Odontogenic Differentiation Induced by TGF-β1 Binding Peptide–Modified Bioglass

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Abstract

Emerging evidence suggests that growth factors are crucial in regenerative endodontic therapy. To achieve the desired effects, the systematic administration of supraphysiologic concentrations of exogenous growth factors is commonly performed, but this is usually associated with high costs, technique, and safety issues. Here, we describe a novel biomaterial that can manipulate endogenous growth factors without the need for adding exogenous growth factors. Transforming growth factor β1 binding peptide (TGFp) was grafted onto the surface of a neutral pH phytic acid–derived bioactive glass (PSC) to synthesize modified bioactive glass (PSC-TGFp). Fourier transform infrared spectroscopy and thermogravimetric analysis results demonstrated that the TGFp was successfully grafted to the surface of the PSC. Scanning electron microscopy and x-ray diffraction showed that PSC-TGFp possessed good in vitro bioactivity. After soaking in simulated body fluid for 24 h, hydroxyapatite formed on the surface of PSC-TGFp. Enzyme-linked immunosorbent assay showed that PSC-TGFp could capture endogenous transforming growth factor β1 from dentin matrix–extracted proteins (DMEP) and release it slowly over 21 d. Cytologic experiments revealed that PSC-TGFp after adsorbing DMEP could enhance the adhesion, migration, viability, and odontogenic differentiation of stem cells from apical papilla. The results highlight that PSC-TGFp may be a promising biomaterial to manipulate endogenous growth factors for regenerative endodontic therapy in the future.

Keywords: transforming growth factor beta 1, regenerative endodontics, odontogenesis, materials science, extracellular matrix proteins, cell adhesion

Introduction

Dental pulp is crucial for maintaining the normal physiologic functions and structures of teeth (Itoh et al. 2018). The emergence of regenerative endodontic therapy has provided an opportunity to regenerate the pulp-dentin complex and promote root development. Pulp revascularization is a novel therapy applied in endodontic treatment that mainly recruits autologous stem cells and utilizes endogenous growth factors to promote root development and tissue regeneration (Duncan et al. 2018). Despite successful radiographic evidence, including apical lesion healing, apex closure, and dentinal wall thickening (Shimizu et al. 2013), the histologic studies have demonstrated that the tissues formed in the canal spaces are periodontal ligament–like, bone-like, or cementum-like tissues rather than pulp-dentin complex (Yang et al. 2016).

Ectopic tissue formation after pulp revascularization may be due to insufficient odontogenic-inducing signals (Chrepa et al. 2020). Previous studies have made some attempts to overcome this issue, such as providing bioengineered microenvironments (Chmilewsky et al. 2014), using a 3-dimensional scaffold (Itoh et al. 2018), and administering chemotactic growth factors (Duncan et al. 2018). Most efforts have focused primarily on delivering exogenous growth factors to the injured site to promote tissue regeneration (Gaharwar et al. 2020). Kim

et al. (2010) delivered several growth factors into the root canal and observed the formation of nerves and blood vessels after subcutaneous implantation in nude mice. Yang et al. (2015) inserted scaffolds loaded with SDF-1α (stromal cell–derived factor 1α) into the canal following the induction of blood clots and observed a thin layer of mineralized tissues

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deposited along the dentin wall in the root canals of beagle dogs. However, the use of exogenous growth factors requires delicate dose control and delivery strategies. Supraphysiologic concentrations and systematic administration are commonly adopted due to the susceptibility of exogenous growth factors to enzymatic and chemical denaturation in the physiologic environment, which are associated with problems including high costs, technique, and safety issues (Crispim et al. 2017; Yu et al. 2018). Moreover, the process of regenerative endodontics is regulated by a cocktail of growth factors. Delivering 1 or 2 growth factors may not provide sufficient signals to induce pulp regeneration. Manipulating and prolonging the activity of endogenous growth factors may be a promising strategy to solve the aforementioned problems (Crispim et al. 2017; Hong et al. 2019).

Dentin is a mineralized tissue that comprises hydroxyapatite crystals and collagenous and noncollagenous proteins. More than 230 proteins have been identified from different dentin matrix samples by proteomic analysis (Park et al. 2009), including transforming growth factor β1 (TGF-β1), insulin-like growth factor, vascular endothelial growth factor, fibroblast growth factor, and other bioactive molecules, indicating that dentin may be a natural complex protein reservoir (Galler et al. 2011). During the process of pulp repair and regeneration, the endogenous growth factors embedded in the dentin matrix could be released and induce cell chemotaxis and differentiation (Luisi et al. 2007). Different approaches have been attempted to obtain more dentin-derived proteins, such as decellularization of the dentin matrix, administration of dentin particles, or solubilization of dentine with EDTA and lyophilization (Tabatabaei et al. 2016; Widbiller et al. 2018).

