Sainan Wang, PhD, Guibin Huang, BS, and Yanmei Dong, PhD

Directional Migration and Odontogenic Differentiation of Bone Marrow Stem Cells Induced by Dentin Coated with Nanobioactive Glass

SIGNIFICANCE

We used nanobioactive glass–coated dentin to induce stem cells' directional migration and differentiation into odontoblasts, which helps regenerate the physiological cell type and tissue structure during pulp-dentin regeneration.

ABSTRACT

Introduction: This study aimed to use nanobioactive glass (nBG) to guide the directional migration of stem cells and odontogenic differentiation on primary dentin, which are important for the functional regeneration of pulp-dentin tissue. **Methods:** Human bone marrow stem cells (BMSCs) were cocultured with 0.5 mg/mL nBG. The cell-biomaterial interaction was monitored using the IncuCyte S3 live cell imaging system (Essen BioScience, Ann Arbor, MI). The adhesion and morphology of BMSCs growing on nBG-coated dentin were assessed at 2 hours and 3 days. The chemotaxis effect of nBG-coated dentin on BMSCs was tested using a 3-dimensional collagen gel model. Subcutaneous transplantation of nBG-treated dentin slices into nude mice was used to investigate cell homing and odontogenic differentiation *in vivo*. **Results:** nBG particles showed good biocompatibility, and they were gradually degraded and relocated during interactions with BMSCs. BMSCs had better initial attachment to an nBG-coated dentin surface than to an untreated dentin surface. Cell migration assays showed that nBG-coated dentin induced significantly more cell migration than untreated dentin. An *in vivo* study revealed that nBG-coated dentin slices facilitated recellularization and revascularization in the root canal and that dentin sialophosphoprotein-positive cells were detected on the surface of the primary dentin. **Conclusions:** nBG recruits stem cells to move toward dentin and further promotes cell adhesion and odontogenic differentiation on primary dentin, which help regenerate the biomimetic structure of pulp-dentin tissue. *(J Endod 2020;46:216–223.)*

KEY WORDS

Bioactive glass; chemotaxis; odontogenesis; pulp regeneration

The regeneration of living pulp tissue that is structurally and functionally identical to the original tissue is an ideal therapeutic solution for pulpal necrosis. Strategies to revitalize affected teeth include pulp revascularization, a cell-free approach that relies on an injectable scaffold functionalized with active molecules[1,2], and a cell-based strategy based on implanted stem/progenitor cells[3,4]. From a functional point of view, regenerative strategies should focus on the structure of the regenerated tissue. However, even if new vital tissue is induced to grow into the pulp space by some pulp regeneration procedures, animal models have revealed that most of the ingrowing tissue resembles bone, the periodontal ligament, or cementlike structures rather than pulp-dentin tissues[5].

To induce regeneration of the pulp-dentin complex, either recruited or implanted stem cells must differentiate into all of the appropriate types of cells, including, in particular, odontoblasts. The regeneration of a fully differentiated odontoblast layer at the pulp-dentin interface is crucial because only newly formed dentin deposited on primary dentin can increase the mechanical resistance of the root and rebuild the dental pulp defense system. In particular, root growth and dentinal wall thickening are the pivotal goals of endodontic treatment of immature teeth with an open apex and thin, fragile dentin walls. Therefore, it is imperative to find ways to induce stem cells to migrate, differentiate into odontoblasts, and deposit a new dentin layer along the primary dentin wall.

In a previous study, we found that bioactive glasses (BGs) induced odontogenesis and promoted dentin formation *in vivo*[6,7]. Furthermore, BGs, especially on the nanometer scale, are known to bond to dentin and induce dentin mineralization via the formation of an apatite layer[8,9]. Therefore, BGs may be good candidates for controlling the fate of stem cells in the root canal space.

Form the Department of Cariology and Endodontology, Peking University School and Hospital of Stomatology, Beijing, China

Address requests for reprints to Dr Yanmei Dong, Department of Cariology and Endodontology, Peking University School and Hospital of Stomatology, Beijing 100081, China.
E-mail address: kqdongyanmei@bjmu.edu.cn
0099-2399/$ - see front matter

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Dentin conditioning may have a profound effect on cellular behavior at the cell-dentin interface[10]. EDTA treatment of dentin removes the smear layer, exposes collagen fibrils, and releases growth factors entrapped in the dentin matrix[11]. EDTA treatment of dentin improves cellular adhesion and differentiation during pulp regeneration procedures[12]. Using bioactive and biodegradable materials to coat dentin has rarely been reported. In this study, nanobioactive glass (nBG) was used to coat EDTA-treated dentin to improve the bioactivity of the dentin surface.

