Nanofibrous topography-driven altered responsiveness to Wnt5a mediates the three-dimensional polarization of odontoblasts

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ARTICLE INFO

Keywords:
Nanofibrous topography
Cytoskeleton
Cdc42
Wnt5a
Odontoblast

ABSTRACT

Cell differentiation with the proper three-dimensional (3-D) structure is critical for cells to carry out their cellular functions in tissues. Odontoblasts derived from neural crest cells are elongated and polarized with the cell process, which is decisive for one directional tubular dentin formation. Here, we report that the fibrous topography of scaffolds directs odontoblast-lineage cells to differentiate to have the 3-D structure of odontoblasts through an altered responsiveness to Wnt family member 5A (Wnt5a). In a pulp-exposure animal model, the scaffolds with the nanofibrous topography supported the regeneration of tubular dentin with odontoblast processes. In cultures of pre-odontoblast cells, the nanofibrous topography heightened the cells on the z-axis. The cells on nanofibrous substrate (FIBER) formed stress fiber cytoskeletons on a conventional tissue culture plate (TCP). Differential activation of Cell division control protein 42 (Cdc42) on FIBER and Ras homolog family member A (RhoA) on TCP led to these differences. The signal from Wnt5a-Cdc42 in the cells on FIBER mediated the phosphorylation of JNK and the polarity growth signaling. Taken together, the nanofibrous topography of the scaffolds led to the 3-D structural differentiation of odontoblasts in vitro and in vivo, implying its application for dentin regeneration. Furthermore, the results on the altered activation of Cdc42 by Wnt5a on FIBER provide evidence that the topography of the scaffolds can cause a distinctive cell responsiveness to their micro-environments.

1. Introduction

Nano-topographic approaches have been developed to simulate physical and biochemical micro-environments that mimic the natural cellular milieu. Physical topographies are associated biologically with cell adhesion. The rationale to these links is the nano- or micro-scale physical topographies of the native ECM. Therefore, structures of engineered scaffolds on relevant scales would be a promising strategy to mimic the natural extracellular matrix (ECM) [1]. A number of approaches, including electrospinning, lithography, embossing, and micromachining, can achieve an engineered ECM with defined topographical features [2]. These approaches can produce micro- or nano-scaled features. The electrospinning technique has gained in popularity due to its ability to produce nanoscale topographies that can influence the cell adhesion, survival, and reorganization outcomes [3,4].

The nanofibrous matrix utilized in tissue engineering has several desirable properties including protein absorption, a high surface area, binding sites for cellular interactions, customized contiguity, the activation of specific intracellular signaling, and different gene expressions. Essentially, these engineered ECM materials can induce cell adhesion, proliferation and differentiation by altering their surface nanotopography. It has been shown that cells respond well to nanotopographic features on synthetic matrix surfaces that induce changes in the cell adhesion and cell differentiation [5–9]. The fibrous structure of electrospin nanofibers can enhance the differentiation of odontoblasts. Previously, it was shown that an electrospun nanofibrous matrix induces the odontogenic differentiation of dental pulp stem cells, specifically dspp expression [6]. Meanwhile, cell differentiation with the proper three-dimensional (3-D) structure is critical for cells to carry out their cellular functions in tissues. Odontoblasts derived from neural crest cells are elongated and polarized with the cell process, which is decisive for one directional tubular dentin formation. We have observed the...
formation of dental tubule-like structures in the teeth of dogs subjected to electrospun nanofiber in an in vivo direct pulp-capping model [10]. Collectively, it can be speculated that the nanotopography of adhesion substrates induces the 3-D structural differentiation of odontoblasts. Previous studies have suggested that cell adhesion leads to cytoskeletal organization which can be controlled by Rho family GTPases [11]. The Rho family of small GTPases consists of RhoA, Rac1, and Cdc42. Among them, Cdc42 induces the formation of filopodia, while Rac1 and RhoA promote the formation of lamellipodia and actin stress fibers, respectively [12–14].

In this study, we investigated the regeneration of the dental tubule structure in a pulp-exposure animal model and the underlying mechanisms on the 3-D polarization of odontoblasts induced by the nanofibrous topography. Methodologically, we implanted electrospun poly(e-caprolactone) nanofibers on the mechanically exposed pulp of the 1st molar of rats. Additionally, we cultured pre-odontoblast MDPC-23 cells on electrospun polystyrene nanofibers (FIBER) and undertook z-axis imaging to examine whether the cells grown on the FIBER could be induced to having the 3-D polarization of odontoblasts for ortho-dentin regeneration by the nanotopographic substrates. Furthermore, we investigated the underlying molecular events by which the nanofibrous topography-induced Wnt5a regulates the rearrangement of the actin-cytoskeleton and cell shapes.

2. Materials and methods

2.1. Preparation of the nanofibrous matrix

A polystyrene nano-fibrous matrix was fabricated by electrospinning. Briefly, the polystyrene beads (Sigma, St. Louis, MO) were dissolved in dimethylformamide (DMF) (Sigma), and a 12% (w/v) polystyrene solution added to a 10-mm syringe with a 30G stainless steel needle. The electric potential and the collector distance were optimized to 30 kV and 20 cm, respectively. The polystyrene nanofiber was collected on a metallic rotating drum. After the electrospinning, the DMF was spread on the dish, and a polystyrene nanofiber sheet was placed over it. The FIBER fixed onto the dish was dried in a vacuum chamber at room temperature. Subsequently, the FIBER on the dish was washed with ethanol (70%) and then dried on a clean bench with UV light overnight at room temperature.

2.2. Animal experiment

For in vivo studies, the mandibular first molars of 6 rats (8-week-old) were operated on. Control and experimental groups were randomly allocated to each animal to avoid possible biases resulting from individual characteristics and pulpal conditions could be eliminated. Investigators performing the animal operation and sampling were not blinded while the investigators that accessed the histological analysis were blinded. All results using animals followed the protocols approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-200512-3-5).