TGF-β1 is a key growth factor in dentin formation and root development and is capable of inducing the behaviors of stem cells (Farges et al. 2003). TGF-β1 is deposited in the peritubular dentin and can be released into the root canal during the process of endodontic procedures after EDTA irrigation (Ivica et al. 2020). Previous studies have demonstrated that TGF-β1 released from dentin could stimulate odontoblast differentiation and promote tissue repair or regeneration (Nakashima and Akamine 2005). TGF-β1 binding peptide (TGFp) is a high-affinity binding peptide for TGF-β1 composed of 13 amino acids (GKGGKGLPLGNSH). Their bonding is noncovalent, and TGF-β1 can be sustained-released after bonding. Previous research suggested that the endogenous TGF-β1 can be enriched to increase the local content on the material surface by grafting TGFp onto polycaprolactone films and promote cell differentiation (Crispim et al. 2017).

Bioactive glass (BG) is a promising biomaterial with excellent biocompatibility and gene activation ability (Jones 2013). Our group has demonstrated the potential of BG to induce regeneration of the pulp-dentin complex (Wang et al. 2014). BG particles can adhere to the dentin surface after immersing or embrocating the dentin with a BG suspension (Sheng et al. 2016; Wang et al. 2020). Therefore, BG suspension could serve as an irrigating solution to promote pulp-dentin complex regeneration by treating the root canal before inducing apex

bleeding during pulp revascularization. In addition, the silicon hydroxyl groups on the surface of BG could react with 3-aminopropyl triethoxysilane (APTES) to introduce the amino group to BG. Based on the reaction of the amino group from APTES and the carboxyl group from TGFp, BG is a suitable material for TGFp modification for regenerative endodontic therapy.

The present work aimed to synthesize a TGFp-modified BG (PSC-TGFp) by grafting TGFp onto the surface of a novel neutral pH phytic acid–derived BG (PSC). We hypothesized that PSC-TGFp could capture the endogenous TGF-β1 from dentin matrix–extracted proteins (DMEP), enrich the local concentration of TGF-β1, and prolong its release, thereby promoting odontogenic differentiation of stem cells from apical papillae (SCAPs).

Materials and Methods

See Appendix for details.

Results

Characteristics of PSC-TGFp

The synthesis process for PSC-TGFp is illustrated in Figure 1A. The Fourier transform infrared spectroscopy results (Fig. 1B) showed that the characteristic absorption peak of APTES at $1,400 \text{ cm}^{-1}$ was observed in PSC-NH$_2$ and PSC-TGFp, representing the bending vibration of C-H, while PSC did not show the corresponding characteristic peak. Thermogravimetric analysis results (Fig. 1C) revealed ~0.5% weight loss between PSC and PSC-NH$_2$ and ~4% weight loss between PSC-NH$_2$ and PSC-TGFp. The x-ray diffraction spectrum (Fig. 1D) confirmed that the characteristic peaks of hydroxyapatite (25.9°, 31.7°, 39.7°, 49.4°) appeared on the surface of PSC, PSC-NH$_2$, and PSC-TGFp after soaking in simulated body fluid for 1 d. Scanning electron microscopy results (Fig. 1E) showed that a large number of plate-like aggregates almost covered the entire surface of PSC, PSC-NH$_2$, and PSC-TGFp, with a typical appearance of hydroxyapatite. These results suggested that TGFp was successfully grafted onto the surface of PSC and that PSC-TGFp possessed good in vitro mineralization ability.