The objective of the present study was to investigate whether BGs affect stem cell distribution and tissue regeneration during the regeneration of the pulp-dentin complex. We used nBG to induce stem cell migration and attachment to primary dentin; we also evaluated the effects of nBG-coated dentin on cell homing and odontogenic differentiation *in vivo*.

MATERIALS AND METHODS

Preparation and Characterization of nBG

nBG sol-gel particles (molar composition: 58% SiO_2, 33% CaO, and 9% P_2O_5) were prepared according to the procedure reported in a previous study[6]. A scanning electron microscope (SU8010; Hitachi, Tokyo, Japan) and a transmission electron microscope (JEM-2100F; JEOL, Tokyo, Japan) showed that nBG exhibited a spherical morphology with a particle size of ~20 nm (Fig. 1*A* and *B*). Energy-dispersive X-ray spectroscopy (SU8010, Hitachi) showed that the surfaces of nBG were composed of large amounts of silicon, some calcium, and phosphate (Fig. 1*C*).

Cell Culture

Human bone marrow stem cells (BMSCs; PCS-500-012, American Type Culture Collection [ATCC], Manassas, VA) were cultured in mesenchymal stem cell human basal medium (PCS-500-030, ATCC) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, St Louis, MO) and the Mesenchymal Stem Cell Growth Kit (PCS-500-041, ATCC). A total of 3 independent samples of BMSCs (donor age range: 20–35 years) were used. The cells were incubated in a humidified incubator with 5% CO_2 at 37°C. The BMSCs were passaged at 80% confluence, with the medium changed every 2 days. Cells at fewer than 4 passages were used in all experiments.

Interaction of nBG with BMSCs

After high-temperature sterilization, nBG powders were prepared as suspensions at a concentration of 0.5 mg/mL in deionized, distilled water. Then, the suspensions were added to 12-well plates (Costar, Cambridge, MA). The plates were air-dried in a laminar airflow cabinet to produce a stable adherent layer of nBG particles. BMSCs were detached using trypsin-EDTA (0.5 g/L trypsin for 3 minutes) and seeded in the nBG-coated plates at 10^5 cells/well. The interactions of the BMSCs with nBG were monitored by the IncuCyte S3 live cell imaging system (Essen BioScience, Ann Arbor, MI). Images were automatically captured every hour for 72 hours under a phase-contrast microscope. The phase object confluence of nBG was determined by counting objects using IncuCyte image analysis software (IncuCyte S3 Software, Essen BioScience).

Dentin Disc and Slice Preparation

A schematic diagram of the preparation process for dentin discs is shown in Figure 1*D*. Human third molars were collected with the approval of the ethics committee, and all patients provided informed consent. The teeth were stored in distilled water containing 0.9% NaCl at 4°C. Dentin discs at the crown were cut at a thickness of 1 mm with a hard histotome (SP1600; Leica, Wetzlar, Germany). After removing the surrounding enamel with diamond burs, the discs were immersed in 17% EDTA (pH = 7.4) for 5 minutes and washed 3 times with deionized water. Then, the dentin discs were randomly divided into nBG and control groups and placed in 48-well plates (Costar, Cambridge, MA). nBG suspensions (0.5 mg/mL) were added to the wells containing the dentin discs and incubated for 10 minutes. Next, the nBG-coated dentin discs were washed 3 times with deionized water. The control discs were placed in wells without the addition of nBG. The dentin discs were used for subsequent cell adhesion and migration experiments.

A schematic diagram of the preparation process for dentin slices is shown in Figure 1*E*. Dentin slices were cut at a thickness of 2 mm from the crown third of the root. The cementum, pulp, and predentin tissues were carefully removed with a drill, and then the root canal was rinsed with 17% EDTA for 5 minutes and washed 3 times with deionized water. Next, the 0.5-mg/mL nBG suspension was brushed onto the internal dentin surface of the slices for 20 seconds. Then, the slices were washed with deionized water for 20 seconds and used for *in vivo* experiments.

Cell Adhesion

BMSCs were first labeled with DiD (ABD Bioquest, Inc, Sunnyvale, CA) as follows. Stock solutions of DiD were prepared in 2.5 mg/mL dimethyl sulfoxide and added to medium at a final concentration of 5 mmol/L to create a working solution. After digestion and centrifugation, the cells were suspended at 10^5/mL in the DiD dye working solution and incubated for 20 minutes at 37°C in a CO_2 incubator. Then, the cells were washed 3 times with fresh medium containing 10% fetal bovine serum and seeded on nBG-coated dentin discs at a concentration of 5×10^3 cells/well. After 2 hours or 3 days in culture, cell morphology was examined by confocal microscopy.