Tooth preparation was performed as previously described [15]. Briefly, after disinfecting the mandibular first molars with 0.5% chlorhexidine, pinpoint pulp exposure was made on the occlusal surface of the mandibular first molars using a round bur (ø = 0.6 mm). After sufficient irrigation, the exposed sites were dried with a cotton pellet. The cavities were either untreated or treated with poly(e-caprolactone) nanofibers. After treatment, the cavities were then filled with a calcium silicate cement (ProRoot) and a glass ionomer cement (Fuji II LC; GC America Inc.). The Unsealed group was unable to maintain FIBER during the recovery period and oral microbiological contamination altered the results. Despite the sealing material's ability to form restorative dentin, FIBER alone cannot suggest results. The samples were cut into blocks after 4 weeks following surgery, fixed in 4% paraformaldehyde, and kept for one day at 4 °C. Decalcification was performed in 10% EDTA (pH 6), and then the samples were embedded in paraffin. Serial sections were used for H&E stained (5-µm-thick).

2.3. Cell culture

The MDPC-23 mice odontoblast-like cell line was used in this study [16]. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (GibcoBRL, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (GibcoBRL, Carlsbad, CA). For each samples, the cells were seeded on non-patterned polystyrene tissue culture plates (TCP) (BD Falcon, Franklin Lakes, NJ) as a control or on the FIBER seeded of 5 × 10^5 cells for each 60-mm dish. After 12-h form cell seeding, the cells were cultured in a differentiation condition (added with 10 mM β-glycerophosphate and 50 µg/ml ascorbic acid (Sigma) in growth medium).

2.4. Scanning electron microscopy

The polystyrene nanofibers were washed with PBS and fixed using 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After freeze-drying, each specimen was dehydrated using serial dipping in increasing concentrations of ethanol, after which each underwent critical point drying. The samples were sputter-coated with a gold-palladium mixture and observed using scanning electron microscopy (SEM) (FE-SEM Hitachi S-4700, Japan) at 12 kV.

2.5. Alizarin red staining

The calcium deposition outcomes of the dental pulp cells on different substrates were analyzed by Alizarin Red S staining. MDPC-23 cells were seeded on six-well cell culture plates without and with the attached FIBER. The next day, the media were changed, and differentiation was allowed to proceed. After seven days of differentiation, the samples were washed three times with cold PBS, fixed with chilled ethanol (70%) for 1 h, washed with deionized water, and stained with 40 mM Alizarin red S (pH 4.2) (Sigma) for 30 min at room temperature. After staining, samples were rinsed with deionized water. Images were captured of the stained samples showing the deposition of calcium.

2.6. Immunostaining and confocal microscopy

To determine the morphological alterations, the cells were seeded on the FIBER or TCP at a density of 1 × 10^5 cells per cm² in 24-well cell culture plates. After incubated the cells 12-h, cells were washed and cultured in differentiation media for 10 days. The cells were washed with PBS and then treated with a freshly prepared working solution of the CellMask™ (Molecular Probes, Eugene, OR) in a warm serum-free media from a 1000X concentrated CellMask™ stain solution for 60 min in an incubator at 37 °C. The cells were washed with PBS and fixed with 4% formaldehyde for 10-min at room temperature, after which they were washed again three times with 1X PBS. The cells were treated with Alexa Fluor™ 488 Phalloidin (Molecular Probes, Eugene, OR) at a dilution of 1:100 for 30 min at room temperature in the dark. After washing the samples with PBS, the samples were mounted using a mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) to identify the nuclei. Confocal laser scanning microscopy images were taken to determine the cell morphology (Zeiss, LSM 700). The images were taken from the bottom to the top of the cells at 3 µm intervals. The experiments were independently repeated three times at least.

2.7. Measurement of the cell height and nuclei polarization

For the cell height and nuclei polarization measurements, the cells were cultured on TCP and FIBER for four days. To determine the cell height, the bottom of the cells was taken at a zero point and adjusted with confocal laser scanning microscopy at a 0.16 µm interval of each stacked
section. The number of stacked intervals from bottom to top for each cell was measured. At the same time, the position of the nucleus was determined at the upper part of the cell based on the height of each respective cell. For quantitative measurements, 100 cells from each group were used for an analysis in each individual experiment, and each experiment was performed at least three times.

2.8. Extraction of total RNAs and RT-qPCR

The cells were cultured on TCP and FIBER in the differentiation medium. Total RNAs were extracted using RNA isoplus reagents (Takara, Kyoto, Japan), and the cDNA was synthesized using a PrimeScritps RT reagent kit (Takara, Kyoto, Japan) according to the manufacturer’s instructions. RT-qPCR was performed on a real-time PCR system using SYBR® Premix Ex TaqTM (Takara, Kyoto, Japan) according to the protocol described in the kit. The relative expression of each target gene transcript was normalized using the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each experiment was performed for three biological replicates. The primers are listed in Supplementary Fig. S11.

2.9. Western blot analysis

The cell lysates were analyzed by western blot to detect the specific proteins. The cell lysates were lysed using a lysis buffer consist of 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 0.2 mM Na3VO4, a phosphatase inhibitor, and a protease inhibitor cocktail tablet (Roche, Basel, Switzerland). The running gels were used for 10–12% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. The membrane blocking performed by 5% skim milk and incubated with each antibody. Immunoreactivity was developed by an enhanced chemiluminescence (ECL) reagent. For the control, anti-β-actin mlgG antibody was used.

2.10. Knockdown assay with RNA interference

ON-TARGET plus SMART pool small interfering RNAs (siRNA) were used to knock down the expression of Wnt5a, Ror2, RhoA, or Cdc42. A scrambled siRNA was used as a negative control. MDPC23 cells were seeded for 5 × 104 cells in 60-mm culture dish with or without FIBER and transfected using transfection reagent with each specific siRNA in accordance with the manufacturer’s guidance (Dharmacon, Lafayette, CO). The cells were harvested after 36 h after the transfection.

