Differentiation of Bone Marrow Mesenchymal Stem Cells Induced by Indirectly Co-cultured Tooth Germ Cells

Heng ZHUANG[1,5], Zhi Hong DENG[4], Jun Nan SHI[2], Jin Hua YU[2,3], Ming JIANG[1,3], Xin NIE[1,3], Yan JIN[1,3]

Objective: *To study the differentiation of bone marrow mesenchymal stem cells (BM-MSCs). When co-cuttured with tooth germ cells (TGCs).*
Methods: *The BM-MSCs were isolated from tibiae and fibula of Sprague-Dawley (SD) rats aseptically and cultured. The fist molar tooth buds were isolated from E17 SD foetus and processed to obtain TGCs. The BM-MSCs were co-cultured with TGCs indirectly. The differentiation potentiality of BM-MSCs and the tooth-forming potentiality of TGCs were assessed.*
Results: *The BM-MSCs started to express odontogenic markers, dentin sialoprotein (DSP) and dentin matrix protein 1 (DMP1) after 7 days co-culture with TGCs. Implants of co-cultured pellets formed osteoid dentin under renal capsules, while control groups self-differentiated into osseous tissue.*
Conclusion: *BM-MSCs could be differentiated by co-cultured with TGCs into dental tissues, although the molecular mechanism requires further study.*
Key words: *co-culture, differentiation, mesenchymal stem cell, tooth germ*

Bone marrow mesenchymal stem cells (BM-MSCs) are a type of adult stem cells that have been recognised for many years[1]. When provided with appropriate inductive factors, including cytokines and growth factors, these clonal, plastic adherent cells are capable *in vitro* of differentiating into many kinds of cells, such as osteoblasts, adipocytes, chondrocytes, myoblasts, and also various non-mesenchymal tissue lineages, including hepatocytes and possibly neural cells[2-8]. It seems that the fate of BM-MSCs is determined by their surroundings[5]. Studies of BM-MSCs in tooth-tissue-engineering have generated a great deal of excitement[9-11]. They indicate that BM-MSCs could give rise to ameloblast-like cells and dental mesenchymal cells, and participate in tooth formation by the induction of early embryonic oral epithelium. Recent advances, including direct cell pellet implantation[9,10,12,13] and tissue engineering in combination with biocompatible scaffolds[14], have allowed us to contemplate new and promising strategies for hard tissue repair, especially for tooth regeneration.

To investigate whether the signals produced from tooth germ cells (TGCs) can determine the final differentiation of BM-MSCs, an indirect co-culture system between BM-MSCs and TGCs was established, and the differentiation of BM-MSCs was evaluated according to molecular and tissue characteristics.

[1] Department of Oral Histology and Pathology,
[2] Department of Operative Dentistry,
[3] Research and Development Center for Tissue Engineering, School of Stomatology, Fourth Military Medical University, Xi'an, P.R. China.
[4] Department of Otolaryngology, Xijing Hospital, Fourth Military Medical University, Xi'an, P.R. China.
[5] Peking University School and Hospital of Stomatology, Beijing, P.R. China.

Corresponding author: Dr. Yan JIN, Department of Oral Histology & Pathology, School of Stomatology, Fourth Military Medical University, 145 West Changle Road, Xi'an, 710032, P.R. China. Tel: 86-29-84776471. Fax: 86-29-83218039. E-mail: yanjin@fmmu.edu.cn

This study was supported by the Chinese National Programs for High Technology Research and Development (863 program) (2002AA205041, 2005AA205241), the National Natural Science Foundationof China (Project No.30572046)

