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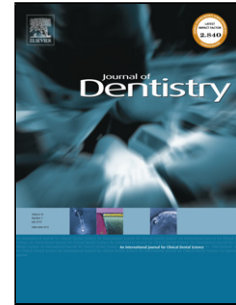
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# Journal Pre-proof

Liposomes loaded with transforming growth factor  $\beta$ 1 promote odontogenic differentiation of dental pulp stem cells

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**Liposomes loaded with transforming growth factor  $\beta$ 1 promote odontogenic differentiation of dental pulp stem cells**

**Short title:** Liposomal TGF- $\beta$ 1 for odontogenic differentiation of dental pulp stem cells

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*Keywords:* Dental pulp stem cells, Liposome, Odontogenic differentiation, Transforming growth factor  $\beta$ 1

### **Abstract**

*Objectives:* This study investigated whether novel liposome formulations loaded with transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) could promote the odontogenic differentiation of human dental pulp stem cells (hDPSCs) for dentine-pulp regeneration.

*Methods:* 0-100 ng/mL of liposomal TGF- $\beta$ 1 was prepared using the thin-film hydration method. Release of TGF- $\beta$ 1 from the liposomes was quantified by an enzyme-linked immunosorbent assay (ELISA). The hDPSCs were treated with different concentrations of liposomal TGF- $\beta$ 1 and cell viability was tested using an MTT assay. "Osteodentine" differentiation capacity was assessed by RT-qPCR, ELISA and Alizarin red S staining.

*Results:* The ELISA results showed that liposomal TGF- $\beta$ 1 achieved a controlled and prolonged release over time. The MTT results demonstrated that the liposomes (100  $\mu$ g/mL) were not cytotoxic to the cells. Liposomal TGF- $\beta$ 1 up-regulated the expression

of “osteodentine” markers, *RUNX-2*, *DMP-1* and *DSPP*, in hDPSCs after 7 days of treatment and resulted in the accumulation of mineralised nodules.

*Conclusion:* This study indicated that liposomes are an effective carrier for delivering TGF- $\beta$ 1 over time. Liposomal TGF- $\beta$ 1 promoted dentinogenesis and increased mineralisation in hDPSCs. This highlights the potential of liposomal TGF- $\beta$ 1 for future use in dentine-pulp regeneration.

*Clinical significance:* Liposomal TGF- $\beta$ 1 may be used as a synergist for promoting dentine-pulp regeneration of immature permanent teeth or as a pulp capping agent for inducing reparative dentine formation.

*Keywords:* Dental pulp stem cells, Liposomes, Odontogenic differentiation, Transforming growth factor  $\beta$ 1

## 1. Introduction

The dentine-pulp complex is critical in maintaining the development, function and homeostasis of the tooth [1]. Dentine plays a crucial role in protecting the inner dental pulp from harmful stimuli and bacterial invasion [2]. Once the dental pulp is infected or removed, there is an increased risk of tooth fracture and loss [3,4]. Pulp necrosis in immature permanent teeth is particularly detrimental and can potentially lead to arrested root development. Hence, it is important to develop a novel approach that will regenerate organised dentine with normal physiological functions for teeth with dentine or pulp damage.



Various growth factors are normally upregulated and secreted during dentinogenesis; in response to tooth injury; or in response to infection by a carious lesion [5]. Amongst these bioactive factors, transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), have been shown to be highly involved in dentine-pulp regeneration [6]. TGF- $\beta$ 1 is a multifunctional cytokine that regulates cell proliferation, differentiation and extracellular matrix synthesis. It has been reported that 6 ng/mL TGF- $\beta$ 1 induces osteo/odontoblastic differentiation of rat dental pulp cells *in vitro*, and also promotes columnar odontoblast-like cell formation *in vivo* [7].

The clinical use of growth factors however is limited, due to their short half-life and susceptibility to protease degradation. High doses are often required to achieve a sustained release and physiological effect [8,9]. Therefore, methods that achieve targeted delivery, whilst also preventing degradation are extremely beneficial. A potential method of achieving this is through the use of lipid-based nanoparticles, known as liposomes. Liposomes consist of a lipid bilayer that allows for the encapsulation of hydrophilic molecules within its aqueous core and hydrophobic molecules within its phospholipid bilayer structure [10]. This delivery system is advantageous due to its biocompatibility (US FDA approved) and has been widely used clinically [11]. Liposomes have been shown to not only increase the uptake of aqueous molecules into cells but also release therapeutics and growth factors in a much more controlled and prolonged manner into the cell microenvironment [12].

Previous studies have reported that stem-cell-based regenerative medicine approaches could provide a potential solution for dentine-pulp complex regeneration [13,14]. Human dental pulp stem cells (hDPSCs) have an inherent differentiation capability and relatively high proliferation and self-renewal potential [15]. Thus, there is significant interest in stimulating these cells for treating tissue damage or tissue loss, such as repairing the dentine-pulp complex.

To further this goal of regenerative endodontics, this study aims to formulate liposomes with different concentrations of TGF- $\beta$ 1 and to investigate the effects of liposomal TGF- $\beta$ 1 on the odontogenic differentiation of hDPSCs for dentine-pulp regeneration.