2.11. Small GTPases activity assay

The activity of small GTPases was determined using a pull-down assay kit following the protocol described by the manufacturer (Cytoskeleton, Denver, CO). Briefly, the cells were cultured on TCP and FIBER to induce cell differentiation. On the fourth day of differentiation, the cells were harvested and lysed using a protease inhibitor in cell lysis buffer. The lysates were centrifuged for 1 min at 14,000 rpm at 4 °C. Protein quantification measurements were taken using a Pierce BCA assay kit (Thermo Scientific, Rockford, IL), and for each sample, 20 μg of protein lysate were used for the total specific small GTPase protein determination. Equal amounts of protein lysate from the remaining supernatants were incubated with Rhotekin-RBD beads for the RhoA activation assay, and equal amounts of protein lysate were mixed with PAK-PBD beads for the Cdc42 activation assay. The samples were incubated on a rotator for 1 h at 4 °C and centrifuged for 1 min at 3000 rpm, after which the supernatant was removed. After washing the beads once with 300 μl each with a wash buffer, the samples were centrifuged at 4000 rpm at 4 °C for 3 min, and the supernatant was removed, and 30 μl of 2x sample buffer were added to each sample. We then thoroughly resuspended the beads.

The samples were boiled for 2 min and assessed by western blot analysis.

2.12. Statistical analysis

All data are statically analyzed as the average and standard deviation (SD). The statistical methods using ANOVA or Student’s t-test for described significance. Differences between groups were considered significant if the p value was less than 0.05.

3. Results

3.1. Nanofiber implantation induces the regeneration of tubular dentin in a pulp-exposure model

An electrospun nanofibrous scaffold with a diameter in the homogeneous range of 200–300 nm was fabricated from a poly(e-caprolactone) solution (Fig. 1A and B) [4,6]. To examine the effects of the nanofibrous topography on the regeneration of tubular dentin in vivo, pulp exposure was mechanically generated in rat molars which were divided randomly into two groups.

After four weeks, amorphous calcified tissue formation was observed in the pulp cavity of the conventionally treated control group. In contrast, newly formed dentin retaining the physiologic tubule structure was found beneath the defect in the FIBER-implanted group (Fig. 1C). As shown in Fig. 1D, immunohistochemistry showed that a strong expression of dentin sialoprotein (DSP), which is a molecular marker of odontoblast differentiation, was observed along with the dentinal tubules in the FIBER group. Notably, the DSP-expressing cell bodies were present under the newly formed dentin, and the cytoplasm extended to the dentinal tubules in the FIBER group, while DSP-expressing odontoblasts were mainly found below or between the amorphous calcium structures in the control group. The length from the pulp to the tip of newly formed tubule was significantly increased in the histological images of the FIBER group (Fig. 1E). Given that elongation and 3-D polarization proceed along with odontoblast differentiation and the asymmetric disposition of organelles and cytoskeletal arrangements are essential for normal dentin formation [17], this figure indicated that the nanofibrous scaffold supported the 3-D structural differentiation of odontoblasts as well as their marker gene expression.

3.2. Nanofibrous topography induces odontoblast polarisation and actin-based filopodia formation

The dental tubule structure of newly formed dentin in the in vivo pulp exposure model suggests that the fibrous topography induces the apical growth of odontoblasts in the FIBER group. It was investigated whether the intracellular organization is affected by the nanotopographic surface to which the cells adhere. Cell polarization and cellular process formation are essential changes during odontoblast differentiation [18,19]. We observed that the MDPC23 pre-odontoblasts grown on the electrospun FIBER only showed significant increases in polarity and branching (Supplementary Fig. S1), but TCP did not. To confirm whether this phenotype was linked to dentin matrix formation, MDPC23 cells cultured on FIBER exhibited intense alizarin-red staining, indicating that FIBER promoted odontoblast differentiation, consistent with our previous report (Fig. 2A) [18,19]. In the TCP group, actin fibers were arranged in a flat configuration throughout the cytosol and formed stress fibers, while condensed and slender antenna-like actin structures were observed in the FIBER group (Fig. 2B).

Physiological odontoblasts show an increase in the cell height and asymmetrical displacement of the nucleus when the cytoplasm is apical basal polarized [20,21]. As shown in Fig. 2C, we observed that the cell height increased significantly (1.8-fold at day 7) when cultured on FIBER and increased further with the passage of the incubation period. The
Fig. 1. Implantation of nanofibrous scaffolds induced the regeneration of tubular dentin in vivo. Scanning electron micrograph of an electrospun nanofibrous matrix at low and high magnification levels showing the random morphology of the nanofibrous substrate (A) Distribution of the fiber diameter (B) H-E staining showing microscopic views of the control (CON) and nanofiber (FIBER) group. Squares indicate positions at high magnification. Asterisks indicate hard tissue in which dentinal tubules are formed. 20X and 40X means objective magnification. (C) The anti-DSP-immunostaining showing microscopic views of the CON and FIBER groups. 20X and 40X means objective magnification. (D) Quantitative analysis of the cytoplasmic length from the pulp to the tip of the tube (E) (N = 100, each group). Data represent the mean ± SD (*, p < 0.05; ***, p < 0.01).
number of cells that exhibited nuclei at the upper part of the cells was also increased significantly in the cells on FIBER compared to those on the control TCP (Fig. 2D).

In the z-axis images from confocal laser microscopy, it was also observed that the cells on the FIBER group appeared cylindrical and had an asymmetric nuclear upper location compared to those on TCP. The height of MDPC23 cells grown on FIBER was significantly increased on the z-axis. In the early stage of differentiation (day 4), the z-axis growth increased by more than 1.6 times compared to TCP (about 15 and 24 μm for TCP and FIBER, respectively), and at the advanced stage of differentiation (day 10), it was increased by more than 1.8 times (about 15 and 27 μm for TCP and FIBER, respectively) (Fig. 2E and F).