Cell Migration Assay Using a 3-dimensional Collagen Gel

Type I collagen gel (3 mg/mL) was laid onto the upper chamber of a transwell (Corning, New York, NY) for cell migration tests. The control group consisted of dentin discs embedded in 2-mm-thick collagen gel. The nBG group consisted of 0.5 mg/mL nBG-coated dentin discs in the collagen gel. After the gels solidified in a CO_2 incubator at 37°C for 30 minutes, BMSCs (10^5 cells/well) were seeded on top of the gels and incubated for 3 days. Then, the gels were fixed in 4% paraformaldehyde for 1 hour at room temperature and then rinsed twice with phosphate-buffered saline (PBS). The fixed gel was stained with 4,6-diamidino-2-phenylindole for 5 minutes and rinsed twice with PBS. The gels were removed from the transwell inserts by cutting the insert membrane along its circumference. The cells that had migrated into the gel were analyzed by confocal microscopy. The number of cells and migration distance in the 3-dimensional gel were ascertained in 3 replicate gels.

Ectopic Subcutaneous Implantation

All animal use, care, and surgical procedures were approved by the Animal Care and Ethics Committee of Peking University (reference no. LA2016027). Prepared human tooth slices coated with nBG as described earlier were used as experimental samples, whereas tooth slices without nBG treatment served as controls. Under general anesthesia with isoflurane, tooth slices were implanted into subcutaneous pockets created by blunt lateral dissection in 8-week-old nude female mice. Each mouse received 2 dentin slices. Four weeks later, the mice were euthanized to

