal procedures were performed in compliance with the regulations and guidelines of the Animal Care Committee of The University of British Columbia. Timed-pregnant C57BL6 mice were used in these studies. Palatal shelves were dissected from E13.0 mouse embryos under sterile conditions and placed in pairs on Millipore filters with their medial edges in contact. The palatal shelves were cultured in BGJb medium at 37°C in 5% CO_2 for up to E13.0+72 h. Experimental groups were treated with 30 μM NDZ, whereas littermate control groups were treated only with the DMSO solvent. The culture media was changed every 24 h.

Histology

Cultured palatal shelves were collected after 72 h and fixed in 4% paraformaldehyde/phosphate buffered saline (PFA/PBS). After rinsing with PBS, the tissues were processed for paraffin embedding. After hematoxylin staining, light microscopic images were captured using a Zeiss Axioplan microscope.

Immunofluorescence

After deparaffinization, rehydration and antigen retrieval using EDTA buffer, 7 μm thickness sections were blocked with 3% BSA/0.1% TritonX-100/PBS for 30 min at room temperature to reduce background staining and

1998, Jin and Snider, 1993). The TGF-β/SMAD2 signaling pathway is involved in the negative regulation of E-cadherin transcription during the EMT process (Dave *et al.*, 2011, Pirozzi *et al.*, 2011, Shirakihara *et al.*, 2007). TGF-β signaling is dependent on the membrane traffic of the internalized TGF-β/receptor complex to the early endosome where receptor-mediated SMAD phosphorylation occurs. Subsequently phosphorylated SMAD reaches the nucleus to facilitate transcription of the target genes (Chen, 2009). The SMAD nuclear translocation has been reported to be dependent on microtubules and the associated motor protein, dynein (Jin *et al.*, 2009). Impairment in trafficking of the TGF-β/receptor complex vesicles and activated SMAD by microtubule disassembly may interfere with the MEE disappearance process due to the improper regulation of E-cadherin transcription. We therefore investigated the effect of NDZ on the TGF-β/SMAD2 signaling pathway. Immunofluorescence analysis successfully clarified that prolonged NDZ treatment blocked activation of SMAD2, which suggests the microtubule disassembly prevents the trafficking of the endocytosed TGF-β/receptor complex vesicles to the early endosomes resulting in a failure to mediate the signals in the MEE during palatal fusion. Quantitative real-time PCR also revealed that NDZ inhibited the transcription factors, Snail and Zeb in the MEE. TGF-β/SMAD promotes EMT via induction of transcriptional repressors of E-cadherin including Snail and Zeb (Dave *et al.*, 2011, Jalali *et al.*, 2012, Pirozzi *et al.*, 2011, Shirakihara *et al.*, 2007). These findings indicated that microtubule disruption in the MEE led to a failure to down-regulate E-cadherin transcription via inhibition of the transcriptional repressors secondary to blockage of signaling from the TGF-β/SMAD2 pathway. Results obtained from TGF-β3 null palatal organ cultures supports that the fate of the adhesion complex is dependent on the TGF-β pathway. NDZ however actively up-regulated E-cadherin mRNA expression rather than simply maintaining the level of expression as a consequence of the failure to down-regulate E-cadherin. Gene expression is

incubated with primary antibodies for 2 h at room temperature. Fluorescence-labeled secondary antibodies were added for 1 h at room temperature. To confirm the specificity of antibodies, additional slides were incubated without the primary antibodies. No fluorescence staining was found in these sections. All sections were examined by using a Nikon Laser Scanning Confocal microscope (C1). We measured fluorescence intensity by defining the MEE remaining area manually, and calculated the mean pixel intensity per unit area of MEE remaining.

Quantitative real time PCR

For each time point, mid-regions of the midline from 2 pairs of palatal shelves were pooled, and 3-5 independent pools were analyzed. Total RNA was extracted with the use of RNeasy micro kit (Qiagen) and reverse transcribed with QuantiTect Rev. Transcription kit (Qiagen) according to the manufacturer's guidelines. SYBR-Green based real-time PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad). Primers used in this study were obtained from PrimerBank (Spandidos *et al.*, 2010). GAPDH expression levels were used as reference for normalization.

Statistical analysis

Statistical analyses were done using SPSS11.5 software. Results were evaluated by one-way analysis of variance (ANOVA) to compare the differences of means among multi-group data followed by a Tukey Kramer's post hoc test. Values are presented as means ± SD. $P < 0.05$ was considered to represent significant differences between groups.

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