Filopodia leads to the adoption of a polarized morphology of the cells [22]. Odontoblasts connect to other cells via junctional complexes, and increased expressions of zona occludens-1 (ZO-1) and claudin-1 have been observed in mature human odontoblasts [23]. Consistent with the cell elongation, the expression levels of ZO-1 and claudin-1 were significantly increased in the cells on FIBER compared to those on TCP (Supplementary Fig. S2). These results indicate that the nanofibrous topography-induced odontoblast differentiation is accompanied by apico-basal polarized, 3-D structural changes.

3.3. Wnt5a induces the 3-D structural changes and actin-cytoskeleton reorganization of pre-odontoblasts

It has been reported that wnt5a can regulates the growth and patterning of teeth during animal development [24]. However, it has not been specifically studied how Wnt5a works for tooth development. In this study, pre-odontoblasts grown on FIBER increased Wnt5a expression, as confirmed by RT-qPCR and western blot analyses after four days of odontoblastic differentiation (Fig. 3A and B). To address the effect of Wnt5a on 3-D apico-basal polarization, the cells were treated with recombinant Wnt5a (rWNT5a) protein or knockdown (KD) siRNA. The cell height on FIBER was significantly increased compared to that on TCP and further increased by the rWNT5a treatment. Consistently, the KD of the Wnt5a expression through siRNA (si-Wnt5a) successfully reduced the Wnt5a expression and induced a significant decrease in the cell height in the FIBER group (Fig. 3C and Supplementary Figs. S3, S4, and S5). Nuclei
Fig. 3. Wnt5a signaling is involved in the morphological alteration during odontoblast differentiation. Quantitative real-time PCR (A); and western blot analysis (B) showing expression of Wnt5a in MDPC-23 cells on TCP and FIBER in osteogenic media for a four day culture period. The height (C) and nuclei polarization (D) of the cells were determined with the treatment of rWnt5a or by the knockdown of Wnt5a. The cells were cultured on TCP and FIBER and either treated with rWnt5a or transfected with siRNA against Wnt5a (si-Wnt5a), after which they were then fixed and stained with phalloidin (green), DAPI (blue), and cell mask membrane (red) staining, with images taken from the bottom to the top of the cells with a 3 μm interval using confocal microscopy to determine the cell morphology after 4 (E) and 10 days (F) of culturing. Immunofluorescence images of MDPC-23 cells against ZO-1 (G) and the relative mRNA expression levels of ZO-1 and Claudin-1 (H) as determined by quantitative real-time PCR analysis. Data represent the mean ± SD. *Significantly different from the control TCP (p < 0.05). #Significantly different from the si-control group (p < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
polarization to the upper part of the cells was increased in cells on FIBER, and this polarization was further increased by the rWNT5a treatment and decreased by KD against Wnt5a (Fig. 3D). When the TCP group was treated with rWNT5a, actin stress fibers became stronger, and the cell height did not change notably (Fig. 3E). When the FIBER group was treated with rWNT5a, the formation of filopodia was significantly increased. Additionally, repression of Wnt5a reduced the cell height along with the dissociation of actin fibers (Fig. 3F). These results suggest that Wnt5a would be closely related to the induction of the 3-D structural changes and actin reorganization.

Molecular markers of planar cell polarization such as Vang1, Scrib1, Prickle1 and Ptk7 [25–27] were increased by the rWNT5a treatment, and the nanotopographic structure exacerbated these changes (Supplementary Fig. S10). When the TCP group was treated with rWNT5a, the expression of ZO-1 was mostly located in the perinuclear area. Meanwhile, the ZO-1 in the cells on FIBER further spread toward cell membranes with rWNT5a treatment, evidence by cytoplasmic colocalization of actin filament and ZO-1(Fig. 3G). The expression levels of ZO-1 and Claudin-1 was increased further with the rWNT5a treatment in cells on FIBER compared to those in TCP and significantly decreased with the KD of Wnt5a (Fig. 3H).

3.4. Ror2 mediates the Wnt5a-induced 3-D structural alteration of pre-odontoblasts

Ror2, an orphan tyrosine kinase, is a transmembrane receptor known to mediate Wnt5a-initiated cell migration and filopodia formation [28, 29]. To verify whether Ror2 is involved in Wnt5a-induced polarized alterations and actin cytoskeleton reorganization, we knocked down Ror2 gene expression using the siRNA of Ror2 (si-Ror2). The cell height was decreased in the Ror2 KD group compared to the si-control treated cells on FIBER. Moreover, the additional rWNT5a treatment could not rescue the Ror2 knockdown effect on the cell height. Actin stress fiber formation in cells on TCP was absent in si-Ror2 without or with the rWNT5a treatment (Supplementary Fig. S6). It was confirmed a Ror2 association with Wnt5a-induced actin-based filopodia formation in cells on FIBER and demonstrated that filopodia protrusions were completely absent during the Ror2 knockdown in samples both without and with the rWNT5a treatment (Fig. 4A). A quantitative analysis of the cell height and nuclei polarization outcomes also showed the consistency with filopodia formation (Fig. 4B and C). As shown in Fig. 4D and E, the knockdown of Ror2 by siRNA abrogated the expression levels of the tight junction molecules of ZO-1 and Claudin-1. Moreover, cells exhibited similar effects on ZO-1 and Claudin-1 mRNA expressions after the knockdown of Ror2 even with Wnt5a treatment.

It has been shown that irrespective of Wnt5a stimulation, the overexpression of Ror2 can induce filopodia formation by actin reorganization and that the knockdown of Ror2 disrupts the formation of filopodia in HEK293T cells [30]. To knock down the Ror2 gene, we used siRNA against Ror2 and determined the knockdown effect by western blot analysis (Supplementary Fig. S4). These results suggest that Ror2-mediated Wnt5a is involved in 3-D polarized changes, which was confirmed by the inhibition of a functioning noncanonical Wnt signaling pathway.