Materials and Methods

Isolation and culture of BM-MSCs

All procedures were carried out according to the guidelines of the Animal Care Committee of Fourth Military Medical University. Approximately 100 g of weighted Sprague-Dawley (SD) male rats were killed by cervical dislocation, and tibiae and fibula were aseptically removed and dissected, free of adherent muscle and fibrous tissue. With the ends of the bone opened, the bone cavity was flushed out with DMEM/F12 medium, which included 50% Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) containing 5 ml Glutamax, 50 units/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml ascorbic acid, and 50% F12 medium (Sigma-Aldrich Corp, St. Louis, MO, USA). The flushed medium was collected and centrifuged. The resultant cell pellet were subsequently suspended in 5 ml DMEM/F12 medium and added to 5 ml Percoll (1.073 g/ml) very gently without mixing, and centrifuged for 20 min at 600 *g*. Mononuclear cells were extracted from the white gradient interface and washed twice with DMEM/F12 medium. Then the cells were cultivated in 75-cm^2 flasks at 10^6 cells/ml in DMEM/F12 medium with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL) in an atmosphere of 5% carbon dioxide. Cells were subcultured at 1×10^4 cells/ml when they reached 80% confluence[15]. The passage 3 subcultured cells were used for identification of ultrastructure under transmission electron microscope (TEM; JEOL, USA), and for evaluation of differentiation potential.

Assessment of potentiality of BM-MSCs

Osteogenic and adipogenic differentiation of BM-MSCs was performed as follows[7,16]: cells were plated at 5×10^4 cells/ml in 24-well plates. For osteogenic differentiation, cells at 80% confluency were stimulated for 2 weeks in DMEM/F12 medium supplemented with 2.0×10^{-4} mol/l ascorbic acid, 7.0×10^{-3} mol/l beta-glycerophosphate, 1.0×10^{-8} mol/l dexamethasone and 10% FBS. Calcium deposition was revealed by von Kossa staining. For adipogenic differentiation, cells were stimulated for 2 weeks in DMEM/F12 medium supplemented with 10% FBS, 5.0×10^{-4} mol/l isobutylmethylxanthine, 1.0×10^{-7} mol/l dexamethasone, and 1.0×10^{-9} mol/l insulin. Lipid droplets were revealed by staining with oil red O.

Cultivation of TGCs and assessment of their tooth-forming potentiality

The first mandibular molar tooth buds were isolated from E17 SD foetus rats and minced into less than 1-mm-sized pieces in 37 ºC Hank's balanced salt solution (HBSS; Gibco-BRL). Tooth bud tissues were digested with type I collagenase (0.66 mg/ml; Sigma-Aldrich) and Dispase I (0.33 mg/ml; Gibco-BRL), dissociated by trituration, and washed 5 times with DMEM/F12 medium containing 10% FBS. Single-cell suspensions were generated by filtration through a Falcon 40-micron cell strainer, washed twice and resuspended in DMEM/F12, plated into 75-cm^2 flasks or 6-well culture plates at 10^6 cells/ml and grown in 5% CO_2 at 37 ºC. The primary cultured TGCs, without being passaged, were used for indirect co-culture with BM-MSCs. The tooth-forming potentiality of E17 TGCs was evaluated by transplanting the pellet of 5×10^6 cultured primary TGCs under adult renal capsules for 2 weeks. The resulting tissues were identified by haematoxylin and eosin (H&E) staining.

Establishment of indirect co-culture system between BM-MSCs and TGCs in vitro

BM-MSCs were seeded onto several slides. When the cells had adhered, the slides were placed on 0.45 μm pore size membrane of Millicell plates (Millipore, USA). Then the Millicell was removed to 6-well culture plates on which TGCs had been seeded for 2 days and the floating cells had been wiped off. The indirect co-culture system was incubated in DMEM/F12 supplemented with 10% FBS and the medium was updated every 3 days. There were no TGCs in control cultures.