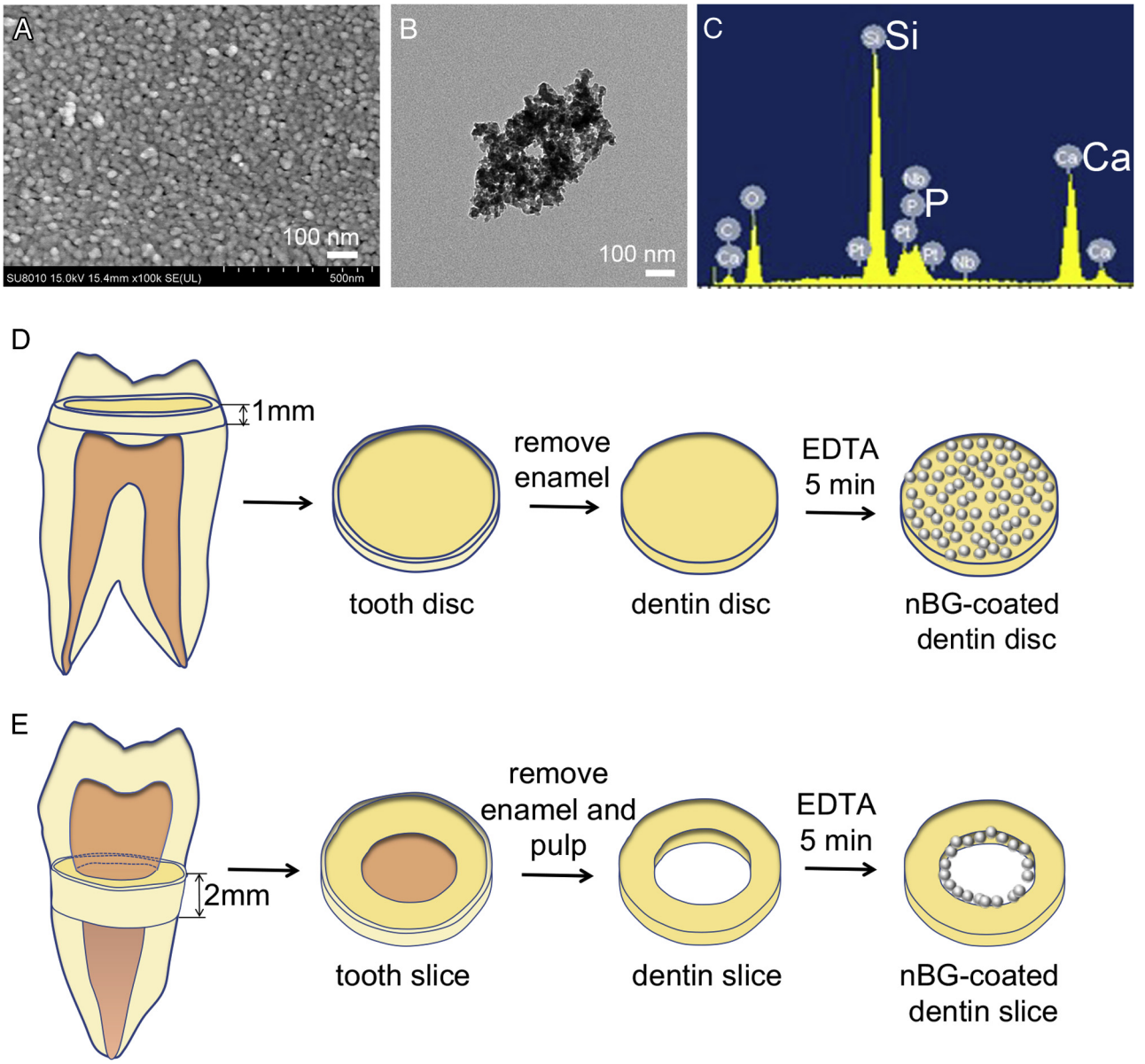

FIGURE 1 – The characteristics of nBG and schematic diagrams of a dentin disc and slice preparation. Morphology of nBG determined by (*A*) scanning electron microscopy and (*B*) transmission electron microscopy. (*C*) Surface elemental composition of nBG determined by energy-dispersive X-ray spectroscopy. (*D*) A schematic diagram of the preparation process for nBG-coated dentin discs. (*E*) A schematic diagram of the preparation process for nBG-coated dentin slices.

retrieve all implants. The specimens were fixed in 4% polyoxymethylene, decalcified, and then processed for Masson trichrome staining according to the manufacturer's recommended protocol (Baso Diagnostic Inc, Zhuhai, Guangdong, China).

Immunofluorescence

For immunofluorescence, randomly selected sections were blocked in 5% goat serum. Primary anti–dentin sialophosphoprotein (DSPP [sc-73632, 1:100; Santa Cruz Biotechnology, Inc, Santa Cruz, CA]) and anti–STRO-1 (sc-47733, 1:100, Santa Cruz Biotechnology) antibodies were incubated overnight at 4°C. After washing with PBS, the sections were incubated with secondary antibodies for 2 hours at room temperature, washed, and counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich). As a negative control, the same procedures were performed without the primary antibodies.

Statistics

Quantitative results are expressed as the mean ± standard deviation. Independent sample *t* tests were calculated with SPSS Windows Version 12.0 software (SPSS Inc, Chicago, IL). *P* values $<.05$ were considered statistically significant.

RESULTS

Interactions of BMSCs with nBG

BMSCs showed a flattened morphology with filopodial extensions at 4 hours (Fig. 2*A*). nBG particles aggregated into clusters after coating the bottom of culture plates (Fig. 2*B*). Cells growing on nBG-coated plates exhibited a morphology similar to that of the control group cells (Fig. 2*C*). After 35 hours in culture, the BMSCs elongated to a fusiform