3.5. Cdc42 and RhoA were antagonistically activated by a reciprocal reduction mechanism

Rho family small GTPases have been revealed as essential regulators of the actin cytoskeleton system and can be activated by Wnt5a/Ror2 [31]. It has been documented that the Rho family of small GTPases, such as RhoA, can regulate the cell shape through cytoskeleton reorganization to form stress fibers [13]. Another of the small GTPases, Cdc42, has important roles in the organization of filopodia structures and in maintaining cell polarization [13,32].

As shown in Fig. 5A, the cell height and nuclei polarization were rather increased in the cells on FIBER after the knockdown of RhoA (si-RhoA), while the cells on TCP did not show any significant alterations in the cellular height or nuclei polarization. We examined the involvement of Cdc42 in the Wnt5a-induced 3-D polarized alterations and actin-based filopodia formation. The cell height was decreased after the knockdown of Cdc42 (si-Cdc42) in the cells on the nanofibrous matrix, and a similar effect was observed with regard to nuclei polarization. Interestingly, the formation of long actin stress fibers was increased in cells on TCP after Cdc42 knockdown and was further increased with the rWNT5a treatment (Supplementary Fig. S8).

During the si-RhoA treatment, the cells on FIBER exhibited an increased filopodia formation, and the cells on TCP exhibited a decreased stress fiber formation (Fig. 5B and C and Supplementary Fig. S7). These alterations of the cell height and nuclei polarization during the knockdown against RhoA were further enhanced in cells on FIBER upon treatment with rWNT5a (Fig. 5D and E).

The knockdown effect of Cdc42 on the cell height and nuclei polarization in the cells on FIBER could not be rescued by the rWNT5a treatment (Fig. 5F and G). Our results showed that filopodia formation was completely absent in cells on FIBER after the knockdown of Cdc42 and that the treatment of rWNT5a did not rescue the effect of the cdc42 knockdown in cells on FIBER (Fig. 5H and I).

3.6. Nanofibrous topography induces the 3-D polarization of odontoblasts through an altered responsiveness to Wnt5a that activates Cdc42

Small GTPases RhoA and Cdc42 may act contrary to each other because the up-regulation of Cdc42 leads to filopodia formation which is accompanied by RhoA down-regulation [33]. To confirm the pivotal role of the nanotopography during the Wnt5a signaling to Cdc42 or RhoA, we conducted pull-down assays. We observed highly active RhoA in the cells on TCP, for which the level was further increased by the rWNT5a treatment (Fig. 6A), while the active Cdc42 was not detected in the TCP group. In contrast, in the FIBER group, the active Cdc42 was high and further increased by the rWNT5a treatment, while active RhoA was not observed in the FIBER group (Fig. 6B). Additionally, Cdc42 and RhoA were antagonistically activated by a reciprocal reduction mechanism. In the FIBER group, knockdown against RhoA induced an increase in Cdc42 activation. On the other hand, knockdown against Cdc42 increased the amount of activated RhoA in both groups (Fig. 6C and D).

The activation of Cdc42 induced the activation of c-Jun N-terminal kinases (JNK), including the MAPK pathway [34]. Interestingly, in the polarized MDPC cells on FIBER, the active Cdc42 increased the phosphorylation of JNK and translocation to nucleus. In addition, the WNT5a treatment further enhanced the activation of JNK signaling (Fig. 6E, Supplementary Fig. S9). Moreover, the expression of planar cell polarity molecules was significantly increased in the FIBER group (Supplementary Fig. S10).

Taken together, it was demonstrated that the FIBER-induced Wnt5a regulated the activation of Cdc42 and that this activation was further increased by the rWNT5a treatment. The cells on TCP showed RhoA activation. These results indicate that the nanofibrous topography was closely related to distinctive activation of Cdc42, which prepared cells for the altered responsiveness to Wnt5a and led to 3-D polarization with filopodia, suggesting that the Wnt5a-Ror2/Cdc42 signaling pathway induces the 3-D structural differentiation of odontoblasts (Fig. 6F).

4. Discussion

Cells derived from common MSCs and differentiated into distinct lineages exhibit various specific morphologies. Adipocyte cells are fat-laden and have a round shape [35], and osteoblast cells range from cuboidal to elongated depending on the matrix deposition activity [36]. These specific morphologies of cells appear to be optimized for their functions in tissue. Odontoblasts exhibit a polarized and tall columnar shape [37]. With regard to dentin formation, cell polarization and the
Fig. 4. Non-canonical Wnt5a signaling is functional through Ror2 during the morphological alteration of MDPC-23 cells. After the knockdown of Ror2 by siRNA, the cells were treated with rWNT5a and immunostained with phallolidin (green), a cell mask membrane (red), and DAPI (blue) to determine the morphology of the cells (A). The height of the cells (B) and the nuclei polarization of cells (C) were determined after four days of culturing. ZO-1 (D) and Claudin-1 (E) gene expression levels were determined through quantitative real-time PCR analyses. Data represent the mean ± SD. *Significantly different from the control ($p < 0.05$). #Significantly different from the si-control group ($p < 0.05$). n. s.: not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Fig. 5. The substrate topography regulates small GTPases activity differentially, which in turn affects cell differentiation. MDPC-23 cells were cultured on FIBER, then transfected with siRNA against RhoA or Cdc42, and allowed to differentiate further with or without the rWNT5a treatment for up to four days. Confocal micrograph of cells from the bottom to top of the cells with a 3 μm interval were taken using confocal microscopy, showing the morphology of the cells (A). The height of the cells after the knockdown of RhoA (B) and with the treatment of rWNT5a (D) and nuclei polarization after the knockdown of RhoA (C) and with the treatment of rWNT5a (E) were measured quantitatively using confocal microscopy. The height of the cells after the knockdown of Cdc42 (F) and with the treatment of rWNT5a (H) and nuclei polarization after knockdown of Cdc42 (G) and with the treatment of rWNT5a (I) was measured using the same method. *Significantly different from the control (p < 0.05). #Significantly different from the si-control group (p < 0.05).
columnar shape facilitate one directional matrix deposition by odonto-
blasts. The cell morphologies are believed to arise from alterations in
cytoskeletal proteins re-organization [38]. Our study investigated the
effects of a nanotopography using a nano-fi-
brous matrix on the morpho-
logical changes and on actin reorganization during odontoblast differ-
entiation via Wnt5a and the activated
Cdc42 [18,39].