## **2. Materials and methods**

### *2.1. Preparation and characterisation of liposomes and liposomal TGF- $\beta$ 1*

Liposomes were prepared by the thin-film hydration method [16]. In brief, lipids were weighed in a round-bottom flask (phosphatidylcholine (Sigma-Aldrich): phosphatidylserine (Sigma-Aldrich): cholesterol (Sigma-Aldrich) at a ratio of 7 : 2 : 1, w/w) and suspended in minimal chloroform, which was then evaporated using a rotary evaporator (BUCHI Interface I-300) at 332 mbar, 50°C for 30 min. A dry film on the bottom of the flask was resuspended in distilled water (control liposomes) or using different concentrations of human recombinant TGF- $\beta$ 1 (PeproTech) in an aqueous solution (10 ng/mL, 50 ng/mL and 100 ng/mL) to give a final lipid concentration of 1

mg/mL. The suspensions were then extruded under nitrogen gas at 8 bar, 3 times through a 400 nm polycarbonate membrane filter, 3 times through a 200 nm filter and 10 times through a 100 nm filter using a Lipex mini-extruder (Northern Lipids). Manufactured liposome and liposomal TGF- $\beta$ 1 were stored in a nitrogen atmosphere at 4 °C until used.

The size and zeta potential of liposome samples were tested by dynamic light scattering using a Malvern Zetasizer ZS (Malvern Zetasizer ZEN3600). 10  $\mu$ L samples were diluted into 1 mL distilled water for measurements.

To test the encapsulation efficiency of TGF- $\beta$ 1 in the liposomes, a human TGF- $\beta$ 1 DuoSet ELISA kit (R&D SYSTEMS) was used to analyse the amount of untrapped TGF- $\beta$ 1 outside of the liposomes. The encapsulation efficiency was calculated using Equation 1, where the total quantity of TGF- $\beta$ 1 used is W and untrapped quantity of TGF- $\beta$ 1 is W1.

$$\text{Encapsulation efficiency (\%)} = (W - W1) / W \times 100 \quad \text{Eq. 1}$$

## 2.2. Release of TGF- $\beta$ 1 from liposomes

The release of TGF- $\beta$ 1 from the liposomal carrier was assessed over time by a DuoSet ELISA kit (R&D SYSTEMS). The total quantity of TGF- $\beta$ 1 in the liposomal TGF- $\beta$ 1 group and free TGF- $\beta$ 1 group was 200 ng. The initial concentration of TGF- $\beta$ 1 in phosphate buffer solution (PBS) was tested after extrusion in both groups. 400  $\mu$ L

samples from both groups were incubated at 37°C for 1, 3, 6, 9, 12, 24, 48 and 72 h and the released TGF- $\beta$ 1 was measured using the TGF- $\beta$ 1 ELISA kit.

### 2.3. Cell culture, characterisation and treatment

hDPSCs (Lonza) were cultured in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM, Gibco, Invitrogen) containing 10% (v/v) fetal bovine serum (Gibco, Invitrogen) at 37°C in 5% (v/v) CO<sub>2</sub>. Cell surface antigens were detected and the expression of specific mesenchymal cell markers analyzed to confirm the mesenchymal phenotype. The cell suspension was incubated with antibodies CD105-FITC, CD73-PE, CD90-FITC, CD31-FITC and CD34-FITC (1: 100, Abcam) respectively for 1 h in the dark on ice and then detected by flow cytometry (Becton-Dickinson). For the multi-lineage differentiation assay, Alizarin red S staining was used to identify the mineralised nodules after incubation in osteogenic medium ( $\alpha$ -MEM supplemented with 10 nM dexamethasone and 100  $\mu$ M  $\beta$ -glycerophosphate); and Oil red O staining was used to identify the lipid vacuoles after incubation in the adipogenic medium ( $\alpha$ -MEM supplemented with 10  $\mu$ g/ml insulin, 1  $\mu$ M dexamethasone, 100  $\mu$ M indomethacin and 0.5 mM 3-Isobutyl-1-methylxanthine) for 3 weeks. Cells were treated using different concentrations of liposomal TGF- $\beta$ 1, with a lipid concentration of 100  $\mu$ g/mL and final encapsulated TGF- $\beta$ 1 concentrations of 0 ng/mL, 1 ng/mL, 5 ng/mL and 10 ng/mL respectively. Free TGF- $\beta$ 1 (5 ng/mL) and PBS treatment were used as controls.

#### 2.4. Cell viability

Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. hDPSCs were seeded at 1,000 cells/well in 96-well plates. Liposomal TGF- $\beta$ 1 was added into the culture medium to give a final concentration of 100  $\mu$ g/mL lipid for all groups with a final concentration of 0 ng/mL, 1 ng/mL, 5 ng/mL and 10 ng/mL of TGF- $\beta$ 1. After 1, 3 and 7 days treatment, 25  $\mu$ L of 5 mg/mL MTT solution (Sigma-Aldrich, USA) was added to each well, followed by incubation for 4 h at 37°C. The MTT solution was removed and 100  $\mu$ L dimethyl sulfoxide was added. The absorbance was then measured at 570 nm using a microplate reader (FLUOstar Omega).