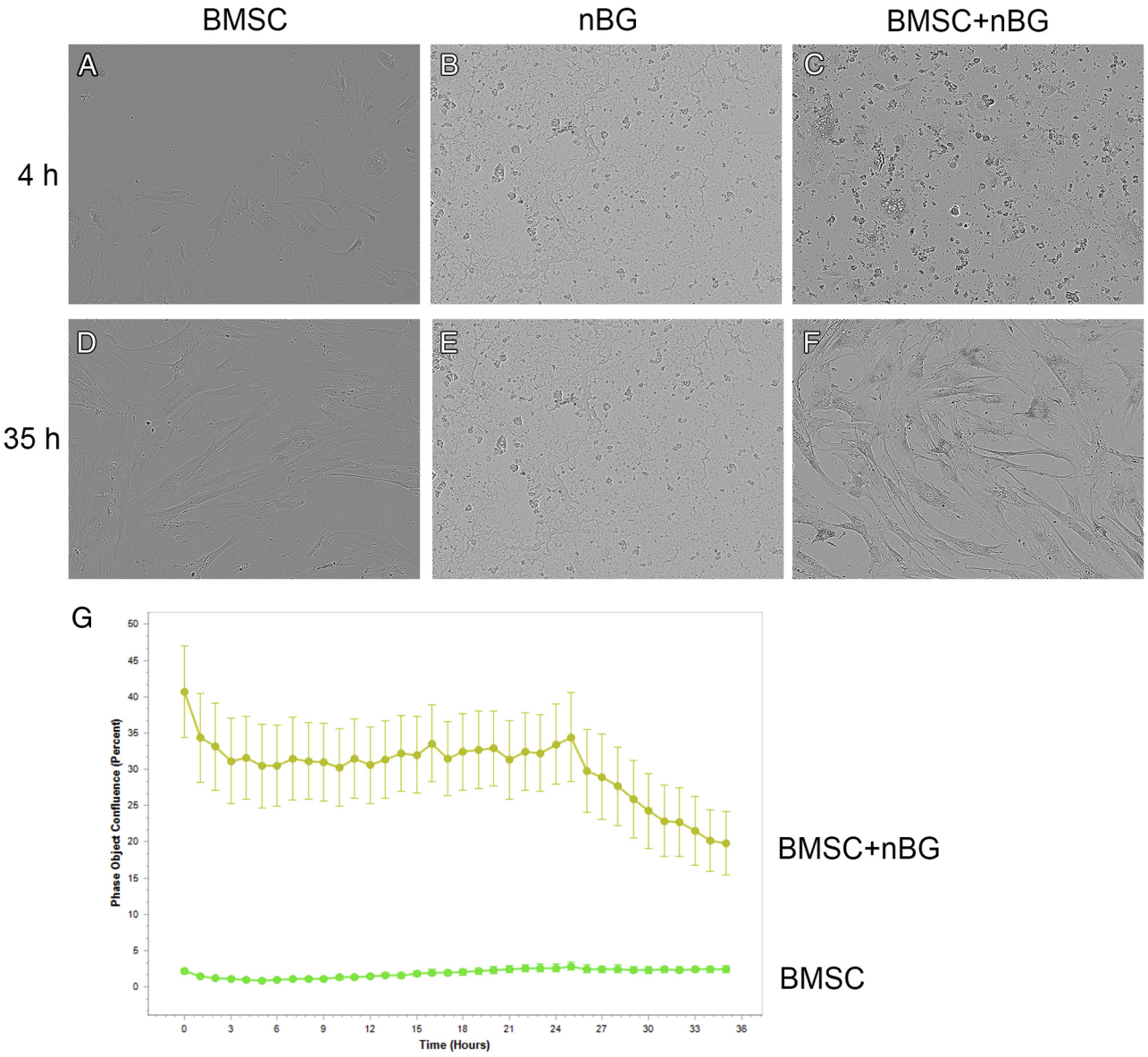

FIGURE 2 – Interaction of nBG with BMSCs. (*A*) BMSCs on a culture plate at 4 hours. (*B*) nBG-coated plate at 4 hours. (*C*) BMSCs on an nBG-coated plate at 4 hours. (*D*) BMSCs on a plate at 35 hours. (*E*) nBG-coated plate at 35 hours. (*F*) BMSCs on an nBG-coated plate at 35 hours. (*G*) The calculated ratios of the nBG particle area to the culture dish area from 0–35 hours.

or polygonal shape (Fig. 2*D*). nBG particles maintained their original appearance (Fig. 2*E*). In the BMSCs and nBG coculture group, after 24 hours, the nBG clusters began to separate into small pieces and relocated under the action of the BMSCs, and some nBG clusters gradually became undetectable under 10× magnification (Supplemental Video S1 is available online at www.jendodon.com). The ratio of the particle area to the culture dish area decreased with time in the BMSCs + nBG group beginning at 24 hours (Fig. 2*G*). At 35 hours, many tiny nBG particles overlapped with the cell cytoplasm, indicating that the cells may have taken up the particles (Fig. 2*F*).

nBG-coated Dentin Promoted Initial Cell Attachment

Scanning electron microscopic analysis showed that after immersion in an nBG suspension, the dentin surface showed adherence of nBG particles (Fig. 3*A* and *B*). Red fluorescence-labeled BMSCs were then seeded on nBG-coated dentin to assess cell adhesion and growth. At 2 hours, cells were detectable on the untreated dentin surface, and most were round in shape (Fig. 3*C*), whereas the cells on the nBG-coated dentin started to spread out (Fig. 3*D*). After 3 days in culture, the cells grew well, and no differences in morphology were detected between the 2 groups (Fig. 3*E* and *F*).

nBG-coated Dentin Promoted BMSC Migration

The chemotaxis effect of nBG-coated dentin on BMSCs was determined using a 3-dimensional collagen migration model (Fig. 4*A* and *B*). nBG-coated dentin discs induced the directional migration of significantly more cells than untreated dentin samples after 3 days of

