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# Mechanical induction of dentin-like differentiation by adult mouse bone marrow stromal cells using compressive scaffolds

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ABSTRACT

Tooth formation during embryogenesis is controlled through a complex interplay between mechanical and chemical cues. We have previously shown that physical cell compaction of dental mesenchyme cells during mesenchymal condensation is responsible for triggering odontogenic differentiation during embryogenesis, and that expression of Collagen VI stabilizes this induction. In addition, we have shown that synthetic polymer scaffolds that artificially induce cell compaction can induce embryonic mandible mesenchymal cells to initiate tooth differentiation both *in vitro* and *in vivo*. As embryonic cells would be difficult to use for regenerative medicine applications, here we explored whether compressive scaffolds coated with Collagen VI can be used to induce *adult* bone marrow stromal cells (BMSCs) to undergo an odontogenic lineage switch. These studies revealed that when mouse BMSCs are compressed using these scaffolds they increase expression of critical markers of tooth differentiation *in vitro*, including the key transcription factors Pax9 and Msx1. Implantation under the kidney capsule of contracting scaffolds bearing these cells in mice also resulted in local mineralization, calcification and production of dentin-like tissue. These findings show that these chemically-primed compressive scaffolds can be used to induce adult BMSCs to undergo a lineage switch and begin to form dentin-like tissue, thus raising the possibility of using adult BMSCs for future tooth regeneration applications.



## 1. Introduction

During embryogenesis, tooth formation results from dynamic reciprocal mechanical and chemical interactions between the epithelium and underlying mesenchyme (Hashmi et al., 2014; Hashmi et al., 2015; Mammoto and Ingber, 2010; Mammoto et al., 2013; Thesleff, 2003). In particular, the embryonic dental epithelium induces underlying mesenchyme to undergo odontogenesis by promoting a cell compaction process, known as mesenchymal condensation, which is also critical for the formation of numerous organs (Hall and Miyake, 1992; Hall and Miyake, 1995; Hall and Miyake, 2000; Hashmi et al., 2015; Mammoto et al., 2011; Smith and Hall, 1990; Thesleff, 2003). Induction of tooth differentiation begins with upregulation of critical mediators of tooth development, such as the transcription factors Pax9 and Msx1 (Hashmi et al., 2014; Hashmi et al., 2015; Maas and Bei, 1997; Mammoto et al., 2011; Ohazama et al., 2004; Thesleff, 1985; Thesleff, 2003; Tucker & Sharpe, 2004). However, once induced by mesenchymal condensation, expression of the extracellular matrix (ECM) protein Collagen VI helps to stabilize the compressed tissue and cell compaction-dependent cell fate determination, thereby supporting odontogenic differentiation (Hashmi et al., 2016; Horibe et al., 2004; Mammoto et al., 2011; Mammoto et al., 2015; Thesleff, 2003; Thesleff et al., 1987). Formation of a mature functional odontogenic population is associated with formation of dentin, which is a calcified tissue layer that is first formed during the late bell stage of tooth development, and required for further development of the hard tissue layers during full tooth formation (Balic and Thesleff, 2015; Karcher-Djuricic et al., 1985).

We have previously demonstrated that undifferentiated embryonic day 10 mandibular mesenchymal cells can be artificially switched into the tooth formation pathway *in vitro* and *in vivo* by mechanically compacting the cells, thereby mimicking mesenchymal condensation (Hashmi et al., 2014; Mammoto et al., 2011). Specifically, when these cells are cultured within in a temperature sensitive, 'shrink-wrap' scaffold that contracts in three-dimensions (3D) when warmed from 34° C to 37°C, the cells undergo odontogenic differentiation *in vitro* and *in vivo* (Hashmi et al., 2014; Mammoto et al., 2011). This scaffold

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was composed of a porous poly(*N*-isopropylacrylamide) (PNIPAAm) hydrogel that was chemically modified with GRGDS peptides to promote cell adhesion and confer biocompatibility (Hashmi et al., 2014).

These results raised the possibility of using similar scaffolds to engineer artificial teeth, however, this approach that uses undifferentiated mesenchymal cells isolated from embryonic tissues would not be viable clinically. We therefore explored whether adult bone marrow stromal cells (BMSCs), which are easily accessible, accepted and known for their reprogramming capabilities, could serve as a potential cell source for our induction strategy. Here, we demonstrate that well-controlled physical compression of adult murine BMSCs using thermoresponsive GRGDS-PNIPAAm compressive scaffolds coated with Collagen VI induce these cells to initiate an odontogenic lineage switch cascade *in vitro* that results in formation of dentin-like tissue *in vivo*.

## 2. Materials and methods

### 2.1. Cell culture

Murine BMSCs, specified as D1 ORL UVA were purchased from ATCC (CRL-12424) and used in all studies prior to passage 6. Cells were cultured at 37 °C under 5% $CO_2$ using Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and 1% penicillin/streptomyocin (Gibco, Carlsbad, CA). Cell medium was changed every 2 days and when cell confluency reached approximately 90%, the cells were passaged using 0.05% EDTA-trypsin (Invitrogen).

