NON-DESTRUCTIVE EVALUATION OF OSTEOGENIC DIFFERENTIATION IN TISSUE ENGINEERING

INTRODUCTION
Bone tissue engineering of autologous osteoprogenitor cells combined with biodegradable scaffolds has appeared as a potential alternative to the bone autograft. In vitro properties of tissue-engineered (TE) constructs directly determine their in vivo bone formation. Thus, evaluation of in vitro TE construct progression can be used to predict the outcome and function of in vivo implantation. Current techniques of assessment are destructive to living tissue and cells, making them unsatisfactory for practical application. Magnetic resonance (MR) microscopy is a non-invasive imaging technique with high spatial resolution and full three-dimensional capabilities. MR parameters of biological tissues can be spatially localized to provide information from living systems. High resolution MR serving as a non-invasive tool has been developed to estimate both in vitro and in vivo characteristics of TE constructs. At the present study, we used MR microscopy to monitor the progression of TE construct differentiation in vitro, and correlate quantitatively MR characteristics to osteogenic potentials of differentiated TE constructs.

MATERIALS AND METHODS
TE constructs preparation and differentiation
Healthy fresh human bone marrow provided commercially (AllCells) was plated at a density of 10^7 nucleated cells per 100 mm petri dish. The cells were incubated in DMEM at 37°C and 5% CO₂. Bone MSCs were isolated by removing unattached cells via the first time medium exchange. MSCs were incubated and proliferated until 80% confluence, then trypsinized and sub-cultured as Passage-1 with an initial density of 2 x 10^5 cells per dish. Trimmed gelatin sponge cubes with the size of 4 x 4 x 4 mm³ were immersed into suspension of MSCs (10^6 cells/ml) under a slight vacuum created by a 10 ml syringe. The constructs of cell-seeded sponges were cultured overnight in basic medium at 37°C and 5% CO₂ and divided into two groups the next day. One group was exposed to osteogenic differentiation medium supplemented with low concentrations of dexamethasone, β-glycerophosphate, and ascorbic acid. The other group was kept in basic medium as control. The medium was changed twice a week. On days 0, 7, 14, 21 and 28, TE constructs were evaluated by MR microscopy and biochemical techniques.

MR evaluation of TE constructs
MR experiments were conducted at 11.7 T in a 56 mm vertical bore magnet using a Bruker DRX Avance spectrometer (Bruker, Billerica, MA). TE constructs were loaded into a 5 mm diameter RF saddle coil and inserted into the Bruker Micro5 imaging probe with triple axis gradients (maximum strength 200 Gauss/cm). High resolution MR images of TE constructs were acquired with the field-of-view of 0.8 cm x 0.8 cm and in plane resolution of 62.5 μm x 62.5 μm for a slice thickness of 0.5 mm. For each sample, the T1, T2 and apparent diffusion coefficient (ADC) were measured.

Biochemical assessment of TE constructs
Following the MR measurements, the TE constructs were washed twice with phosphate buffered saline (PBS, pH 7.4). For alkaline phosphatase (ALP) staining, the samples were fixed in 10% formalin for 15 min, and then stained using fast red BB/naphthol AS-MX as substrate. Standard histological procedures were used for mineralization and osteocalcin staining. The constructs were fixed in 10% formalin for 1 hr, embedded in paraffin, and sectioned at 5 μm. Sections were stained with von Kossa staining and immunohistochemical staining using anti-human osteocalcin antibody. In order to measure ALP activity and calcium content quantitatively, TE constructs were immersed in 0.5 ml of 1% Triton-X100 for 15 min, followed by homogenization with a tissue mixer and sonication. ALP activity and calcium content of the lysates were measured using commercial kits (Raichem, San Diego, CA).

RESULTS
The progression of osteogenic differentiation in TE constructs created by seeding human bone MSCs on gelatin sponges was reflected by consecutive variations of MR imaging and parameters (Fig. 1). For the TE constructs exposed to differentiation medium, the signal intensity of MR image constantly decreased. MR parameters of constructs decreased significantly over four weeks period of experiment. The values of T1, T2 and ADC of differentiated constructs were decreased by 8.06%, 65.6% and 39.5%, respectively. In contrast, TE constructs exposed to basic medium as control expressed minute changes on MR images. The levels of T1, T2 and ADC of differentiated TE constructs were significantly lower than those of the control.

Biochemical examination revealed that ALP activities of osteogenic TE constructs were obviously increased shown by a strongly positive result of ALP staining (Fig. 2A). In contrast, ALP activity was hardly observed in the controls exposed to basic medium (Fig. 2B). Mineral deposition and osteocalcin were observed in the four week differentiated constructs after von Kossa and anti-human osteocalcin staining (Fig. 2C and D).

Few mineralized nodules and anti-osteocalcin reactions were found in the control group (Fig. 2D and F). Quantitative analysis revealed that the level of ALP activities of differentiated TE was significantly higher than that of control constructs after two weeks of differentiation (Fig. 2G). The calcium content of TE constructs were continuously increased after exposure to differentiation medium, which were significantly higher than those of control groups at each observation time point (Fig. 2H). ALP activities and calcium contents of TE constructs were found to well correlate to MR parameters. ALP to T1 (correlation coefficient r = 0.57, n=30, p<0.01), T2 (r = 0.78, n=30, p<0.01) and ADC (r = 0.81, n=30, p<0.01). Calcium to T2 (r = 0.90, n=30, p<0.01) and ADC (r = 0.91, n=30, p<0.01) (Fig. 3).

DISCUSSION
Different characteristics of bone marrow cells of individual donor, MSCs’ responsiveness of osteogenic stimuli, and properties of scaffolds can vary the properties of in vitro TE constructs and consequently may influence the efficiency of their in vivo bone formation. A system with capabilities of non-invasive evaluation TE constructs is demanded for optimizing osteogenic differentiation for each individual patient. The present study demonstrated that MR characteristics effectively respond the progression of osteogenic differentiation of TE constructs. Temporal up-regulation of ALP activity, mineral deposition and osteocalcin of differentiated TE constructs were accompanied by reduction of MR parameters of T1, T2 and ADC over the differentiation time period. MR parameters highly correlated to the osteogenic potentials of TE constructs measured by biochemical techniques. These preliminary results strongly supported that MR microscopy can be a promising tool to evaluate and monitor osteogenic differentiation in TE constructs for practical clinical application.

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