#### 2.5. Alizarin red S staining

hDPSCs were treated with control liposomes and liposomal TGF- $\beta$ 1 in osteogenic media ( $\alpha$ -MEM supplemented with 10 nM dexamethasone and 100  $\mu$ M  $\beta$ -glycerophosphate) for 21 days, fixed with 10% (w/v) formaldehyde for 30 min and stained with 2% (w/v) Alizarin Red S (Sigma-Aldrich, USA, pH = 4.2) for 20 min at room temperature. The cells were washed three times with distilled water and photos were taken at 20 $\times$  magnification.

#### 2.6. Quantitative real-time PCR

The hDPSCs were seeded in 6-well plates at  $5 \times 10^4$  cells/well and treated with different concentrations of liposomal TGF- $\beta$ 1 (0 – 10 ng/mL TGF- $\beta$ 1; liposomal concentration 100  $\mu$ g/mL) or free TGF- $\beta$ 1 (5 ng/mL) for 7 days. Total RNA was extracted from the cells using a QIAGEN RNeasy Mini Kit. RNA yield was quantified using a NanoVue spectrophotometer (GE Healthcare). cDNA was synthesized from 1  $\mu$ g RNA using M-MLV Reverse Transcriptase (Promega). “Osteodentine” marker expression was analysed by SYBR Green quantitative PCR reaction (QuantStudio 6 Flex) and was performed at 95°C for 20 sec, 55°C for 20 sec, 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec. The gene-specific primers for runt related transcription factor 2 (*RUNX-2*), dentin matrix protein-1 (*DMP-1*) and dentin sialophosphoprotein (*DSPP*) were used to evaluate the odontogenic differentiation of hDPSCs, using  *$\beta$ -actin* as the reference gene. The results were analyzed by  $\Delta\Delta$ CT method according to MIQE guidelines [17]. The efficiency of all primers was validated through qRT-PCR of Human Reference Total RNA dilutions (Agilent Technologies). Sequences of primers used in RT-qPCR are shown in Table 1.

**Table 1** Sequences of primers used in RT-qPCR

### 2.7. *DMP-1* expression tested by ELISA

The cell culture medium was collected after treatment with control liposomes, 5 ng/mL liposomal TGF- $\beta$ 1 and 5 ng/mL free TGF- $\beta$ 1, respectively at 7 days. The human

DMP-1 ELISA Kit (Abcam) was used to determine DMP-1 concentrations following the manufacturer's instruction. The concentration of DMP-1 was calculated by the standard curve.

### 2.8 Statistical analysis

All data were presented as the mean  $\pm$  standard deviation. Statistical analysis was performed using a post-hoc multiple comparison test to compare the differences between each two groups.  $P < 0.05$  was considered statistically significant difference.

Gene	Primer sequence (5'-3') (F: Forward, R: Reverse)	Expected PCR product size (bp)	Efficiency (%)	NCBI no.
<i><math>\beta</math>-actin</i>	F: CTT TTT TGT CCC CCA ACT TGA R: TGG CTG CCT CCA CCC A	68	108.81%	NM_001101.3
<i>RUNX-2</i>	F: CCCGTGGCCTTCAAGGT R: CGTTACCCGCCATGACAGTA	135	108.07%	XM_017011396.1
<i>DMP-1</i>	F: AAG CAG ACA GCG AAT CCA GT R: CTG CTG AGC TGC TGT GAG AC	114	107.26%	XM_011531706.2
<i>DSPP</i>	F: GAA GAT GCT GGC CTG GAT AA R: TCT TCT TTC CCA TGG TCC TG	164	95.16%	NM_014208.3

## 3. Results

### 3.1. Cell characterisation

As shown in Fig.1, hDPSCs had a spindle-shaped morphology and were similar to the fibroblast-like cells (Fig.1 A). The cells expressed the mesenchymal stromal cell markers CD105 (95.68%), CD73 (98.17%), CD90 (87%) and lacked expression of

CD31 (0.1%) and CD34 (0.1%) (Fig.1 B). Mineral deposits and lipid vacuoles could be observed after 3 weeks of osteogenic induction or adipogenic differentiation, respectively (Fig.1 C and D).

### 3.2. Characterisation of control liposomes and liposomal TGF- $\beta$ 1

The average diameter of the control liposomes was  $108.9 \pm 26.6$  nm with an average polydispersity index (PDI) of 0.262. The average diameter of the liposomal TGF- $\beta$ 1 samples (10 ng/mL, 50 ng/mL, 100 ng/mL) were  $104.2 \pm 18.9$  nm,  $107.8 \pm 26.6$  nm, and  $120.8 \pm 34.9$  nm respectively and their PDI were 0.216, 0.366, 0.256 respectively. The zeta potential of the control liposomes and all groups of liposomal TGF- $\beta$ 1 were similar, ranging from -17 and -20 mV. For the 10 ng/mL and 50 ng/mL liposomal TGF- $\beta$ 1 groups, the encapsulation efficiencies were 25.2% and 39.5% respectively. The encapsulation efficiency for the 100 ng/ml TGF- $\beta$ 1 concentration was not significantly increased compared to other conditions, at 44.9% (Table 2).