Immunocytochemistry

From day 1 to day 14, co-cultured BM-MSCs were processed for immunocytochemistry to evaluate the expression of odontogenic markers each day. The details were as follows: slides were fixed in 4% formaldehyde for 30 min, then treated by 2.5 ml/l Triton X-100 at 37 ºC for 15 min, fresh 3% H_2O_2 for 20 min, and blocked by 5% bovine serum albumin in PBS. After incubation at 4 ºC with primary antibodies, including rabbit polyclonal antibodies for dentin sialoprotein (DSP) and dentin matrix protein 1 (DMP1) (Santz Cruz, USA), slides were incubated with biotinylated goat anti-rabbit IgG 1:500 (Vector Labs, Burlingame, CA, USA). Biotinylated secondary incubations were followed by streptavadin-horseradish peroxidase (Vectastain ABC kit, Vector Labs). Detection of signals was completed by use of the DAB kit following the manufacturer's directions. Cells were then counter-stained with haematoxylin and mounted. Bright-field images were captured with an E4500 digital camera (Nikon, Japan).

Transplantation under adult renal capsules

Implants of experimental and control groups (E, C1, C2) were transplanted under renal capsules, with 20 implants

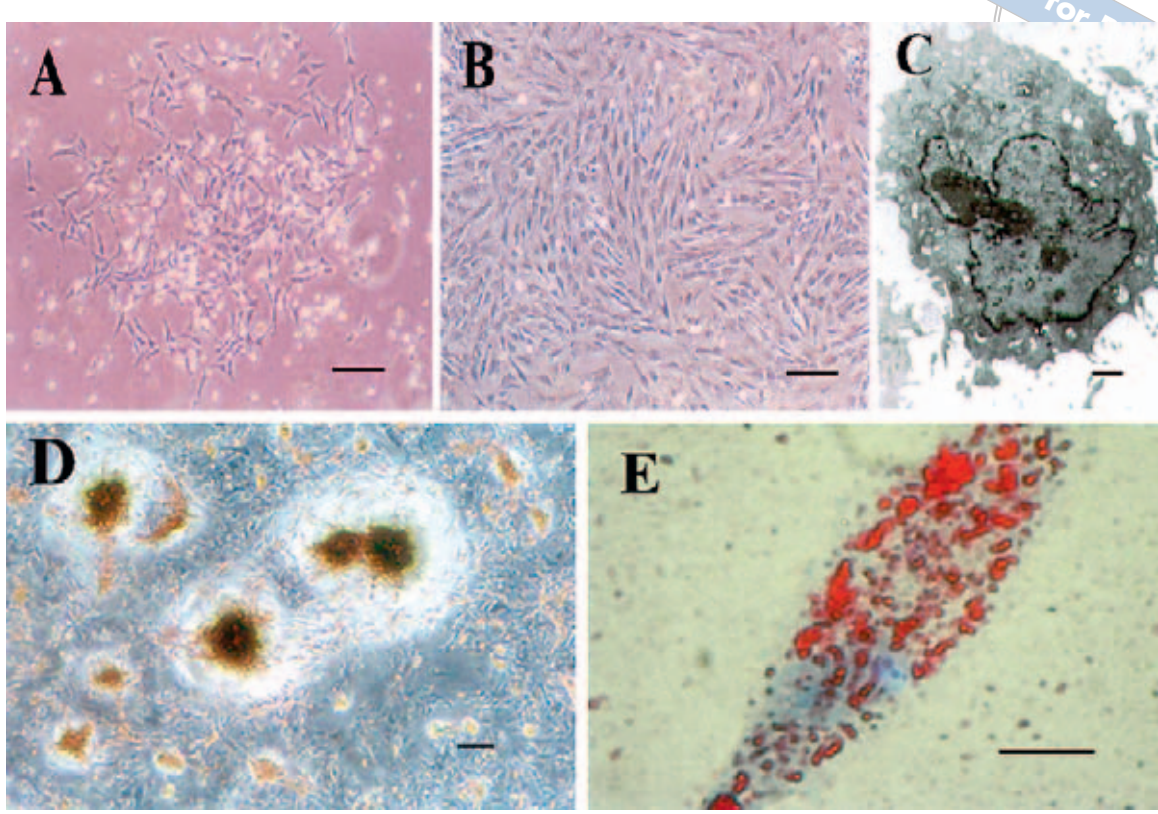

Fig 1 Characteristics of cultured bone marrow mesenchymal stem cells (BM-MSCs).
Figs 1A and 1B Spindle-shaped cells in primary culture and subculture (scale bar 30 μm).
Fig 1C BM-MSCs with large nucleus in proportion to their cytoplasms under transmission electron microscope (scale bar 1 μm).