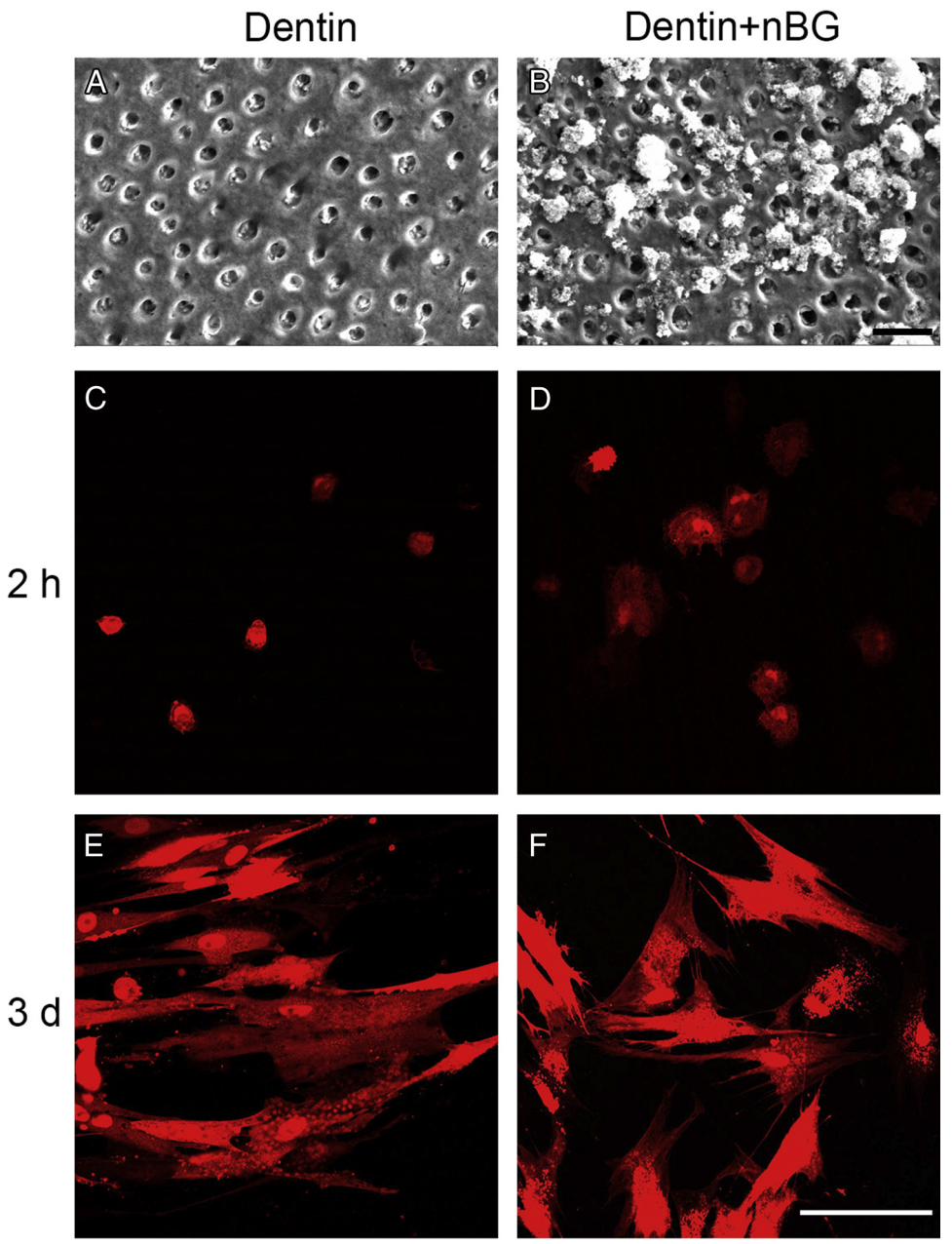

FIGURE 3 – Cell adhesion on an nBG-coated dentin surface. (*A*) Scanning electron microscopy of an untreated dentin surface. (*B*) Scanning electron microscopy of an nBG-coated dentin surface. Cell morphology of BMSCs on a dentin surface (*C*) without or (*D*) with nBG coating at 2 hours evaluated by a confocal microscope. Cell growth on a dentin surface (*E*) without or (*F*) with nBG coating at 3 days evaluated by a confocal microscope.

culture (Fig. 4*C*–*E*). The migration distances of the 2 groups did not significantly differ (Fig. 4*F*).

nBG-coated Dentin Induced Odontogenic Differentiation in an Ectopic Transplantation Model

Endodontically prepared dentin slices were coated with or without nBG, transplanted under the dorsum of nude mice, and left for 4 weeks. Masson trichrome staining revealed that relatively loose connective tissue with sparse cells and blood vessels was generated in the root canal of the untreated dentin slices (Fig. 5*A* and *B*). In contrast, newly formed tissue with abundant cells and vasculature was evident in the nBG-coated dentin slices (Fig. 5*E* and *F*). Remarkably, we found some DSPP-positive columnar odontoblastlike cells attached to the primary dentin surface after nBG coating (Fig. 5*G*), whereas few DSPP-positive cells were present in the control group (Fig. 5*C*). Immunofluorescence staining for STRO-1 visualized more stem cells near the dentin in the nBG-coated group (Fig. 5*H*), whereas more STRO-1–positive cells occurred in the central area of the root canal in the control group (Fig. 5*D*), suggesting that more stem cells attracted to the nBG-coated dentin surface.