#### **Table 2** Characterisation of control liposomes and liposomal TGF- $\beta$ 1

PDI, polydispersity index; SD, standard deviation.

### 3.3. Release profile of TGF- $\beta$ 1 from liposomes

The initial concentration of TGF- $\beta$ 1 in the liposomal TGF- $\beta$ 1 group and free TGF- $\beta$ 1 group was 9.62 ng/mL and 7.25 ng/mL respectively after the extrusion process. Over 24 h, the TGF- $\beta$ 1 concentration in the free TGF- $\beta$ 1 group decreased dramatically to



less than a quarter of the initial concentration. For the liposomal TGF- $\beta$ 1 group, the TGF- $\beta$ 1 concentration remained above 6 ng/mL over 9 h, which indicated its longer release and stability over time. After 9 h, the concentration of TGF- $\beta$ 1 reduced gradually until 72 h (Fig. 2).

### 3.4 Cell viability after liposomal TGF- $\beta$ 1 treatment

Groups	Average size $\pm$ SD (nm)	PDI	Zeta potential	Encapsulation efficiency (%)
Control Liposomes	108.9 $\pm$ 26.6	0.262	-18.8 $\pm$ 5.7	-
Liposomal TGF- $\beta$ 1 (10 ng/mL)	104.2 $\pm$ 18.9	0.216	-18.7 $\pm$ 3.9	25.2 $\pm$ 3.4
Liposomal TGF- $\beta$ 1 (50 ng/mL)	107.8 $\pm$ 26.6	0.366	-20.2 $\pm$ 6.9	39.5 $\pm$ 1.4
Liposomal TGF- $\beta$ 1 (100 ng/mL)	120.8 $\pm$ 34.9	0.256	-17.2 $\pm$ 5.1	44.9 $\pm$ 1.2

The MTT results demonstrated that the cell viability of control liposomes and liposomal TGF- $\beta$ 1 groups was above 90% at 7 days. This confirmed that 100  $\mu$ g/mL liposomes were biocompatible and had no cytotoxic effects on hDPSCs. For the free TGF- $\beta$ 1 group, the cell viability was 118% at 7 days, which was higher than the other groups ( $P < 0.05$ ), reflecting the TGF- $\beta$ 1 induced proliferation of hDPSCs (Fig. 3).

### 3.5. Liposomal TGF- $\beta$ 1 promoted mineralisation of hDPSCs

There was increase in Alizarin red S staining, reflective of mineralisation, in hDPSCs treated with liposomal TGF- $\beta$ 1 compared with the control group, particularly for the 5 ng/mL liposomal TGF- $\beta$ 1 group (Fig. 4A). Cells cultured in normal medium without osteogenic induction are shown in supplementary Fig. 1.

### 3.6 Liposomal TGF- $\beta$ 1 induced the expression of odontoblastic genes and protein

According to the Alizarin red S staining data, 5 ng/mL liposomal TGF- $\beta$ 1 showed the most obvious increase in mineralisation in all groups (Fig. 4A). Therefore, this concentration (5 ng/mL) was taken forward for comparison between the liposomal TGF- $\beta$ 1 and free TGF- $\beta$ 1. *RUNX-2* expression in hDPSCs treated with liposomal TGF- $\beta$ 1 (1 ng/mL and 5 ng/mL) were similar and approximately 1.9-fold greater than the control group after 7 days ( $P < 0.05$ ). Free TGF- $\beta$ 1 caused a 1.3-fold increase in *RUNX-2* expression compared with the control group ( $P < 0.05$ ), but interestingly, the induction observed with liposomal TGF- $\beta$ 1 was significantly higher than that seen with free TGF- $\beta$ 1 ( $P < 0.05$ ). An increase in the expression of *DMP-1* and *DSPP* for the cells treated with liposomal TGF- $\beta$ 1 was also observed. For liposomal TGF- $\beta$ 1 (1 ng/mL), both *DMP-1* and *DSPP* expression were around 3.0-fold greater than the control ( $P < 0.05$ ). For the liposomal TGF- $\beta$ 1 (5 ng/mL), there was a 3.7-fold increase in *DMP-1* expression and 2.2-fold increase in *DSPP* expression compared with the control. There was no significant difference in the expression of these genes between the higher liposomal TGF- $\beta$ 1 concentration (10 ng/mL) and the control group ( $P > 0.05$ ).

(Fig. 4B-D). ELISA data showed that the concentration of DMP-1 in the 5 ng/mL liposomal TGF- $\beta$ 1 group was significantly greater than that in other groups. ( $P < 0.05$ ) (Fig. 4 E).