PSC-TGFp Adsorbed and Sustained the Release of DMEP

The initial TGF-β1 concentration in the DMEP supernatant was approximately 11.5 ± 1.6 ng/mL (mean ± SD). To detect the maximum protein loading of PSC or PSC-TGFp, DMEP were concentrated to contain 100 ng/mL TGF-β1. Bicinchoninic acid results (Fig. 2A) showed that the total protein concentration in DMEP solution was $3,158.5 \pm 399.6$ μg/mL. After PSC or PSC-TGFp was incubated with DMEP, the protein amounts significantly decreased ($P < 0.05$) and showed no difference ($P > 0.05$) between the PSC group ($1,398.4 \pm 303.5$ μg/mL) and PSC-TGFp group ($1,306.4 \pm 271.6$ μg/mL), demonstrating
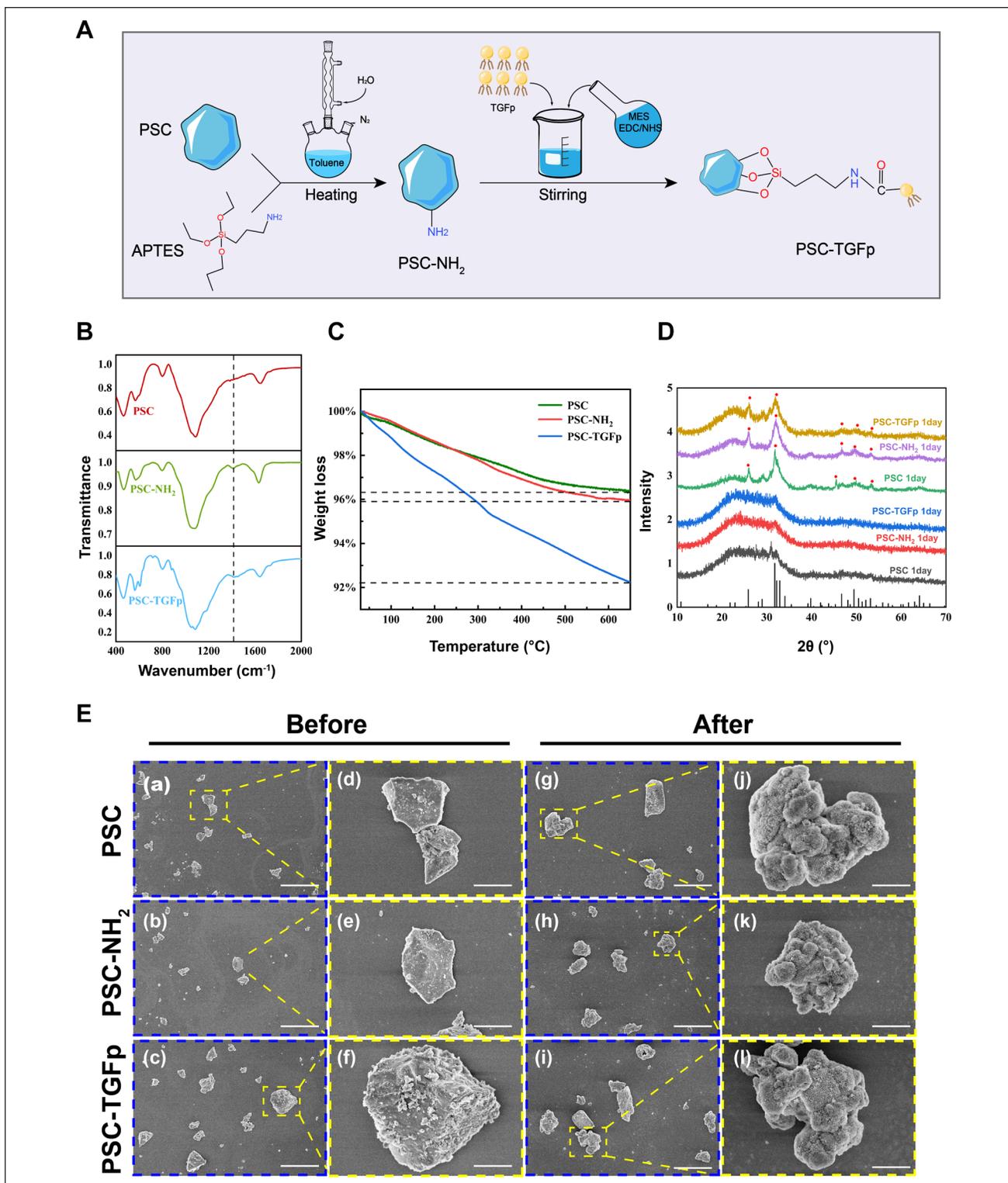
Figure 1. (**A**) A schematic of PSC-TGFp synthesis. Characteristics of bioactive glass before and after modification. Surface modification confirmed by (**B**) Fourier transform infrared spectroscopy and (**C**) thermogravimetric analysis. (**D**) X-ray diffraction pattern and (**E**) scanning electron microscopy of PSC, PSC-NH$_2$, and PSC-TGFp after immersion in simulated body fluid for 24 h (scale bar = 25 μm in blue box, 5 μm in yellow box). PSC, phytic acid–derived bioactive glass; TGFp, transforming growth factor β1 binding peptide.

that PSC and PSC-TGFp could adsorb similar amounts of total proteins. Enzyme-linked immunosorbent assay results (Fig. 2B) showed that the TGF-β1 concentration in the original DMEP was 102.2 ± 16.9 ng/mL. After incubation, the remaining TGF-β1 in the supernatant of the PSC group was 60.9 ± 9.4 ng/mL, and that in the PSC-TGFp group was 22.4 ± 9.4 ng/mL.