Several signaling pathways and different transcription factors regu-
late the differentiation of odontoblasts leading to tooth formation during

the development process. Among them, bone morphogenetic proteins
(BMP), Wnts, Hedgehog family proteins (Hh), and fibroblast growth
factors (FGF) have vital roles in tooth development. Alterations of these
pathways during odontogenesis can disrupted tooth development. The
evidence from animal models indicates that the Wnt signaling pathway
has an important role in tooth morphogenesis and that continuous tooth
generation is induced by activated Wnt signaling [18,39]. Wnt5a is the
most widely studied member of the Wnt family proteins and is critical

Fig. 6. Nanofiber-induced Wnt5a regulates actin-based filopodia formation through the Cdc42/JNK axis. The activations of the Rho family of small GTPases RhoA (A) and Cdc42 (B) were increased upon the rWNT5a treatment on TCP and FIBER. RhoA and Cdc42 activate each other complimentary, as determined by a pull-down assay analysis of cells on TCP and FIBER and confirmed by the knockdown of RhoA and Cdc42 (C, D). Immunofluorescence images of MDPC cells showed an increase in the phosphorylation of the JNK level on FIBER and a further increase upon treatment with rWNT5a (E). Schematic illustration of Wnt5a-induced apical polarization of MDPC through the Cdc42/JNK axis (F).
during the developmental process of various organs. The Wnt5a knock-out mice exhibit small and abnormal patterned teeth with delayed odontoblast differentiation [18]. Wnt5a has gained importance with regard to its role in odontoblast differentiation in mice and humans and is involved in various cellular functions through the regulation of multiple signaling pathways [18,40,41]. It may be possible that cells that exhibit actin-based filopodia protrusions can carry Wnt proteins to the neighboring cells to induce odontoblast differentiation [42]. Moreover, small GTPases are therefore ideally placed to facilitate a significant feature of non-canonical Wnt signaling to induce polarization and actin cytoskeleton reorganization, leading to changes in the cell morphology.

An important part of tissue engineering is to create a more favorable ECM microenvironment to guide cell differentiation and tissue regeneration. The topography of synthetic substrates has been shown to guide the differentiation of stem cells [43,44]. The ability to mimic the physical and mechanical properties of the natural ECM is an essential requirement for tissue engineering applications [45,46]. Nanofibrous matrices hold great potential for a mimetic natural ECM, which can modulate cell responses, leading to tissue regeneration [46,47]. Recent progress in nanofabrication techniques are significant and widely used to construct substrates of differing topographies that mimic a fibrillar structure of natural ECM, providing essential support for cellular functions. Using two different materials, in animal experiment were treated with poly(ε-caprolactone) nanofibers, but in vitro experiment were conducted on polystyrene nanofiber can be suggest the apical growth of odontoblast in vitro and vivo occurred due to the difference in the 3-D topology of nanofibers, not the properties of the materials.

We demonstrated that FIBER induced Wnt5a expression followed by odontoblast polarization, which exhibited increased heights and exhibited a columnar appearance with increased actin-based filopodia formations. The junctional ZO-1 and transmembrane claudin-1 expression levels were also increased in cells on FIBER. The actin-based filopodia formation on the nanofibrous matrix was found to be under the control of Cdc42 activation, while stress fiber formation on TCP was connected to the activated RhoA. All these features on FIBER were further augmented by the recombinant Wnt5a protein and abrogated by siRNAs against Wnt5a, Ror2, or Cdc42. Taken together, it was found that the nanofibrous topography-driven altered responsiveness of small GTPase Cdc42 to Wnt5a mediates the three-dimensional polarization of odontoblasts.

5. Conclusion

Our results demonstrate that nano-topographical cues in the form of a nanofibrous substrate can significantly induce the 3-D structural differentiation of odontoblasts. In a pulp-exposure model, the nanofibrous scaffold supported the 3-D structural differentiation of odontoblasts as well as their marker gene expression. It was confirmed that MDPC-23 pre-odontoblast cells grown on FIBER had a higher cell height, a more asymmetrically located nucleus and cell processes than those on TCP; hence, FIBER provided a more favorable guide for dentin regeneration. Mechanistically, the nanofibrous topography induced the distinctive activation of Cdc42, which prepared cells for the altered responsiveness to Wnt5a and led to a specific differentiated morphotype. This study using nanofibrous substrates also implicates that the topography of scaffoldings is closely related to the altered cell responsiveness to their micro-environment and cell adhesion.

Credit author statement

Saeed Ur Rahman: Investigation, Methodology, Visualization, Writing – original draft. Woo-Jin Kim: Investigation, Validation, Visualization, Writing – original draft. Shin Hye Chung: Resources. Kyung Mi Woo: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

Authors would like to acknowledge the financial support from National Research Foundation of Korea (NRF-2018R1A5A224418, NRF-2019R1A2C2008113).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mtbio.2022.100479.

References

[1] M. Akhmanova, E. Osidak, S. Domogatsky, S. Rodin, A. Domogatskaya, Physical, spatial, and molecular aspects of extracellular matrix of in vivo niches and artificial scaffolds relevant to stem cells research, Stem Cell. Int. 2015 (2015) 1–35, https://doi.org/10.1155/2015/167025.

[2] J.J. Norman, T.A. Desai, Methods for fabrication of nanoscale topography for tissue engineering scaffolds, Ann. Biomed. Eng. 34 (2006) 89–101, https://doi.org/10.1007/s10439-005-9005-4.

[3] J. Quirós, K. Boltes, R. Rosal, Bioactive applications for electrospun fibers, Polym. Rev. 56 (2016) 631–667, https://doi.org/10.1080/15583724.2015.1136641.