Fig 1D Under osteogenic induction, BM-MSCs demonstrated deposition of mineralised matrix shown by von Kossa staining (scale bar 20 μm).
Fig 1E Under adipogenic induction, lipid droplets in BM-MSCs were revealed by staining with oil red O (scale bar 3 μm).

in each group. Experimental group (E) comprised BM-MSCs that had been cultured in indirect co-culture systems for 10 days, were centrifuged to form pellets consisting of 5×10^6 cells and then transplanted under renal capsules. Control groups included the pellets of BM-MSCs (5×10^6 cells) that had not been co-cultured with TGCs (C1), and for C2 the pellets (5×10^6 cells) consisted of a 1:1 ratio of BM-MSCs and TGCs, which had never been cultured together. Adult female syngeneic rats aged 2 months were used as hosts. After 2 weeks all the resulting tissues were fixed and decalcified, embedded in paraffin, sectioned at 5 μm intervals, and stained by H & E staining.

Results

Characteristics of cultured BM-MSCs

After the primary BM-MSCs were first cultured for 3-5 days, a few cells showed as adherent, elongate, and polygon- or spindle-shaped cells. Several clones formed gradually in 7-10 days (Fig 1a). The subcultured cells at 80% confluence showed swirling growth style (Fig 1b). The cells showed a large nucleus in proportion to their cytoplasms, a typical structure of stem cells, under a TEM (Fig 1c). Under osteogenic induction, the cells demonstrated deposition of mineralised matrix by von Kossa staining (Fig 1d). Under adipogenic induction, lipid droplets in the cytoplasm of BM-MSCs were revealed by staining with oil red O (Fig 1e). The results in-