It can be inferred that 1 mg of PSC could adsorb approximately 4 ng of TGF-β1, while 1 mg of PSC-TGFp could capture nearly 8 ng of TGF-β1; this difference was significant ($P < 0.05$). TGF-β1 release analysis showed an apparent initial burst release within 24 h and reached a plateau after 3 d in the PSC + DMEP group. The PSC-TGFp + DMEP group did not show an initial burst release and slowly released TGF-β1 until 21 d (Fig. 2C). Figure 2D shows that the TGF-β1 concentration released from dentin disks was 6.3 ± 0.9 ng/mL. After incubation, the remaining TGF-β1 in the supernatant of the PSC group was 2.7 ± 0.6 ng/mL, and that in the PSC-TGFp group was 0.3 ± 0.2 ng/mL, indicating that PSC-TGFp could adsorb more TGF-β1 released from EDTA-treated dentin discs directly. These results indicate that PSC-TGFp could not only sequester more TGF-β1 from DMEP but also effectively slow its release.

PSC-TGFp + DMEP Increased Cell Viability

PSC-TGFp + DMEP significantly increased the viability of SCAPs as compared with the control, PSC, PSC-TGFp, and PSC + DMEP groups on days 4 and 7 ($P < 0.05$; Fig. 3A). PSC + DMEP significantly reduced SCAPs viability on day 1, and the inhibitory effects vanished on days 4 and 7. There was no significant difference in cell viability among the other groups ($P > 0.05$).

PSC-TGFp + DMEP Promoted Cell Adhesion and Migration

Western blot (Fig. 3B, C) showed that the expression levels of vinculin and p-paxillin, which are focal adhesion-associated proteins, were upregulated by PSC + DMEP and PSC-TGFp + DMEP as compared with the control, PSC, and PSC-TGFp. Moreover, PSC-TGFp + DMEP had a more significant promoting effect than PSC + DMEP. Cytoskeletal staining at low (Appendix Fig. 1A) and high (Fig. 3D) magnification showed that the cells in the control group had a triangular or polygonal shape, while the cell morphology in the PSC, PSC-TGFp, PSC + DMEP, and PSC-TGFp + DMEP groups was more spread with more pseudopods sticking out. The cell number (Appendix Fig. 1B) in the PSC, PSC-TGFp, PSC + DMEP, and PSC-TGFp + DMEP groups was significantly higher than that in the control group ($P < 0.05$), and the PSC-TGFp + DMEP group had the highest cell number. As shown in Figure 3E, PSC-TGFp + DMEP induced significantly more SCAPs migration than 1% fetal bovine serum (FBS; control group), PSC, PSC-TGFp, and PSC + DMEP, comparable to the 10% FBS group. These results demonstrated that PSC-TGFp + DMEP had the strongest promoting effect on cell adhesion and migration.
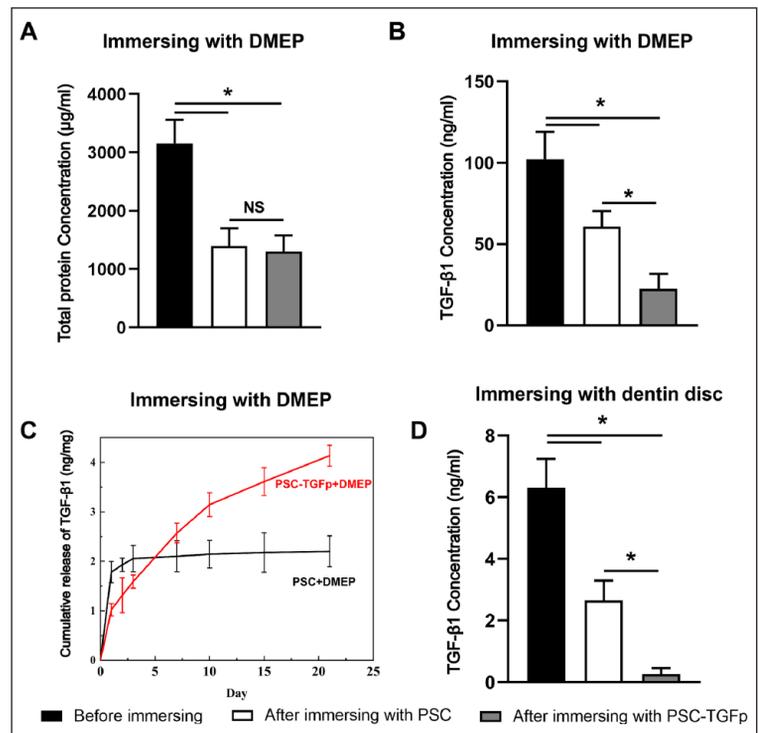

Figure 2. Adsorption and release of DMEP by PSC-TGFp. (**A**) Total protein and (**B**) TGF-β1 concentrations in DMEP supernatant before and after incubation with PSC and PSC-TGFp detected by bicinchoninic acid and ELISA, respectively. (**C**) TGF-β1 release curves for PSC and PSC-TGFp. (**D**) TGF-β1 concentrations in medium supernatant before and after PSC and PSC-TGFp incubation with EDTA-treated dentin discs detected by ELISA. *$P < 0.05$. Data are presented as mean ± SD. DMEP, dentin matrix–extracted proteins; PSC, phytic acid–derived bioactive glass; TGFp, transforming growth factor β1 binding peptide.