#### 4. Discussion

Liposomes have been widely used for the encapsulation and release of bioactive substances in tissue engineering and regeneration [18]. Size and zeta potential of liposomes are important parameters for assessing their stability and function. Hwang *et al.* demonstrated that liposomes filtered through 100 nm, 200 nm or 400 nm polycarbonate membrane filters became large unilamellar vesicles (LUVs) from multilamellar vesicles (MLVs). This process resulted in liposomes with a relatively narrow and mono-disperse particle diameter distribution [19]. A similar liposomal preparation method was used in this study to ensure that the liposomal size was close to approximately 100 nm with acceptable stability. For zeta potential, all groups of liposomes and liposomal TGF- $\beta$ 1 were between -17 and -20 mV, demonstrating no change after the inclusion of TGF- $\beta$ 1. The highly negative charge of the liposomal TGF- $\beta$ 1 formulations is indicative of a stable liposome suspension in solution. Similar zeta potential values of liposomes have been estimated in other studies which demonstrate that formulation stability was achieved at zeta potential values greater than  $\pm 30$  mV [20,21]. In this system, liposomal TGF- $\beta$ 1 was relatively stable in solution, however long-term shelf-life stability needs to be assessed. Liposome-mediated

delivery of therapeutics faces additional obstacles *in vivo*, such as entry into the cell, endosomal escape, and lysosomal degradation [22]. Niu *et al.* reported that liposomes containing glycocholate could deliver and protect recombinant human insulin against enzymatic degradation [23]. Additionally, it was demonstrated that the complement system mediated the clearance of liposomes in rats *via* uptake by macrophages and degradation in blood circulation [24]. Further *in vivo* studies are required to establish the likely *in vivo* degradation pathways of this formulation and the feasibility of clinical implementation. Studies are also required to assess the scalability of the formulation and long-term shelf-life.

In general, exogenous TGF- $\beta$ 1 is not systemically administered *in vivo* because it is short-lived when exposed to a physiological environment. Carriers are usually required to protect soluble growth factors from proteolysis or antibody neutralization and to locally deliver growth factors at effective concentrations and controlled rates. Many kinds of carriers are used to overcome the above-mentioned disadvantages [16,25,26]. In our study, liposomes were demonstrated to be an excellent, biocompatible carrier to deliver TGF- $\beta$ 1 to cells, with sufficient TGF- $\beta$ 1 encapsulation and prolonged TGF- $\beta$ 1 release.

As a member of TGF- $\beta$  superfamily, TGF- $\beta$ 1 is one of the most abundant growth factors in dentine, and has many reported roles in wound healing, extracellular matrix production and mineralized tissue repair [27]. One of the most well characterized effects of TGF- $\beta$ 1 is the promotion of cell proliferation, which has been shown in

precartilagenous stem cells [28], bone marrow mesenchymal stem cells [29], adipose-derived stem cells [30], as well as DPSCs [31]. It has been reported that a combination of a chitosan scaffold with TGF- $\beta$ 1 enhanced the proliferation and osteogenic differentiation of multipotent stem cells derived from human exfoliated deciduous teeth [32]. The effective concentration of TGF- $\beta$ 1 for stem cell proliferation is likely to be different and dependent on cell type and treatment time. Tabatabaei *et al.* reported that 5 ng/mL and 10 ng/mL free TGF- $\beta$ 1 showed a significant increase in cell viability at 24 h compared with the control group [33]. In this study, all liposomal TGF- $\beta$ 1 groups had no negative effect on cell viability and free TGF- $\beta$ 1 (5 ng/mL) showed a positive effect on DPSC proliferation.

TGF- $\beta$ 1 in dentine also plays important roles in dentine matrix maintenance and in promoting odontogenic differentiation. Many clinically used conditioning agents have been shown to release TGF- $\beta$ 1, which could induce odontogenic differentiation of hDPSCs [34,35]. It was also reported that 330 pg/mL of TGF- $\beta$ 1 could be released from 100  $\mu$ g/mL demineralized dentin matrix (DDM), which could induce the osteogenic differentiation of DPSCs [16]. Comparatively, in this study, the odontogenic differentiation of hDPSCs induced by liposomal TGF- $\beta$ 1 seemed to be greater than that of liposomal DDM judged by the induction of biomineralization. This may be related to the relatively higher concentration of TGF- $\beta$ 1 used during cell treatment, compared to the amount released by DDM.

DMP-1 and DSPP are important dentine matrix phospho-proteins that belong to the small integrin-binding ligand N-linked glycoproteins (SIBLING) family and possess key roles in dentine-pulp regeneration and hard tissue biomineralization. Li *et al.* reported that 6 ng/mL TGF- $\beta$ 1 could increase the expression of dentine sialoprotein (*DSP*) and osteopontin (*OPN*) *in vitro* after 7 days [7]. However, Luisi *et al.* found that the exposure of 1 ng/mL TGF- $\beta$ 1 to human dental pulp cells was not sufficient to induce expression of the dentin matrix components bone sialoprotein (*BSP*) and *DSPP* by RT-PCR [36]. As a result, the function of TGF- $\beta$ 1 on dental pulp cells is still inconclusive. In this study, free TGF- $\beta$ 1 (5 ng/mL) had no effect on increasing the expression of *DSPP* and *DMP-1*, whereas lower concentrations of liposomal TGF- $\beta$ 1 (1 ng/mL and 5 ng/mL) were found to be effective. Our data demonstrated that the continuous and low concentration TGF- $\beta$ 1 release from liposomes was effective for inducing odontogenic differentiation of DPSCs.