[4] Q. Yao, J.G. Cosme, T. Xu, J.M. Miszk, P.H. Piccioli, H. Fong, H. Sun, Three dimensional electrospun PCL/PHA blend nanofibrous scaffolds with significantly improved stem cells osteogenic differentiation and cranial bone formation, Biomaterials 115 (2017) 115–127, https://doi.org/10.1016/j.biomaterials.2016.11.018.

[5] R.J. McMurray, N. Gadegaard, P.M. Taimbouri, K.V. Burgess, L.E. McNamara, R. Tare, K. Muravski, E. Kingham, R.O. Oref, M.J. Dalby, Nanoscale surfaces for the long-term maintenance of mesenchymal stem cell phenotype and multipotency, Nat. Mater. 10 (2011) 637.

[6] S.U. Rahman, J.H. Oh, Y.-D. Cho, S.H. Chung, G. Lee, J.-H. Baek, H.-M. Ryoo, K.M. Woo, Fibrous Topography-Potentiated Canonical Wnt Signaling Directs the Odontoblastic Differentiation of Dental Pulp-Derived Stem Cells, ACS applied materials & interfaces, 2018, pp. 17526–17541, https://doi.org/10.1021/acsami.7b19762.

[7] J. Wang, X. Liu, X. Jin, H. Ma, J. Hu, L. Ni, P.X. Ma, The odontogenic differentiation of human dental pulp stem cells on nanofibrous poly (L-lactic acid) scaffolds in vitro and in vivo, Acta Biomater. 6 (2010) 3856–3863.

[8] E.K. Yim, S.W. Pang, K.W. Leong, Synthetic nanstructures inducing differentiation of human mesenchymal stem cells into neuronal lineage, Exp. Cell Res. 313 (2007) 1820–1829.

[9] E.K. Yim, E.M. Darling, K. Kulangara, F. Gualk, K.W. Leong, Nanotopography-induced changes in focal adhesions, cytoskeletal organization, and mechanical properties of human mesenchymal stem cells, Biomaterials 31 (2010) 1299–1306.

[10] W. Lee, J.H. Oh, J.C. Park, H.L. Shin, J.H. Baek, H.M. Ryoo, K.M. Woo, Performance of electrospun poly(ε-caprolactone) fiber meshes used with mineral trioxide aggregate in a pulp capping procedure, Acta Biomater. 8 (2012) 2986–2995, https://doi.org/10.1016/j.actbio.2012.04.032.

[11] X.D. Ren, W.B. Kissos, M.A. Schwartz, Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton, EMBO J. 18 (1999) 578–585.

[12] R. Sendjela, W. Jiang, G.-C. Chen, M. Carto, J. Settlement, Modulation of Rho GTPase signaling regulates a switch between adipogenesis and myogenesis, Cell 113 (2003) 147–158.

[13] S. Etienne-Manneville, A. Hall, Rho GTPases in cell biology, Nature 420 (2002) 629.

[14] M.L. Kutys, K.M. Yamada, An extracellular-matrix-specific GEF–GAP interaction regulates Rho GTPase crosstalk for 3D collagen migration, Nat. Cell Biol. 16 (2014) 909.

[15] Y.S. Lee, Y.H. Park, D.S. Lee, Y.M. Se, J.H. Lee, J.H. Park, H.W. Chaung, S.H. Park, W.J. Shon, J.C. Park, Tubular dentin regeneration using a CPNE7-derived functional peptide, Materials 13 (2020), https://doi.org/10.3390/ma13204618.

[16] C.T. Hanks, Z.L. Sun, D.N. Fang, C.A. Edwards, J.C. Wataha, H.H. Ritchie, W.T. Butler, Cloned 376 cell line from CD-1 mouse fetal molar dental papilla, Connect. Tissue Res. 37 (1998) 233–249, https://doi.org/10.1080/0300829890024424.

[17] X. Yuan, X. Cao, S. Yang, IFB10 is required for stem cell proliferation, differentiation, and odontoblast polarization during tooth development, Cell Death Dis. 10 (2019) 63, https://doi.org/10.1038/s41419-018-0951-9.
[18] M. Lin, L. Li, C. Liu, H. Liu, F. He, F. Yan, Y. Zhang, Y. Chen, Wnt5a regulates growth, patterning, and odontoblast differentiation of developing mouse tooth, Dev. Dynam. 240 (2011) 432–440.

[19] L. Täderhane, S. Koivumäki, V. Paakkonen, J. Iiviesuo, Y. Soini, T. Salo, K. Metsäkön, J. Tuukkanen, Polarity of mature human odontoblasts, J. Dent. Res. 92 (2013) 1011–1016.

[20] P. Hilkens, P. Gervois, V. Fanton, J. Vanomelingen, W. Martens, T. Struys, C. Politi, I. Lambrichts, A. Bronckaers, Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells, Cell Tissue Res. 352 (2013) 65–78.

[21] T. Kökten, T. Becavin, L. Keller, J.-A. Weikert, S. Kuchler-Bopp, H. Lesot, Immunomodulation stimulates the innervation of engineered tooth organ, PLoS One 9 (2014), e86011.

[22] M.K. Hoelzle, T. Svitkina, The cytoskeletal mechanisms of cell-cell junction formation in endothelial cells, Mol. Biol. Cell 23 (2012) 310–323.

[23] J. Xu, M. Shao, H. Pan, H. Wang, L. Cheng, H. Yang, T. Hu, Novel role of zonula occludens-1: a tight junction protein closely associated with the odontoblast differentiation of human dental pulp cells, Cell Biol. Int. 40 (2016) 787–795, https://doi.org/10.1002/cbi.201617.

[24] M. Lin, L. Li, C. Liu, H. Liu, F. He, F. Yan, Y. Zhang, Y. Chen, Wnt5a regulates growth, patterning, and odontoblast differentiation of developing mouse tooth, Dev. Dynam. 240 (2011) 432–440, https://doi.org/10.1002/ddy.22550.