PSC-TGFp + DMEP Promoted Odontogenic Differentiation

The relative mRNA expression levels (Fig. 4A) of odontogenic marker genes, including dentin sialophosphoprotein (*DSPP*) and dentin matrix protein 1 (*DMP1*), were markedly elevated in the PSC, PSC-TGFp, PSC + DMEP, and PSC-TGFp + DMEP groups ($P < 0.05$). DSPP and DMP1 expression in the PSC-TGFp + DMEP group was significantly higher than that in the other groups, implying that PSC-TGFp + DMEP had the strongest promoting effect on odontogenic differentiation. The Western blot results (Fig. 4B, C) were consistent with the polymerase chain reaction results. Alizarin red S staining (Fig. 4D) showed that no mineralized nodules were generated in the control group, while scattered mineralized nodules were formed in the odontogenic-inducing medium group. The number and size of nodules in the 4 experimental groups were significantly higher than those in the odontogenic-inducing medium group, and the most mineralized nodules were formed in the PSC-TGFp + DMEP group. The semiquantitative calcium concentrations (Fig. 4E) were consistent with the staining results, indicating that PSC-TGFp + DMEP had the strongest mineralization-inducing effects ($P < 0.05$).
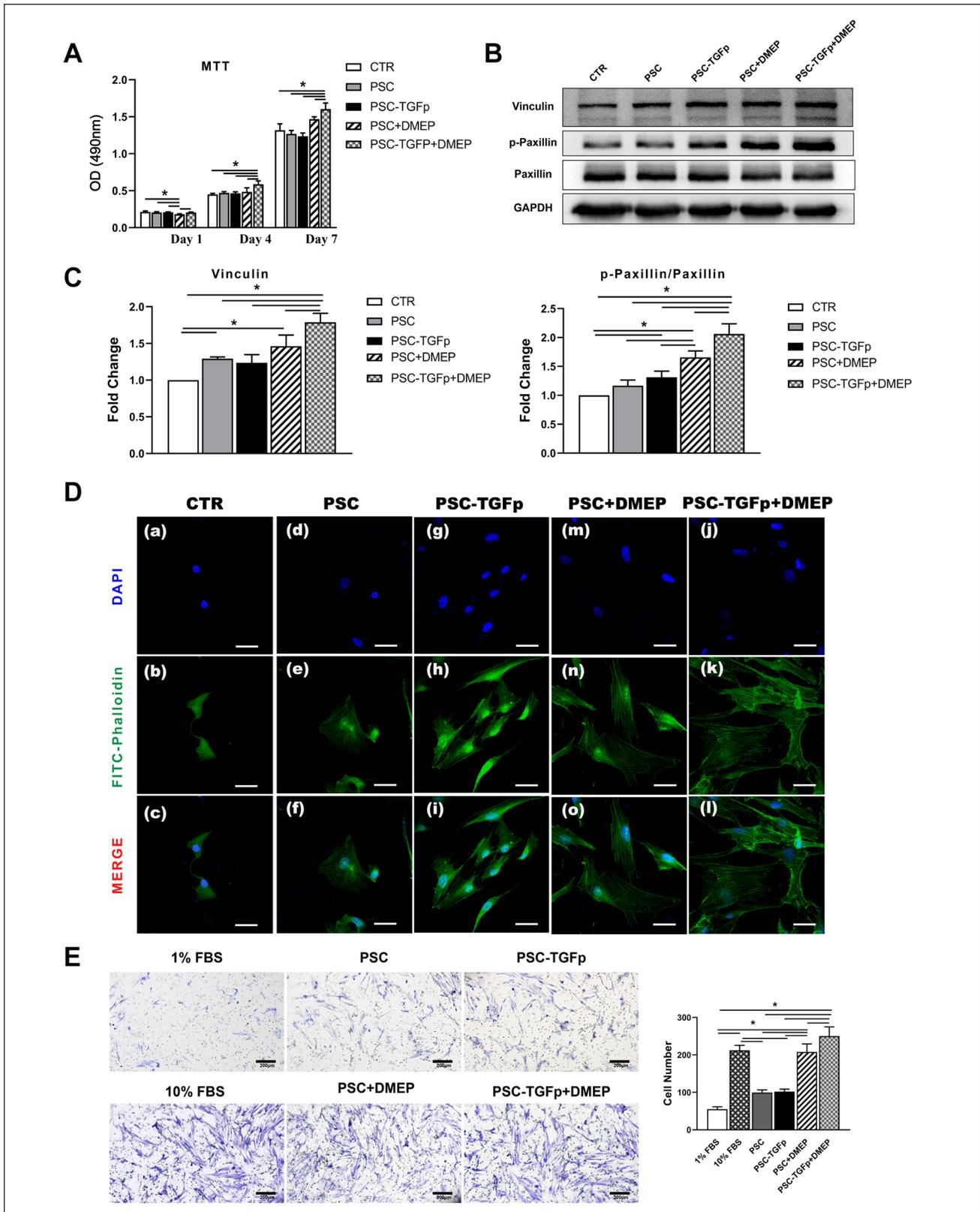
Figure 3. (**A**) MTT results of SCAPs cultured with PSC, PSC-TGFp, PSC + DMEP, and PSC-TGFp + DMEP. (**B**) Western blot results of vinculin and p-paxillin protein levels in SCAPs cultured with and without PSC, PSC-TGFp, PSC + DMEP, and PSC-TGFp + DMEP. GAPDH was used as a loading control. (**C**) Quantification of Western blot results by ImageJ software. (**D**) Cytoskeleton reorganization was observed under a confocal laser scanning