The exact mechanism by which liposomes loaded with TGF- $\beta$ 1 or other growth factors have their effect still remain to be delineated. Additionally, the combination of applying liposomal TGF- $\beta$ 1 in degradable biomaterials could further promote dentine-pulp complex regeneration and help achieve the therapeutic effects of growth factors *in vivo* in the future.

## 5. Conclusion

This work has shown that liposomes can deliver TGF- $\beta$ 1 over prolonged periods and maintain therapeutic concentrations within the cell microenvironment. Liposomal TGF- $\beta$ 1 was biocompatible and induced odontogenic differentiation of hDPSCs. This study highlights the potential for using this technology to successfully promote dentine-pulp regeneration in the clinical setting.

### Disclosures

All authors declare no conflicts of interest.

### AUTHOR DECLARATION

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

### Credit Author Statement

**Liming Jiang:** Investigation, methodology, data analysis, original draft manuscript preparation. **Wayne Ayre.:** Supervision, manuscript preparation and reviewing. **Genevieve Melling:** Data analysis, manuscript reviewing. **Bing Song:** Supervision, manuscript reviewing. **Xiaoqing Wei:** methodology, draft manuscript reviewing. **Alastair J Sloan:** Conceptualisation, supervision, manuscript reviewing and editing. **Xu Chen:** Supervision, manuscript reviewing

### Declaration of interests

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.

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## References

- [1] M. Zhang, F. Jiang, X. Zhang, S. Wang, Y. Jin, W. Zhang, et al., The effects of platelet-derived growth factor-BB on human dental pulp stem cells mediated dentin-pulp complex regeneration, *Stem. Cells. Transl. Med.* 6 (12) (2017) 2126-2134.
- [2] M. Nakashima, K. Iohara, Regeneration of dental pulp by stem cells, *Adv. Dent. Res.* 23 (2011) 313-319.
- [3] S. Eramo, A. Natali, R. Pinna, E. Milia, Dental pulp regeneration via cell homing, *Int. Endod. J.* 51(4) (2018) 405-419.
- [4] J. Yang, G. Yuan, Z. Chen, Pulp Regeneration: current approaches and future challenges, *Front. Physiol.* 7 (2016) 58.
- [5] A. Piattelli, C. Rubini, M. Fioroni, R. Strocchi, Transforming growth factor-beta 1 (TGF-beta1) expression in normal healthy pulps and in those with irreversible pulpitis, *Int. Endod. J.* 37 (2) (2004) 114-119.
- [6] J. Botelho, M.A. Cavacas, V. Machado, J.J. Mendes, Dental stem cells: recent progresses in tissue engineering and regenerative medicine, *Ann. Med.* 49 (8) (2017) 644-651.
- [7] Y. Li, X. Lü, X. Sun, S. Bai, S. Li, J. Shi, Odontoblast-like cell differentiation and dentin formation induced with TGF- $\beta$ 1, *Arch. Oral. Biol.* 56 (11) (2011) 1221-1229.
- [8] K.F. Bruggeman, A.L. Rodriguez, C.L. Parish, R.J. Williams, D.R. Nisbet, Temporally controlled release of multiple growth factors from a self-assembling peptide hydrogel, *Nanotechnology.* 27 (38) (2016) 385102.

- [9] R. Mittermayr, P. Slezak, N. Haffner, D. Smolen, J. Hartinger, A. Hofmann, et al., Controlled release of fibrin matrix-conjugated platelet derived growth factor improves ischemic tissue regeneration by functional angiogenesis, *Acta. Biomater.* 29 (2016) 11-20.
- [10] G. Bozzuto, A. Molinari, Liposomes as nanomedical devices, *Int. J. Nanomedicine.* 10 (2015) 975-999.
- [11] V.P. Torchilin, Recent advances with liposomes as pharmaceutical carriers, *Nat. Rev. Drug. Discov.* 4 (2) (2005) 145-160.
- [12] M.L. Etheridge, S.A. Campbell, A.G. Erdman, C.L. Haynes, S.M. Wolf, J. McCullough, The big picture on nanomedicine: the state of investigational and approved nanomedicine products, *Nanomedicine.* 9 (1) 2013 1-14.
- [13] T.A. Mitsiadis, G. Orsini, L. Jimenez-Rojo, Stem cell-based approaches in dentistry, *Eur. Cell. Mater.* 30 (2015) 248-257.
- [14] D.B. Bhuiyan, J.C. Middleton, R. Tannenbaum, T.M. Wick, Bone regeneration from human mesenchymal stem cells on porous hydroxyapatite-PLGA-collagen bioactive polymer scaffolds, *Biomed. Mater. Eng.* 28 (6) (2017) 671-685.
- [15] C. Rombouts, C. Jeanneau, A. Bakopoulou, I. About, Dental pulp stem cell recruitment signals within injured dental pulp tissue, *Dent. J. (Basel).* 4 (2) (2016) 8.
- [16] G.E. Melling, J.S. Colombo, S.J. Avery, W.N. Ayre, S.L. Evans, R.J. Waddington, et al., Liposomal delivery of demineralized dentin matrix for dental tissue regeneration, *Tissue. Eng. Part. A.* 24 (13-14) (2018) 1057-1065.