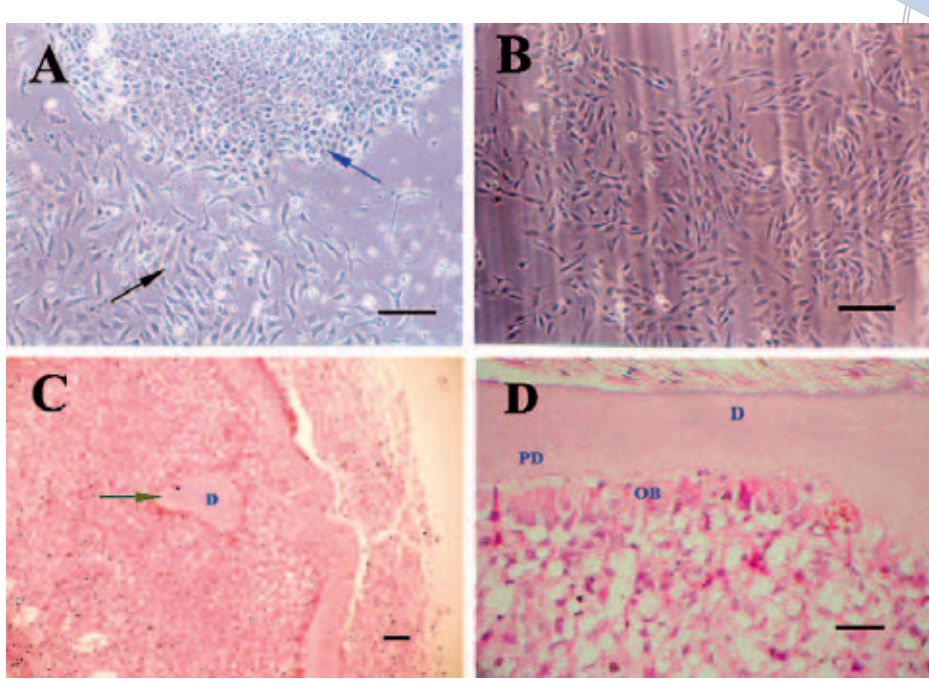

Fig 2A to D Tooth-forming potentiality of tooth germ cells (TGCs).
Fig 2A Cultured primary TGCs were heterogeneous (scale bar, 30 μm).
Fig 2B Subcultured cells were mesenchymal-like cells (scale bar, 30 μm).
Figs 2C and 2D Dentin-pulp like structures were identified using H&E staining after TGCs pellets were transplanted under adult renal capsules for 2 weeks, with distinctive dentin (D), predentin (PD) and odontoblasts (OB) (scale bars: c, 150 μm; d, 50 μm).

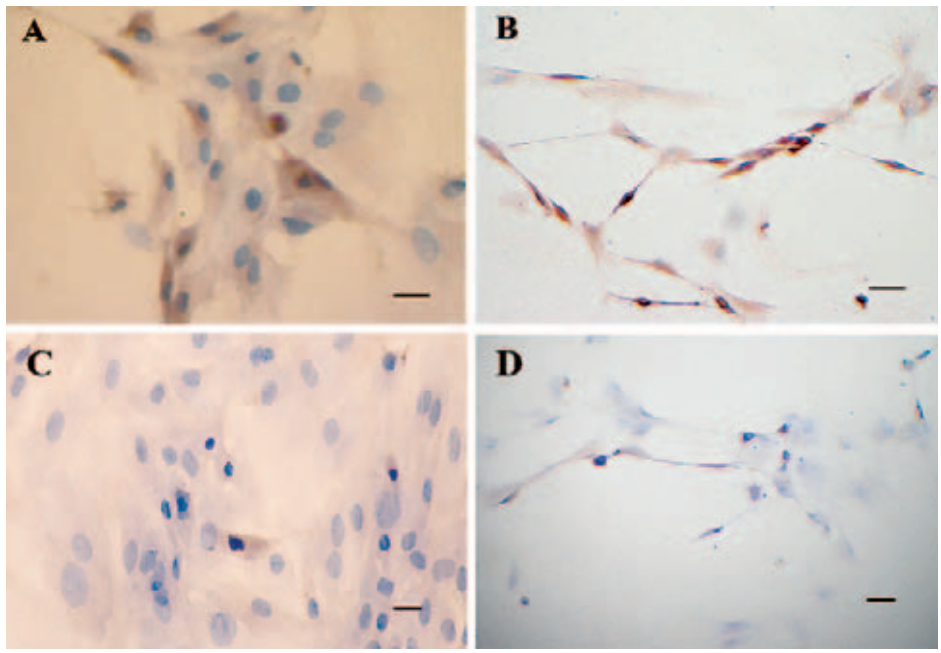

Fig 3A to D Expression of odontogenic molecules in co-cultured bone marrow mesenchymal stem cells (BM-MSCs). From day 7, some of the co-cultured BM-MSCs started to exhibit positive expression of (a) dentin sialoprotein (DSP) and (b) dentin matrix protein 1 (DMP1), by use of DAB kit, while those in control cultures did not (c and d) (scale bars, 10 μm).