DISCUSSION

Our BMSCs + nBG coculture experiments revealed that nBG clusters separated into small particles and colocalized with the

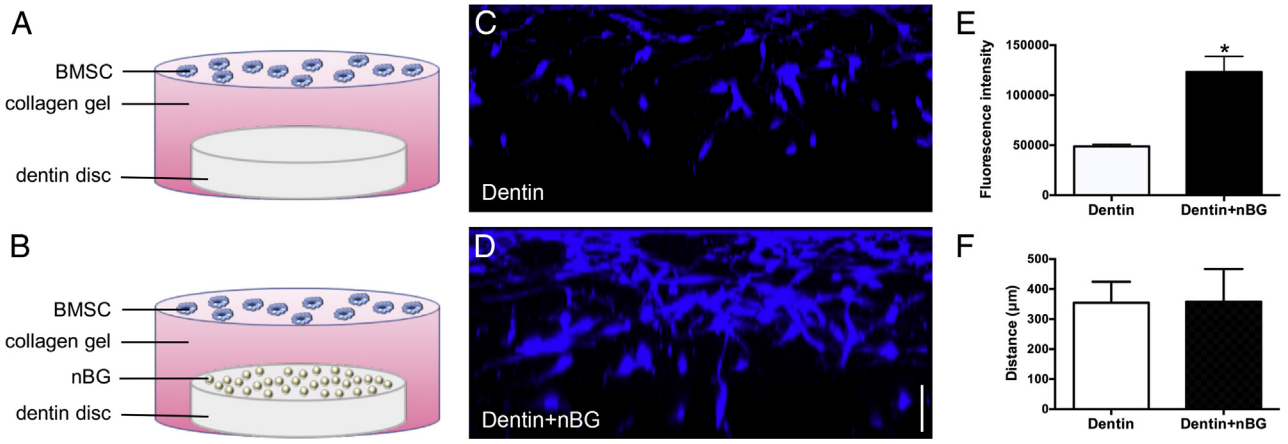

FIGURE 4 – A schematic diagram of 3-dimensional migration models for (*A*) the control group and (*B*) the nBG group. 4,6-Diamidino-2-phenylindole staining of a collagen gel with (*C*) untreated dentin slices or (*D*) nBG-coated dentin slices. Quantification of the (*E*) cell numbers and (*F*) migration distances in *C* and *D*. * indicates significant difference in the comparison between the dentin+nBG group and the dentin group ($P < .05$).

BMSCs after incubation for 24 hours, indicating that some of the nBG particles might have disaggregated and been taken up by the BMSCs. Rejman et al[13] reported that the mouse melanoma cell line B16-F10 took up BG particles up to 500 nm in size but that no uptake was observed with 1-μm particles. nBG particles with a size range from 61–174 nm can be internalized by MC3T3 cells and are encapsulated into lysosomes, indicating that small nanoparticles are transported and degraded by the endolysosomal pathway, reducing the cytotoxicity of the nanoparticles[14]. Furthermore, these works revealed that small BG particles (61–174 nm) at low doses might have potential biomedical applications such as functioning as bone tissue engineering scaffolds or gene- and drug-delivery carriers[14]. Large particles (>174 nm) at high concentrations exhibit increased cytotoxicity. In this study, the size of the nBG particles we used was ~20 nm, so these particles were probably internalized by the cells that exhibited good biological responses. Our previous studies also showed that 0.1 mg/mL nBG promoted cell proliferation and odontogenic differentiation[6]. nBG at a concentration of 0.5 mg/mL promotes mineral formation on the dentin surface, which can completely seal dentin tubules when nBG is used to brush the dentin surface[8].

nBG particles immediately attach after they are brushed onto dentin. BG is a calcium phosphosilicate-based material that has been shown to rapidly form hydroxyapatite on the dentin surface in dentin hypersensitivity studies[15]. Hydroxyapatite is similar in composition to dentin and interacts with the collagen fibrils of dentin. After BG is applied to the root canal, it is possible for the particles to bind to the internal dentin surface of the root canal where dentin regeneration is needed rather than remaining floating in the root canal. After BG particles are degraded or endocytosed by cells, newly generated mineral tissue may undergo remodeling and replace this material. Suspension in a gel with suitable viscosity may result in better dispersal and retention of BG powders on dentin than suspension in water[16]. The nBG formulation we used attached easily to the dentin surface and could also be trapped in dental tubules; furthermore, nBG is more resistant to acid attack than micro-BG or traditional 45S5 BG[8].