### 2.2. Peptide and gel synthesis and cell seeding

Polynipaam gels were synthesized and modified with GRGDS peptide as described previously (Hashmi et al., 2014). These lyophilized hydrogels contract volumetrically by approximately 45% when heated from 34 °C to 37 °C in medium containing 10% fetal bovine serum, and their average pore size changes from approximately 2398 ± 211 μm to 1618 ± 108 μm, respectively, as described previously (Hashmi et al., 2014). These gels upon sterilization were coated overnight with Collagen VI at a concentration of 100 μg/ml. A cell pellet consisting of approximately $1 \times 10^6$ Murine BMD-MSCs was carefully injected within the pores of the gel construct using a 25 gauge syringe needle under a dissection microscope, and placed in an incubator set at 34 °C and 5% $CO_2$ overnight. These gels were either kept in the 34 °C incubator as a control in their non-shrinking form or moved to an incubator set at 37 °C with 5% $CO_2$ where they subsequently shrank.

### 2.3. Immunohistochemistry

Samples of interest were stained with ALP and Alizarin Red S as described previously (Hashmi et al., 2014). Immunostaining with Anti-Pax9, Anti-Msx1, Anti-Runx2, Anti-Collagen II, Anti-Sox9 and Anti-DSPP, which were all purchased from Abcam, Anti-Barx1 (Sigma-Aldrich) and secondary antibody Alexa Fluor 488 (Thermo Fisher) was conducted as previously described (Mammoto et al., 2011). Controls included use of secondary antibody alone. A toluidine blue stock solution was prepared by dissolving and mixing toluidine blue O (Sigma-Aldrich) (1 g) in 70% alcohol (100 ml). A 1% sodium chloride solution was prepared and adjusted to a pH ~ 2.3 using HCl. The toluidine blue stock solution (5 ml) was then mixed with 1% sodium chloride solution (45 ml), ensuring that the pH remained at ~2.3. Samples were stained with the working blue solution, washed in distilled water 3 times, dehydrated using 2 changes each of 95% alcohol and 100% alcohol, and cleared with 3 changes of xylene prior to mounting the coverslip over the sample section with Permount.

### 2.4. Scanning electron microscopy (SEM)

Backscattered scanning electron (BSE) imaging and elemental mapping was performed on two environmental SEMs (Tescan VegaGMU and Tescan MiraGMU, Brno Czech Republic) equipped with energy dispersive spectrometers (Bruker Nano X-Flash, Billereca, Massachusetts, USA).

### 2.5. Quantitative PCR

Quantitative real time PCR was performed with the SYBR Green RT-PCR kit (Qiagen) using primers for murine Pax9 and Msx1 on the CFX96 RT-PCR system (Bio-Rad) (PCRBIOR). Murine Cyclophilin was used as the housekeeping gene. The sequences for the primers used are shown below:

| | Forward | Reverse | GenBank accession number |
|---|---|---|---|
| Mouse Cyclophilin (house keeping gene) | 5′-CAGACGCCA CTGTCGCTTT-3′ | 5′-TGTCTTTGGAA CTTTGTCTGCAA-3′ | NM_008907.1 |
| Mouse Pax9 | 5′-CATTCGGCT TCG CATCGTG-3′ | 5′-CTCCCGGCAAA ATCGAACC-3′ | XM_011244013.2 |
| Mouse Msx1 | 5′-TGCTGCTAT GACTTCTTTG CC-3′ | 5′-GCTTCCTGTGA TCGGCCAT-3′ | NM_010835.2 |

### 2.6. Animal experiments

The Animal Care and Use Committee of Boston Children's Hospital approved all the *in vivo* animal studies. These studies were conducted as previously described (Hashmi et al., 2014; Mammoto et al., 2011; Nakao et al., 2007; Ohazama et al., 2004). Briefly, gels were placed overnight in an incubator set at 34 °C with 5% $CO_2$ prior to implanting under the kidney capsule of mice for 2 weeks. After 14 days, gels were harvested for histological analyses as described previously (Mammoto et al., 2011). We performed 3 independent experiments (using 3 different cell lots) and included 3 replicates for each condition.

### 2.7. Statistical analyses

Students' *t*-test was used to determine statistical significance. All results are presented in mean +/− the standard error of the mean.

## 3. Results

We first set out to determine whether artificial compaction of adult BMSCs induced using thermoresponsive GRGDS-PNIPAAm hydrogels influence expression of Pax9 and Msx1, two key odontogenic markers *in vitro*. To increase the likelihood of inducing tooth differentiation, we also coated these scaffolds with Collagen VI (100 μg/ml) based on its role in supporting odontogenic differentiation *in vivo* (Hashmi et al., 2016; Mammoto et al., 2015). Murine BMSCs were cultured within these scaffolds overnight at 34°C, and then maintained for 48 h at either the same temperature (uncompressed) or shifted to 37°C to trigger scaffold compression and cell compaction (compressed) (Fig. 1). Immunofluorescence microscopic analysis revealed that Pax9 and Msx1 protein levels were much higher in MSCs that were cultured in the compressed gel compared to those in the uncompressed gel (Fig. 2A). Quantitative PCR independently confirmed that both Pax9 and Msx1 mRNA levels increased significantly ($p \leq 0.05$) in compressed *versus* uncompressed BMSCs (Fig. 2B). Importantly, these differences in expression were not due to changes in temperature in the different cultures as neither Pax9 nor Msx1 were induced when the BMSCs were cultured