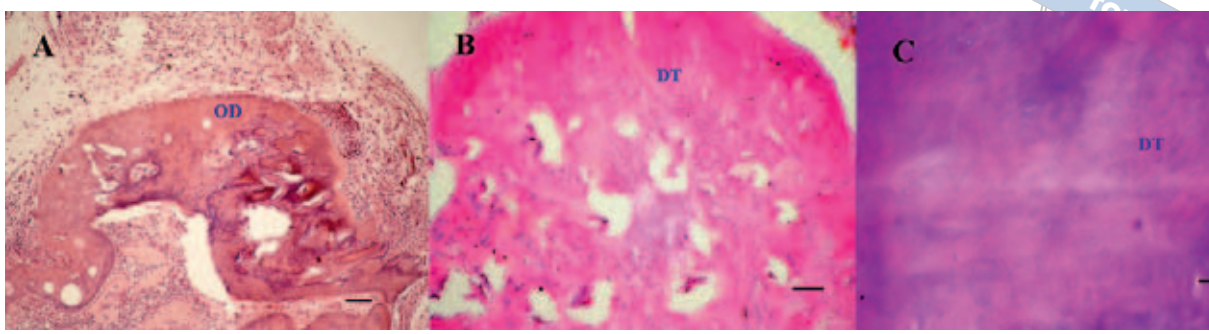

Fig 4 Histological analysis of 2-week experimental implants (H&E stain).
Figs 4A to 4C Implants of bone marrow mesenchymal stem cells (BM-MSCs) pellets alone which had been indirectly co-cultured with tooth germ cells (TGCs) for 10 days in vitro formed osteoid dentin. In some areas dentinal tubules could be observed (D, dentin; BT, bone tissue; OD, osteoid dentin; DT, dentinal tubules. Scale bars: a, 400 μm; b and c, 50 μm).

dicate differentiation potential of the cultured BM-MSCs and the cellls could be applied to tooth-forming potentiality.

Tooth-forming potentiality of TGCs
The primary cultured TGCs appeared to be heterogeneous, consisting of fibrous, mesenchymal-like cells (indicated with black arrow) and clusters of smaller, epithelial-like cells (indicated with blue arrow) in DMEM/F12 medium as shown in Fig 2a. However, the subcultured TGCs only showed mesenchymal-like cells (Fig 2b). After the TGCs were transplanted under adult rat renal capsules for 2 weeks, dentin-pulp like structures were observed with H&E staining, with distinctive dentinal tubules, predentin and odontoblasts (Figs 2c and 2d). In the pulp chamber of some explants, a pack of disorganised dentin surrounded by odontoblasts could be observed (see arrow in Fig 2c).

Effects of indirect co-culture with TGCs on differentiation of BM-MSCs
From day 7, some of the co-cultured BM-MSCs started to exhibit positive expression of odontogenic molecules, DSP and DMP1, as shown in Figs 3a and 3b, while those in control cultures did not (Figs 3c and 3d). At 2 weeks, three types of implants comprising experimental and control groups were excised and analysed. By visual inspection, aggregated BM-MSCs that had been cultured in indirect co-culture systems for 10 days in vitro, were white-coloured and rigid. Histological analyses showed that 12 out of 20 (60%) resulting tissues formed evident osteoid dentin, whose structure resembled the primary osseous tissue. Disorganised odontoblast-like cells were embedded within the osteoid matrix. In some areas, dentinal tubules could be observed (Figs 4a to 4c). Prolongations of secreting cells were arranged irregularly, and this abundance of exocytic vesicles indicated the active synthesis and secretion of the matrix by these cells.

All of the control implants exhibited white-coloured mineralised tissue. C1 pellets formed a porous calcified substance resembling osseous tissue (Figs 5a and 5b), and C2 pellets generated bone tissue and dentinal structures, with obvious borderline, as shown in Figs 5c and 5d.

Discussion

Under certain physiological or experimental conditions, BM-MSCs can be induced to become cells with special functions, such as the beating cells of the heart muscle or the insulin-producing cells of the pancreas[17-20]. The inductive signals produced from the oral epithelium also can affect the BM-MSCs population. Cultured TGCs, composed of dental mesenchymal cells and epithelial cells, could give rise to bioengineered tooth structures[14,21]. This suggests that dissociated cells retain their established programme of developing into a tooth, communicating with each other through soluble factors or cell-to-cell contact. These multiple signals released from TGCs may be able to pre-differentiate BM-MSCs.