We also found that nBG-coated dentin promoted the directional migration and adhesion of cells; these features are important because cell homing is a critical step in endodontic regeneration. The effect of nBG was probably attributable to it increasing the

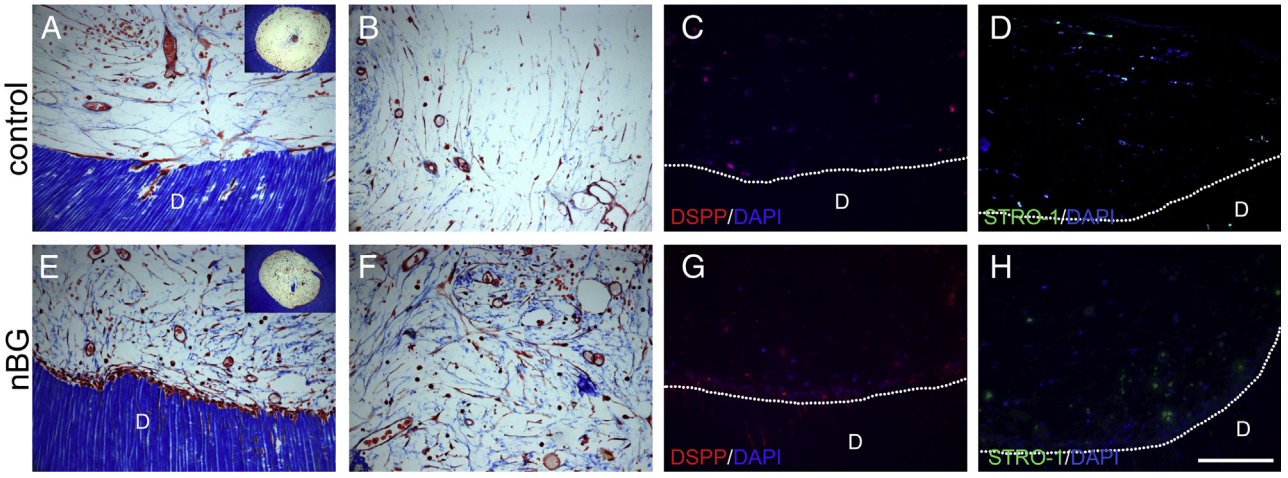

FIGURE 5 – Regeneration of pulplike tissue after ectopic transplantation of nBG-coated dentin slices. (*A* and *B*) Masson trichrome staining of dentin slices 4 weeks after transplantation. Immunohistochemical staining for (*C*) DSPP and (*D*) STRO-1 in samples from the control group. (*E* and *F*) Masson trichrome staining of nBG-coated dentin slices 4 weeks after transplantation. Immunohistochemical staining for (*G*) DSPP and (*H*) STRO-1 in samples from the nBG group. D, dentin.

release of ions. Studies have reported that localized increases in calcium promote chemotaxis in many cells, including BMSCs and hemopoietin progenitors, in a concentration-dependent manner[17–19]. BGs in the local environment have been shown to regulate several families of genes that control the cell cycle, mitosis, and differentiation[20,21]. The morphology of BMSCs on nBG-coated dentin changed from a rounded phenotype to a spread, polygonal phenotype in a short time, which is indicative of good adhesion. Cells are sensitive to topography, so the increases in surface area and roughness induced by nBG coating and further mineralization may provide a maximum area for protein adsorption and filopodia extension[22]. It has been reported that BG ceramics strongly adsorb proteins from body fluids because of their surface charge and texture[23,24]. The adsorption of serum fibronectin onto BG may be responsible for the enhancement in osteoblast adhesion[25]. Nanophase ceramics enhance osteoblast adhesion by adsorbing large quantities of vitronectin[26]. In this study, proteins from the serum in the culture medium or released by dentin might have been absorbed by the nBG coating, further promoting cell migration and adhesion.

An ectopic transplantation model was used for testing cell homing and odontogenic differentiation *in vivo*. The use of biomaterials based on calcium phosphates is a promising strategy for improving tissue regeneration[27]. The cell-guiding features of hydroxyapatite-coated cellulose sponges facilitate the homing of bone marrow–derived stem and progenitor cells more efficiently than those of untreated cellulose sponges[19]. Our results showed that significantly more cells and blood vessels regenerated in nBG-coated dentin slices than uncoated dentin slices, and, more importantly, DSPP-positive cells were detected on the primary dentin. These findings showed that in addition to recruiting stem cells via directional migration, nBG-coated dentin induced odontogenic differentiation. The capacity of nBG to mediate directional migration and odontogenic induction in stem cells in a designated location, along the primary dentin, is essential for the successful functional regeneration of dentin-pulp tissue.

CONCLUSIONS

In conclusion, our results suggest that nBG-coated dentin enhances the chemotaxis, attachment, and odontogenic differentiation of BMSCs. nBG may be used to control the stem cell distribution and cell fates in the root canal. Further work is needed to evaluate the effect of nBG-coated dentin on the regeneration of the pulp-dentin complex in an *in situ* regenerative animal model.

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The authors deny any conflicts of interest related to this study.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found in the online version at www.jendodon.com (https://doi.org/10.1016/j.joen.2019.11.004).

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