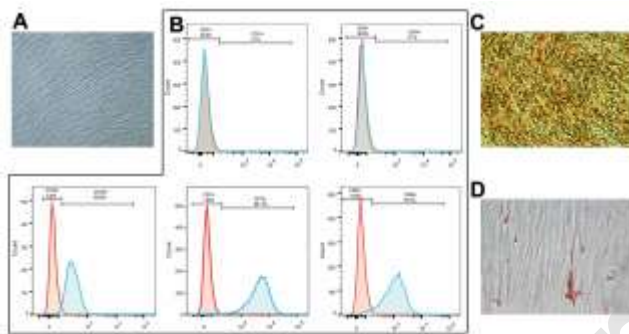
- [17] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, et al., The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, *Clin. Chem.* 55 (4) (2009) 611-622.
- [18] A. Madni, M. Sarfraz, M. Rehman, M. Ahmad, N. Akhtar, S. Ahmad, et al., Liposomal drug delivery: a versatile platform for challenging clinical applications, *Pharm. Pharm. Sci.* 17 (3) (2014) 401-426.
- [19] S.Y. Hwang, H.K. Kim, T.B. Choo, J. Hien, E.K. Lee, Effects of operating parameters on the efficiency of liposomal encapsulation of enzymes, *Colloids. Surf. B. Biointerfaces.* 94 (2012) 296-303.
- [20] W.C. Tsai, S.S.H. Rizvi, Simultaneous microencapsulation of hydrophilic and lipophilic bioactives in liposomes produced by an ecofriendly supercritical fluid process, *Food. Res. Int.* 99(Pt 1) (2017) 256-262.
- [21] B. Heurtault, P. Saulnier, B. Pech, J.E. Proust, J.P. Benoit, Physico-chemical stability of colloidal lipid particles, *Biomaterials.* 24 (23) (2003) 4283-4300.
- [22] C. Zylberberg, K. Gaskill, S. Pasley, S. Matosevi, Engineering liposomal nanoparticles for targeted gene therapy, *Gene. Ther.* 24 (8) (2017) 441-452.
- [23] M. Niu, Y. Lu, L. Hovgaard, W. Wu, Liposomes containing glycocholate as potential oral insulin delivery systems: preparation, in vitro characterization, and improved protection against enzymatic degradation, *Int. J. Nanomedicine.* 6 (2011) 1155-1166.

- [24] T.M. Huong, H. Harashima, H. Kiwada, In vivo studies on the role of complement in the clearance of liposomes in rats and guinea pigs, *Boil. Pharm. Bull.* 22 (5) (1999) 515-520.
- [25] Q. Min, J. Liu, J. Li, Y. Wan, J. Wu, Chitosan-poly lactide/hyaluronic acid complex microspheres as carriers for controlled release of bioactive transforming growth factor- $\beta$ 1, *Pharmaceutics*. 10 (4) (2018) E239.
- [26] H. Furuya, Y. Tabata, K. Kaneko, Bone regeneration for murine femur fracture by gelatin hydrogels incorporating basic fibroblast growth factor with different release profiles, *Tissue. Eng. Part. A*. 20 (9-10) (2014) 1531-1541.
- [27] S.J. Avery, L. Sadaghiani, A.J. Sloan, R.J. Waddington, Analysing the bioactive makeup of demineralised dentine matrix on bone marrow mesenchymal stem cells for enhanced bone repair, *Eur. Cell. Mater.* 34 (2017) 1-14.
- [28] L. Cheng, C. Zhang, D. Li, J. Zou, J. Wang, Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) induces mouse precartilaginous stem cell proliferation through TGF- $\beta$  receptor II (TGFRII)-Akt- $\beta$ -catenin signaling, *Int. J. Mol. Sci.* 15 (7) (2014) 12665-12676.
- [29] F. Zhang, T. Ren, J. Wu, J. Niu, Small concentrations of TGF- $\beta$ 1 promote proliferation of bone marrow-derived mesenchymal stem cells via activation of Wnt/ $\beta$ -catenin pathway, *Indian. J. Exp. Biol.* 53 (8) (2015) 508-513.
- [30] F. Josh, M. Tobita, R. Tanaka, H. Orbay, K. Ogata, K. Suzuki, Concentration of PDGF-AB, BB and TGF- $\beta$ 1 as valuable human serum parameters in adipose-derived stem cell proliferation, *J. Nippon. Med. Sch.* 80 (2) (2013) 140-147.