In our experiment, the potentiality of the BM-MSCs was verified by inducing the cells into different lineages, including osteoblasts and adipocytes. It was demonstrated that dissociated E17 TGCs could be recombined, and formed dentin-pulp like structures after growing in hosts for some time. Although disorganised TGCs were

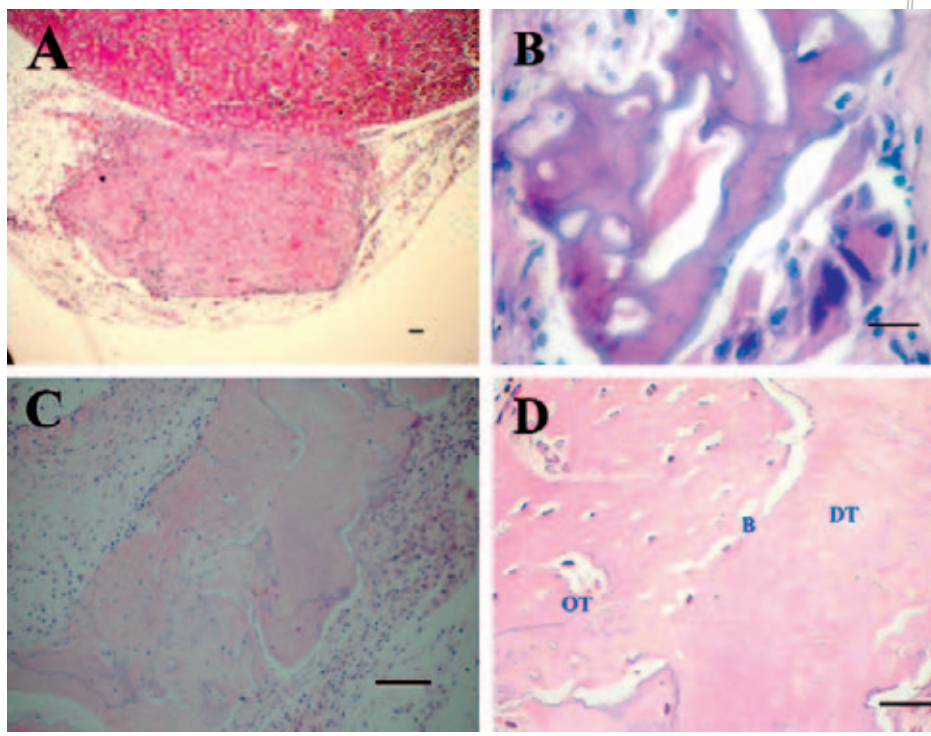

Fig 5 Histological analysis of 2-week control implants (H&E stain) (scale bars: a and c, 200 μm; b and d, 50 μm).
Figs 5A and 5B Pellets of bone marrow mesenchymal stem cells (BM-MSCs), which had not been induced by tooth germ cells (TGCs), formed a porous calcified substance resembling osseous tissue.
Figs 5C and 5D Mixed cell pellets, comprising BM-MSCs and TGCs in 1:1 ratio, generated bone tissue and dentinal structures respectively with obvious borderline. (OT, osseous tissue; DT, dentinal tissue; B, borderline).

cultured together *in vitro* for several days, they could send and receive multiple molecular signals from each other, developing in the direction of tooth. In the co-culture settings these soluble signals, i.e. multiple molecular signals or growth factors released from dental mesenchymal cells and epithelial cells, could act upon BM-MSCs by binding to specific matrix receptors on the cell surface, such as transmembrane proteins. In this way, specific genes of BM-MSCs could be switched on, as suggested by the positive expression of DSP and DMP1 in co-cultured BM-MSCs from day 7.