[25] S.M. Joa, V.E. Arana-Chavez, Expression of connexin 43 and ZO-1 in differentiating ameloblasts and odontoblasts from rat molar tooth germs, Histochem. Cell Biol. 119 (2003) 21–26.

[26] J. Xu, M. Shao, H. Pan, H. Wang, L. Cheng, H. Yang, T. Hu, Novel role of zonula occludens-1: a tight junction protein closely associated with the odontoblast differentiation of human dental pulp cells, Cell Biol. Int. 40 (2016) 787–795, https://doi.org/10.1002/cbi.201617.

[27] M. Hoshino, S. Hashimoto, T. Muramatsu, M. Matsuki, H. Ogiuchi, M. Shimono, Claudin rather than occludin is essential for differentiation in rat incisor odontoblasts, Oral Dis. 14 (2008) 606–612.

[28] M. Nishita, S.K. Yoo, A. Nomachi, S. Kani, N. Sougawa, Y. Ohta, S. Takada, A. Kikuchi, Y. Minami, Filopodia formation mediated by receptor tyrosine kinase Ror2 is required for Wnt5a-induced cell migration, J. Cell Biol. 175 (2006) 555–562, https://doi.org/10.1083/jcb.200607127.

[29] A. Nomachi, M. Nishita, D. Inaba, M. Enomoto, M. Hamasaki, Y. Minami, Receptor tyrosine kinase Ror2 mediates Wnt5a-induced polarized cell migration by activating c-Jun N-terminal kinase via actin-binding protein filamin A, J. Biol. Chem. 283 (2008) 27973–27981, https://doi.org/10.1074/jbc.M802325200.

[30] M. Nishita, S.K. Yoo, A. Nomachi, S. Kani, N. Sougawa, Y. Ohta, S. Takada, A. Kikuchi, Y. Minami, Filopodia formation mediated by receptor tyrosine kinase Ror2 is required for Wnt5a-induced cell migration, J. Cell Biol. 175 (2006) 555–562.

[31] X. Wu, T. Yan, L. Hao, Y. Zhu, Wnt5a induces ROR1 and ROR2 to activate RhoA in esophageal squamous cell carcinoma cells, Cancer Manag. Res. 11 (2019) 2803–2815, https://doi.org/10.2147/CMAR.S190099.

[32] D. Bar-Sagi, A. Hall, Ras and Rho GTPases: a family reunion, Cell 103 (2000) 227–238.

[33] L.A. Roberts, H. Glenn, C.S. Hahn, B.S. Jacobson, Cdc42 and RhoA are differentially regulated during arachidonate-mediated Hella cell adhesion, J. Cell. Physiol. 196 (2003) 196–205.

[34] N. Lamarche, N. Tapon, L. Stowers, P.D. Burbelo, P. Aspanstrom, T. Bridges, J. Chant, A. Hall, Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade, Cell 87 (1996) 519–529, https://doi.org/10.1016/s0092-8674(00)81371-9.

[35] F.M. Gregoire, C.M. Smas, H.S. Sul, Understanding adipocyte differentiation, Physiol. Rev. 78 (1998) 783–809.

[36] V.I. Sikavinis, J.S. Temenoff, A.G. Mikos, Biomaterials and bone mechanotransduction, Biomaterials 22 (2001) 2581–2593.

[37] N. Kawashima, T. Okiji, Odontoblastic specialized hard-tissue-forming cells in the dentin-pulp complex, Congenital. Anom. 56 (2016) 144–153.

[38] P.-Y. Wang, Y.-S. Lian, R. Chang, W.-H. Liao, W.-S. Chen, W.-B. Tsai, Modulation of PEI-mediated gene transfection through controlling cytoskeleton organization and nuclear morphology via nanogrooved topographies, ACS Biomater. Sci. Eng. 3 (2017) 3283–3291.

[39] L. Peng, G. Dong, P. Xu, L. Ren, C. Wang, M. Aragon, X. Zhou, L. Ye, Expression of Wnt5a in tooth germs and the related signal transduction analysis, Arch. Oral Biol. 55 (2010) 108–114.

[40] M.T. Veeman, J.D. Axelrod, R.T. Moon, A second canon: functions and mechanisms of j-catenin-independent Wnt signaling, Dev. Cell 5 (2003) 367–377.

[41] L. Peng, L. Ren, G. Dong, C. Wang, P. Xu, L. Ye, X. Zhou, Wnt5a promotes differentiation of human dental papilla cells, Int. Endod. J. 43 (2010) 404–412.

[42] E. Stanganello, A.I. Hagemann, B. Mattes, C. Sinner, D. Meyen, S. Weber, A. Schug, E. Raz, S. Scholpp, Filopodia-based Wnt transport during vertebrate tissue patterning, Nat. Commun. 6 (2015) 5846.

[43] B. Trappmann, J.E. Gautrot, J.T. Connelly, D.G. Strange, Y. Li, M.L. Oyen, M.A.C. Stuart, H. Boehm, B. Li, V. Vogel, Extracellular-matrix tethering regulates stem-cell fate, Nat. Mater. 11 (2012) 642.

[44] J. Fu, Y.-K. Wang, M.T. Yang, R.A. Desai, X. Yu, Z. Liu, C.S. Chen, Mechanical regulation of cell function with geometrically modulated elastomeric substrates, Nat. Methods 7 (2010) 733.

[45] D.E. Discher, D.J. Mooney, P.W. Zandstra, Growth factors, matrices, and forces combine and control stem cells, Science 324 (2009) 1673–1677.

[46] M. Lutolf, J. Hubbell, Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering, Nat. Biotechnol. 23 (2005) 47.

[47] X. Gao, J. Song, Y. Zhang, X. Xu, S. Zhang, P. Ji, S. Wei, Bioinspired design of polycaprolactone composite nanofibers as artificial bone extracellular matrix for bone regeneration application, ACS Appl. Mater. Interfaces 8 (2016) 27594–27610.