microscope (scale bar = 10 μm). Green, cytoskeleton; blue, DAPI. (**E**) SCAPs migration after exposure to 1% FBS, 10% FBS, PSC, PSC-TGFp, PSC + DMEP, and PSC + DMEP (scale bar = 100 μm). The cell number was counted with ImageJ software. *P < 0.05. Data are presented as mean ± SD. DMEP, dentin matrix–extracted proteins; FBS, fetal bovine serum; PSC, phytic acid–derived bioactive glass; SCAPs, stem cells from apical papilla; TGFp, transforming growth factor β1 binding peptide.
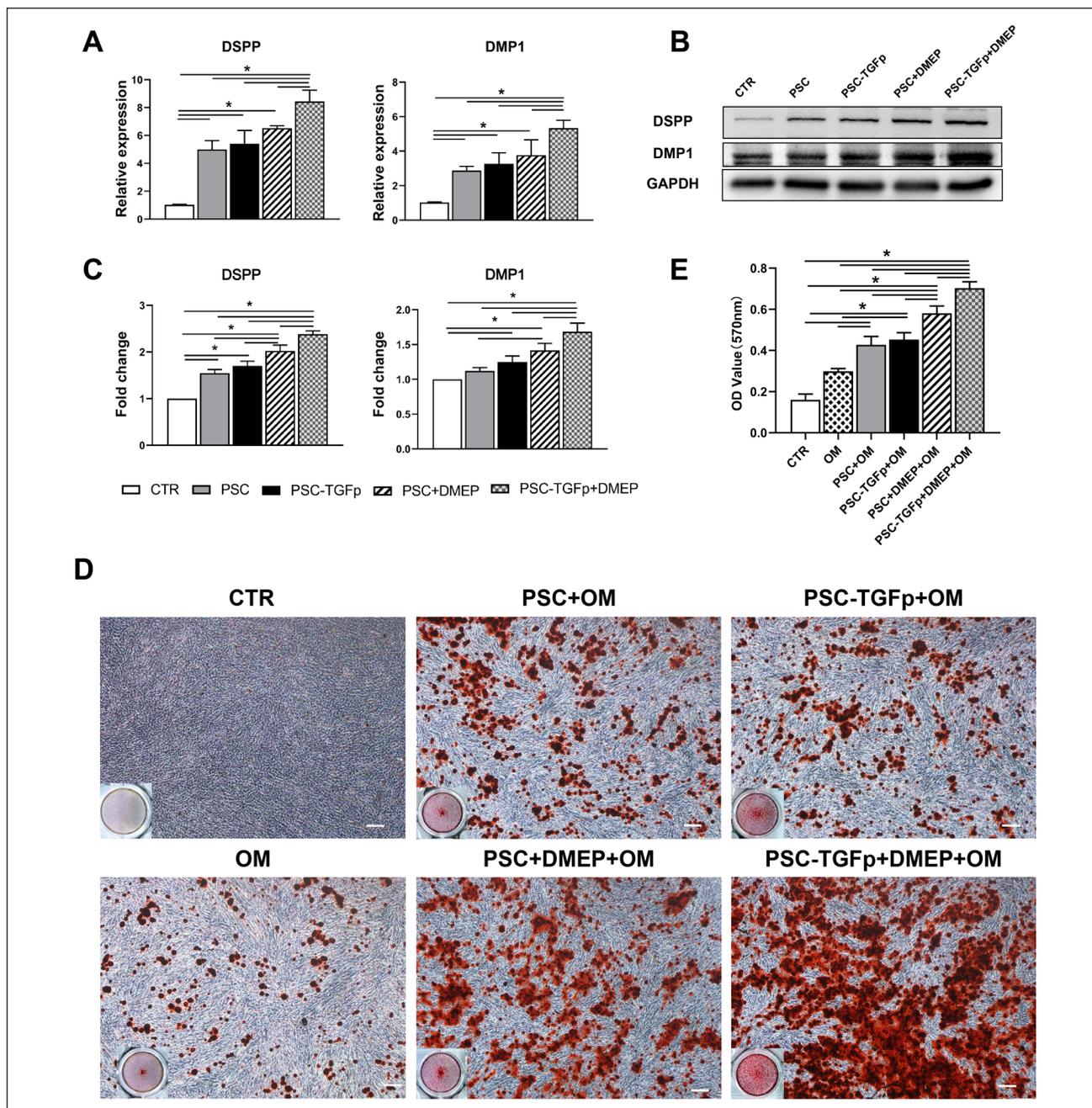
Figure 4. Effects of PSC-TGFp + DMEP on the odontogenic differentiation and mineralization of SCAPs. (**A**) Real-time polymerase chain reaction results of odontogenic-related gene (*DSPP, DMP1*) expression in SCAPs cultured with or without PSC, PSC-TGFp, PSC + DMEP, and PSC-TGFp + DMEP. GADPH was used as an internal control. Gene expression was described as fold changes relative to the control group at the same time point. Values show the mean ± SD of 3 experiments. (**B**) Western blot results of DSPP and DMP1 protein levels in SCAPs cultured with and without PSC, PSC-TGFp, PSC + DMEP, and PSC-TGFp + DMEP. GAPDH was used as a loading control. (**C**) Quantification of Western blot results by ImageJ software. (**D**) Alizarin red staining of mineralized nodules after 2 wk of culture with or without PSC, PSC-TGFp, PSC + DMEP, and PSC-TGFp + DMEP (scale bar = 100 μm). (**E**) Calcium concentrations in groups by cetylpyridinium chloride detection. *$P < 0.05$. DMEP, dentin matrix–extracted proteins; OM, odontogenic-inducing medium; PSC, phytic acid–derived bioactive glass; SCAPs, stem cells from apical papilla; TGFp, transforming growth factor β1 binding peptide.