After indirect co-culture with TGCs *in vitro* for 10 days, BM-MSCs pellets were implanted under the renal capsule, where they lost the inductive source from TGCs and formed osteoid dentin structures. Odontoblast-like cells and some dentinal tubules indicate that the differentiation of BM-MSCs was determined by signals from TGCs. Resulting tissue of control implants of BM-MSCs alone showed that untreated pellets had a tendency to self-differentiate into osseous tissue. Mixed-cell pellets generated bone tissue and dentinal structures, with an evident dividing line, indicating that BM-MSCs could self-differentiate rapidly *in vivo* before inductive signals from TGCs were sent to them. Although mixed cells were heterogeneous, migration and aggregation could occur through recognition among homogeneous cells.

In summary, the present study demonstrated that multiple inductive signals released from heterogeneous TGCs might bring some pivotal molecules of BM-MSCs into play and direct their functional polarisation. Although the molecular mechanism regulating the differentiation remains largely unknown and requires further study, it is possible the BM-MSCs were specifically induced by the selection of pre-differentiating cells.

References

1. Conget PA, Minguell JJ. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. J Cell Physiol 1999;181:67-73.
2. Makino S, Fukuda K, Miyoshi S et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 1999;103: 697-705.
3. Black IB, Woodbury D. Adult rat and human bone marrow stromal stem cells differentiate into neurons. Blood Cells Mol Dis 2001;27:632-636.
4. Krause DS, Theise ND, Collector MI et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 2001;105:369-377.
5. Orlic D, Kajstura J, Chimenti S et al. Bone marrow cells regenerate infracted myocardium. Nature 2001;410:701-705.
6. Jiang Y, Jahagirdar BN, Reinhardt RL et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002;418:41-49.
7. Lee HS, Huang GT, Chiang H et al. Multipotential mesenchymal stem cells from femoral bone marrow near the site of osteonecrosis. Stem Cells 2003;21:190-199.
8. Ianus A, Holz GG, Theise ND, Hussain MA. In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. J Clin Invest 2003;111:843-850.
9. Ohazama A, Modino SA, Miletich I, Sharpe PT. Stem-cell-based tissue engineering of murine teeth. J Dent Res 2004;83:518-522.
10. Modino SA, Sharpe PT. Tissue engineering of teeth using adult stem cells. Arch Oral Biol 2005;50:255-258.
11. Hu B, Unda F, Bopp-Kuchler S et al. Bone marrow cells can give rise to ameloblast-like cells. J Dent Res 2006;85:416-421.
12. Yu JH, Shi JN, Deng ZH et al. Cell pellets from dental papillae can reexhibit dental morphogenesis and dentinogenesis. Biochem Biophys Res Commun 2006;346:116-124.
13. Yu JH, Deng ZH, Shi JN et al. Differentiation of dental pulp stem cells into regular-shaped dentin-pulp complex induced by tooth germ cell conditioned medium. Tissue Eng 2006;12:3097-3105.
14. Duailibi MT, Duailibi SE, Young CS et al. Bioengineered teeth from cultured rat tooth bud cells. J Dent Res 2004;83:523-528.
15. Sotiropoulou PA, Perez SA, Salagianni M et al. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem Cells 2006;24:462-471.
16. Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-147.
17. Kopen GC, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci USA 1999;96:10711-10716.
18. Watt FM, Hogan BL. Out of Eden: stem cells and their niches. Science 2000;287:1427-1430.
19. Okumoto K, Saito T, Hattori E et al. Differentiation of bone marrow cells into cells that express liver-specific genes in vitro: implication of the Notch signals in differentiation. Biochem Biophys Res Commun 2003;304:691-695.
20. Moriscot C, de Fraipont F, Richard MJ et al. Human bone marrow mesenchymal stem cells can express insulin and key transcription factors of the endocrine pancreas developmental pathway upon genetic and/or microenvironmental manipulation in vitro. Stem Cells 2005;23:594-603.
21. Young CS, Terada S, Vacanti JP et al. Tissue engineering of complex tooth structure on biodegradable polymer scaffolds. J Dent Res 2002;81:695-700.