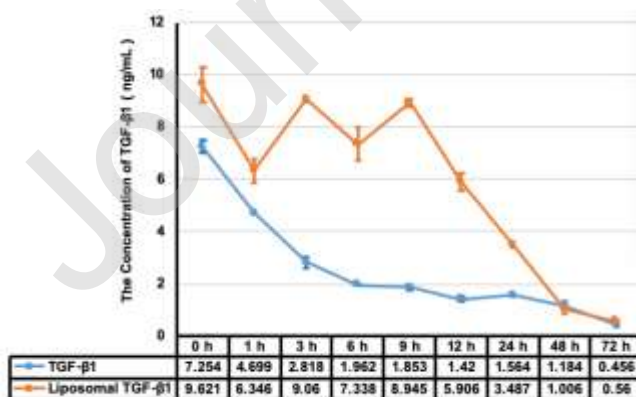
- [31]H. Salkın, Z.B. Gönen, E. Ergen, D. Bahar, M. Çetin, Effects of TGF- $\beta$ 1 overexpression on biological characteristics of human dental pulp-derived mesenchymal stromal cells, *Int. J. Stem. Cells.* 12 (1) (2019) 170-182.
- [32]M. Farea, A. Husein, A.S. Halim, N.A. Abdullah, K.I. Mokhtar, C.K. Lim, et al., Synergistic effects of chitosan scaffold and TGF- $\beta$ 1 on the proliferation and osteogenic differentiation of dental pulp stem cells derived from human exfoliated deciduous teeth, *Arch. Oral. Biol.* 59 (12) (2014) 1400-1411.
- [33]F.S. Tabatabaei, M. Torshabi, Effects of non-collagenous proteins, TGF- $\beta$ 1, and PDGF-BB on viability and proliferation of dental pulp stem cells, *J. Oral. Maxillofac. Res.* 7 (1) (2016) e4.
- [34]P. Laurent, J. Camps, I. About, Biodentine(TM) induces TGF- $\beta$ 1 release from human pulp cells and early dental pulp mineralization, *Int. Endod. J.* 45 (5) (2012) 439-448.
- [35]L. Sadaghiani, H.B. Gleeson, S. Youde, R.J. Waddington, C.D. Lynch, A.J. Sloan, Growth factor liberation and DPSC response following dentine conditioning. *J. Dent. Res.* 95 (11) (2016) 1298-1307.
- [36]S.B. Luisi, J.J. Barbachan, J.A. Chies, M.S. Filho, Behavior of human dental pulp cells exposed to transforming growth factor-beta1 and acidic fibroblast growth factor in culture, *J. Endod.* 33 (7) (2007) 833-835

## Figure Legends

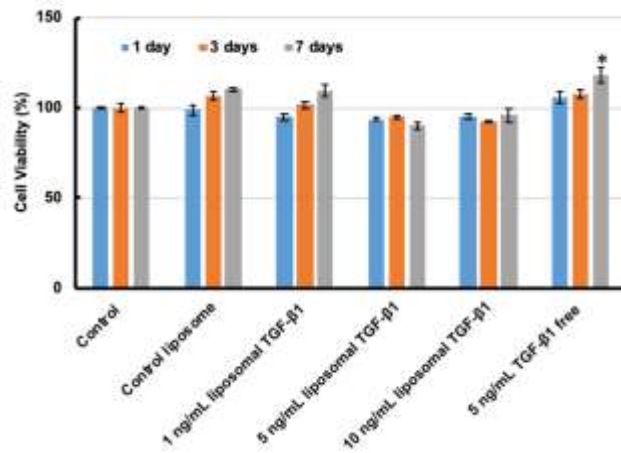
**Fig. 1.** Characterisation of hDPSCs. (A) The morphology of hDPSCs was spindle-shaped; (B) hDPSCs expressed the mesenchymal stem cell surface markers, including CD105 (95.68%), CD73 (98.17%), CD90 (87%) and lacked the markers CD31 (0.1%), CD34 (0.1%); (C) The mineral deposits in hDPSCs was indicated by Alizarin red S staining; (D) The neutral lipid vacuoles in hDPSCs was indicated by Oil red O staining. The images were taken at 40× magnification.



**Fig. 2.** TGF- $\beta$ 1 release profile from liposomes. TGF- $\beta$ 1 was measured by an ELISA to assess the release profile from liposomes over time. Liposomal TGF- $\beta$ 1 could maintain more stable TGF- $\beta$ 1 concentrations in solution than the free TGF- $\beta$ 1 over the first 9 h.  $n = 3 \pm$  standard error of the mean.



**Fig. 3.** Cell viability of hDPSCs treated with liposomal TGF- $\beta$ 1. Cell viability of hDPSCs treated with control liposomes, liposomal TGF- $\beta$ 1 and free TGF- $\beta$ 1 was assessed using an MTT assay over 7 days.  $n = 3 \pm$  standard error of the mean.  $*P < 0.05$  (compared to control).



**Fig. 4.** Liposomal TGF- $\beta$ 1 induced hDPSCs odontogenic differentiation and mineralisation. A. Mineralisation of hDPSCs was assessed by Alizarin red S staining. Representative pictures were taken at  $20\times$  magnification. B-D. The mRNA expression of hDPSCs treated by liposomes ( $100 \mu\text{g/mL}$ ), liposomal TGF- $\beta$ 1 ( $0\text{--}10 \text{ ng/mL}$  TGF- $\beta$ 1,  $100 \mu\text{g/mL}$  liposomes) and free TGF- $\beta$ 1 ( $5 \text{ ng/mL}$ ) were assessed by RT-qPCR for 7 days. B. Runt-related transcription factor (RUNX-2); C. Dentin matrix protein 1 (DMP-1); D. Dentin sialophosphoprotein (DSPP). E. The concentration of DMP-1 was tested by ELISA.  $n = 3 \pm$  standard error of the mean.  $* P < 0.05$ .