Discussion

Growth factors are susceptible to enzymatic and chemical denaturation in the physiologic environment, and immobilizing them on biomaterials can prevent proteolytic degradation and prolong their life span (Lee et al. 2011). Growth factor immobilization can be classified into covalent binding and noncovalent binding. Previous studies have suggested that covalent binding often hides the active sites of growth factors, changes the molecular conformation, and blocks the translocation,

resulting in decreased biological activity (Lee et al. 2011; Crispim et al. 2017). Noncovalent binding includes direct electrostatic adsorption and interactions between growth factors and biological molecules, such as growth factor–binding peptide, heparin, or extracellular matrix. Direct electrostatic adsorption may be the simplest strategy for capturing growth factors. However, this approach is often associated with an initial burst release. To maintain the long-term effects, an excess dose of growth factors is commonly adopted (Lee et al. 2011). Previous research has shown that the initial burst release of growth factors inhibits cell proliferation and differentiation, resulting in clinical complications such as ectopic bone formation or abnormal bone resorption (James et al. 2016; Mller-Siegert et al. 2017). Therefore, synthesizing a special material that interacts with the particular domain of growth factors and avoids the initial burst release may be key to optimizing their effects. Here, we adopted a noncovalent binding strategy by grafting the TGFp onto the surface of BG. Adsorption and release results suggested that PSC-TGFp can capture endogenous TGF-β1 from DMEP and eliminate the initial burst release better than PSC. The cytologic results further demonstrated the feasibility and advantage of this growth factor enrichment and sustained-release strategy.

BG is a promising endodontic tissue material due to its excellent biocompatibility, bioactivity, and odontogenic-inducing ability (Wang et al. 2014; Cui et al. 2017). Due to the silicone hydroxyl groups on the surface, BG can be used as a suitable carrier for grafting functional groups that can bind growth factors (Lee et al. 2016). In the present work, we found that unmodified PSC could adsorb TGF-β1. We considered that the adsorption mechanism between unmodified PSC and TGF-β1 is direct electrostatic adsorption. TGF-β1 has an isoelectric point of 9.5 and is positively charged in a physiologic environment (pH 7.4), while the surface of BG is negatively charged (Lee et al. 2016). After the amino group in APTES was introduced into PSC, the negative charge of PSC changed to a positive charge, resulting in less electrostatic adsorption with TGF-β1. The TGFp could mimic the binding domain of TGF-β1 and showed high affinity toward TGF-β1 due to the selective noncovalent binding (Kaspar and Reichert 2013; Crispim et al. 2017). We believe that this noncovalent, selective, and high-affinity binding between TGFp and TGF-β1 might be the reason why PSC-TGFp could capture more TGF-β1 and sustain its release in the long term.

Endogenous growth factors usually consist of multiple growth factors, which may have synergistic promoting effects. For example, plasma rich in growth factors is extracted from autologous blood and then delivered to the injured site to induce mesenchymal stem cell proliferation and differentiation and accelerate bone repair (Solakoglu et al. 2020). Concentrated growth factors produced from peripheral blood can promote the proliferation, migration, and differentiation of SCAPs and be a promising biomaterial applied in regenerative endodontic therapy (Hong et al. 2019). Various soluble proteins, such as dentin phosphoprotein, dentin sialoprotein, TGF-β1, and fibroblast growth factor, are embedded in the dentin matrix during

the process of dentin mineralization (Galler et al. 2011). These proteins can regulate the biological behaviors of stem cells, making dentin a valuable reservoir of endogenous growth factors for endodontic regeneration (Chun et al. 2011). Our PSC-TGFp could adsorb large amounts of total protein, suggesting that it adsorbed not only TGF-β1 but also other dentin-derived proteins, indicating a stronger odontogenic effect.

TGF-β1 is often used as a marker substance because it presents in incomparably higher amounts than other growth factors (Widbiller et al. 2018). Previous studies have shown that DMEP solution containing 1,000 pg/mL of TGF-β1 led to an initial increase in cell proliferation and decreased after day 5. In addition, this solution could significantly promote cell migration, enhance DSPP and DMP1 expression, and induce mineralization (Widbiller et al. 2018). The cytologic experiments used 0.1 mg/mL of PSC-TGFp + DMEP, which could release approximately 800 pg/mL of TGF-β1 into the medium. Our previous research showed that PSC could maintain a neutral pH when in contact with fluid and did not influence cell viability at a low concentration (Cui et al. 2017). In this study, neither 0.1 mg/mL of PSC nor PSC-TGFp affected cell viability. PSC-TGFp combined with DMEP increased cell viability on days 4 and 7, indicating excellent biocompatibility. Our previous studies have shown that BG can increase the expression of odontogenesis-related markers, including DSPP and DMP1 (Wang et al. 2014). In this study, real-time polymerase chain reaction and Western blot results showed that PSC-TGFp + DMEP could promote the expression of odontogenic genes and proteins as compared with PSC, PSC-TGFp, or PSC + DMEP. These results demonstrated that the BG and DMEP might have a synergistic promoting effect. Alizarin red staining results also proved this hypothesis.

Growth factors usually have specific temporal and spatial expression patterns during tooth development, indicating that the concentration and distribution of growth factors could determine the fate of stem cells (Yang et al. 2016; Duncan et al. 2018). Different from other tissues, the root canal is a relatively closed space and communicates only with the outside environment through the apical foramen such that during endodontic regeneration, an insufficient amount of growth factors can be obtained directly from the body fluid. If endogenous growth factors, which are released from dentin after EDTA irrigation, could be enriched to the root dentin surface, the created concentration gradient may promote cell migration toward the root dentin wall and directional differentiation (Zhujiang and Kim 2016). We suppose that by treating the root canal with our modified BG suspension before inducing apex bleeding in pulp revascularization, the materials may adhere to the surface of the dentin, capture dentin-derived TGF-β1 and other growth factors, and slowly release them. The created concentration gradient of these endogenous growth factors may induce stem cell migration and mineralization on the primary dentin, avoiding the ectopic mineralization that is common in pulp revascularization. However, due to the narrow space of the root canal, it is still challenging to ensure that the material is distributed homogeneously on the surface of the root dentin. Further

research is needed to precisely control the physical and chemical properties of the modified BG, making it suitable for in vivo application. This study provides an essential experimental basis for the use of biomaterials to manipulate endogenous growth factors and may have broad application prospects.

Conclusion

The present study shows that TGFp-modified BG could capture endogenous TGF-β1 and other growth factors from DMEP, thereby enhancing cell viability, adhesion, migration, and odontogenic differentiation. PSC-TGFp may be a promising material for regenerative endodontic therapy by capturing and enriching endogenous growth factors.

Author Contributions

J. Wu, S. Wang, Y. Dong, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; S. Mao, L. Xu, D. Qiu, contributed to conception, design, and data acquisition, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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